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# The supra-additive hyperactivity caused by an amphetamine–chlordiazepoxide mixture exhibits an inverted-U dose response: Negative implications for the use of a model in screening for mood stabilizers

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ABSTRACT

One of the few preclinical models used to identify mood stabilizers is an assay in which amphetamine-induced hyperactivity (AMPH) is potentiated by the benzodiazepine chlordiazepoxide (CDP), an effect purportedly blocked by mood stabilizers. Our data here challenge this standard interpretation of the AMPH–CDP model. We show that the potentiating effects of AMPH–CDP are not explained by a pharmacokinetic interaction as both drugs have similar brain and plasma exposures whether administered alone or in combination. Of concern, however, we find that combining CDP (1–12 mg/kg) with AMPH (3 mg/kg) results in an inverted-U dose response in outbred CD-1 as well as inbred C57Bl/6N and 129S6 mice (peak hyperactivity at 3 mg/kg CDP + 3 mg/kg AMPH). Such an inverted-U dose response complicates interpreting whether a reduction in hyperactivity produced by a mood stabilizer reflects a "blockade" or a "potentiation" of the mixture. In fact, we show that the prototypical mood stabilizer valproic acid augments the effects of CDP on hypolocomotion and anxiolytic-like behavior (increases punished crossings by Swiss–Webster mice in the four-plate test). We argue that these data, in addition to other practical and theoretical concerns surrounding the model, limit the utility of the AMPH–CDP mixture model in drug discovery.



Bipolar disorder is a severely debilitating psychiatric disorder affecting as much as 5% of the population worldwide (c.f., Emilien et al., 2007). Despite this widespread prevalence, little is known about the pathophysiology underlying the disease. Similarly, little is understood about what neurobiological mechanisms are responsible precisely for therapeutic actions of the mood stabilizers used to treat bipolar disorder. A significant factor contributing to our limited understanding in this field is a paucity of well-validated animal models (Cryan and Slattery, 2007; Einat, 2006, 2007; Gould and Einat, 2007). One animal model that purportedly predicts efficacy of mood stabilizers is an assay in which a mixture of D-amphetamine (AMPH, a psychostimulant) plus chlordiazepoxide (CDP, a benzodiazepine) is administered, resulting in heightened levels of hyperactivity relative to levels triggered by either compound alone. The "mutual potentiation" (Rushton and Steinberg, 1966, page 1312) of AMPH, which blocks uptake and facilitates release of dopamine at the transporter, and CDP, which facilitates binding of GABA to $GABA_A$ receptors, was originally characterized behaviorally in the 1960s. Despite the fact that the biological mechanisms explaining the potentiative effects of the AMPH–CDP mixture remain unknown, this mixture-induced hyperactivity is generally referenced as an animal model of mania and mood stabilizers are proposed to block the mixture effect (Arban et al., 2005; Aylmer et al., 1987; Cao and Peng, 1993; Foreman et al., 2008; Kozikowski et al., 2007; Lamberty et al., 2001).

Although the AMPH–CDP mixture model may hold some apparent value as a model for bipolar disorder, in that patients exhibit increased locomotor activity (Young et al., 2007), many studies have been unsuccessful in their attempts to satisfactorily validate this model. As recently explored by Arban et al. (2005), studies showing the ability of a mood stabilizer to reduce mixture-induced hyperactivity often neglect to determine the effect of combining the mood stabilizer with the benzodiazepine in the absence of the psychostimulant. As such, it is impossible to interpret whether or not the reduction in mixture-induced hyperactivity caused by the mood stabilizer simply reflects an ability to potentiate the hypolocomotive effects of the benzodiazepine. Indeed, Arban et al. (2005) show that combining an ineffective dose of the mood stabilizer carbamazepine with an ineffective dose of CDP together significantly decreases locomotor activity. These authors

*Abbreviations:* AMPH, D-amphetamine; CDP, Chlordiazepoxide; LMA, Locomotor activity; MDD, Major depressive disorder.

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also show that CDP plus valproic acid decreased activity relative to vehicle; however, it was unclear if this represented true potentiation because valproic acid alone also reduced activity. Together, the results of Arban et al. provide a cautionary tale regarding the implementation and interpretation of the AMPH–CDP mixture model.

Given the controversy arising around the AMPH–CDP mixture model, we seek here to assess what potential this model may hold for drug discovery efforts. To do so, we determined if the AMPH–CDP mixture effect was simply due to a pharmacokinetic interaction between CDP and AMPH. In addition, we behaviorally tested a wide range of CDP doses (1–12 mg/kg) in combination with a constant dose of AMPH (3 mg/kg), in CD-1, C57Bl/6N, and 129S6 strains. The assessment of a wide range of doses was prompted by a brief notation in the original AMPH–CDP publication that the potentiative effects of the AMPH–CDP mixture were observed over a range of doses "except at the extremes" (Rushton and Steinberg, 1966, page 1313). If an inverted-U dose response indeed exists, this would immediately complicate interpreting whether a potential mood stabilizer actually "blocks" vs "potentiates" the effect of the mixture. The outbred CD-1 and inbred C57Bl/6N mouse strains were chosen based on previous use of these strains in the model (e.g., Arban et al., 2005; Foreman et al., 2008) and the inbred 129S6 strain was chosen in order to characterize a strain of mice that, by comparison, exhibits relatively low levels of spontaneous locomotor activity. Finally, we conducted experiments designed to clarify if the prototypical mood stabilizer valproic acid does, in fact, augment the effects of CDP not only on locomotor activity but also anxiolytic-like behavior as measured in the four-plate test.

## 1. Methods

### 1.1. Subjects

8–12 week old male CD-1 (Charles River), Swiss–Webster (Charles River), C57Bl/6N (Taconic), and 129S6 mice (formerly 129SvEv; Taconic) were group-housed (4 per cage) and allowed to acclimate to the housing facility for 1 week prior to testing. All mice were maintained on a 12:12 light:dark cycle with ad libidum access to chow and water. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Pub 85-23, revised 1996) and were fully approved by the Institutional Animal Care and Use Committee of Wyeth Research. Please note figure legends for the number of subjects in each experimental group.

### 1.2. Drug preparation

All drugs were obtained from Sigma-Aldrich (Sigma-Aldrich; St. Louis, MO 63178). Chlordiazepoxide (CDP) was dissolved in saline at a concentration of 0.03–1.2 mg/ml (corrected for active moiety: 89.2%) and administered at a dose of 0.3–12 mg/kg (where indicated). D-amphetamine (AMPH) was dissolved in saline at a concentration of 0.3 mg/ml (corrected for active moiety: 73.4%) and administered at a dose of 3 mg/kg. This dose was chosen based on dose response curves in pilot experiments (data not shown). Valproic acid was dissolved in saline at a concentration of 5.4–30 mg/ml and administered at a dose of 54–300 mg/kg. All drugs were injected intraperitoneally (i.p.).

### 1.3. Behavior

*Locomotor activity* was recorded under indirect room light using Accuscan infrared beam activity monitors with enclosed 20.3 cm × 20.3 cm Plexiglas chambers (Columbus Instruments, Columbus, OH). Data were collected for 30 min. Sessions were limited to 30 min for two reasons. First, Arban et al. (2005), to whom we wished to compare results, employed 30-minute sessions. Second, our own preliminary studies that measured activity for 60 min suggested that the augmenting effect of CDP began to diminish approximately 40 min into the session. To measure total distance traveled, Accuscan Versamax and Versadat software (Columbus Instruments, Columbus, OH) were used to convert the infrared beam breaks into distance (centimeters). *Stereotypy* data were also collected in this automated fashion and calculated by these software packages based on contiguous breaks of the same single beam. When considering these data, it is important to consider that automated measurement of stereotypy is considered to be poor relative to manual scoring. CD-1, C57Bl/6N, and 129S6 mice were tested in parallel (i.e., in the same sessions) across 24 chambers. In studies examining the effect of the AMPH–CDP mixture in non-habituated subjects, mice were injected 10 or 18 min prior to the session. There was no difference in locomotor activity between these pretreatment intervals; therefore, data were collapsed for subsequent analyses.

*Anxiolytic-like behavior* was measured using the four-plate test. The four-plate apparatus consists of a Plexiglas chamber (18 × 25 × 16 cm) floored with four identical rectangular metal plates (8 × 11 cm), which are separated from one another by a gap of 4 mm and connected to a computerized device that can deliver electric shocks (0.8 mA, 0.5 s) (Aron et al., 1971). In this test, Swiss–Webster mice are placed into the chamber and following a brief (18 s) habituation period, the animal's innate motivation to explore the novel environment is suppressed by the delivery of a mild foot shock every time the animal crosses any of the boundaries (gaps) while moving from one plate to another (referred to as a 'punished crossing'). Following any punished crossing, there is a 3-second time out where the mouse may cross the electric plates without receiving another shock. An experimenter blind to the dosing conditions administers shocks, and a computer records the total number of punished crossings an animal makes during a 1-minute testing period. Clinically effective classes of anxiolytic compounds such as benzodiazepines, selective serotonin reuptake inhibitors (SSRIs), or 5-$HT_{1A}$ antagonists produce increases in punished crossings in this paradigm, which is indicative of anxiolytic-like activity as opposed to analgesia (Ripoll et al., 2006). In tests assessing the effect of valproic acid and CDP in this model, drugs were administered 30 min prior to the session.

### 1.4. Pharmacokinetic analyses

The pharmacokinetics of CDP and AMPH were investigated in male CD-1 mice after single intraperitoneal doses of 3 mg/kg of each drug, given alone or in combination. This was to test any potential pharmacokinetic interaction between the compounds when co-administered as being responsible for the observed supra-additive locomotor effects. The compounds were administered in 0.9% saline (10 mL/kg) after an overnight fast and blood and brain samples were collected before and at 1, 10, 30 and 60 min after dosing. Blood was collected in EDTA and plasma was obtained after centrifugation at 14000 rpm for 10 min at 4 °C. The wet brains were weighed and homogenized after addition of 1.2 mL of water. Both the plasma and brain homogenate samples were stored at −70 °C before and after analysis. An aliquot of the samples (50 μL) was extracted by protein precipitation. To the aliquot was added 20 μL of a 5 μg/mL solution of the internal standard (a proprietary compound) and 400 μL of acetonitrile. The mixture was shaken for 5 min, centrifuged at 3400 rpm for 5 min and an aliquot (5 μL) of the supernatant was assessed by Liquid Chromatography/Mass Spectrometry/Mass Spectrometry.

### 1.5. Data analyses

Behavioral data were analyzed using Sigmastat (v3.5; Systat, Point Richmond, CA 94804). Summed locomotor activity in the open field (centimeters traveled) and anxiolytic-like behavior in the four-plate test

(number of punished crossings) were analyzed by ANOVA for effect of drug treatment, and post hoc comparisons were made by Least Significant Difference tests. In analyses of behavioral data, statistical outliers greater than two standard deviations from the mean were removed from analyses. Statistical outliers were identified in the C57BL/6N study (1 from the amphetamine +6 mg/kg CDP group, 1 from the 3 mg/kg CDP group, and 2 from the amphetamine group), the 129S6 study (1 from the 3 mg/kg CDP group, 1 from the amphetamine +3 mg/kg CDP group, and 1 from the amphetamine +6 mg/kg CDP group), the CDP + VPA locomotor activity study (1 from the 300 mg/kg VPA + 17 mg/kg CDP group, 1 from the 100 mg/kg VPA group, and 2 from the 17 mg/kg CDP group), the four-plate VPA dose response (1 from vehicle) and the four-plate CDP dose response (1 from vehicle). Pharmacokinetic analyses were performed by the non-compartmental method using the software WinNonlin 4.1. Brain penetration was assessed by a comparison of the exposure ($AUC_{last}$) of the compound in the brain to the exposure in the plasma. Significance was determined at $p<0.05$ and all data are expressed as mean ± SEM.

## 2. Results

### *2.1. AMPH–CDP mixture exhibits an inverted-U dose response in both inbred and outbred strains*

To determine if the supra-additive effect of the AMPH–CDP mixture were comparable between outbred and inbred strains of mice, we tested the locomotor effect of combining a wide range of CDP doses (1–12 mg/kg, ip) plus AMPH (3 mg/kg, ip) in the inbred C57Bl/6N and 129S6 (formerly 129/SvEv) strains as well as the outbred CD-1 strain. In CD-1 mice (Fig. 1A), although 1–12 mg/kg CDP alone does not significantly affect locomotor activity (LMA), 1–8 mg/kg CDP significantly increases the hyperlocomotion triggered by 3 mg/kg AMPH ($F_{(13,248)}=34.08$, $p<0.001$; Post hoc, AMPH vs vehicle, $p<0.001$; 1, 3, 6, and 8 mg/kg CDP + AMPH vs AMPH alone, each $p<0.005$–$0.001$). Surprisingly, the highest doses of 10 and 12 mg/kg CDP does not increase the AMPH hyperlocomotion and, in fact, results in significantly lower LMA relative to the combination of 1–8 mg/kg CDP plus AMPH (Post hoc, 10 mg/kg CDP + AMPH vs 3 and 6 mg/kg CDP + AMPH, each $p<0.005$–$0.001$; 12 mg/kg CDP + AMPH vs 1, 3, 6, and 8 mg/kg CDP + AMPH, each $p<0.01$–$0.001$). This inverted-U dose response does not appear to be due to sedation as 10 and 12 mg/kg CDP alone does not reduce LMA (Fig. 1A). It is important to point out that the lack of hypolocomotion is not due to a floor effect, as we are readily able to detect significant decreases in locomotor activity within these apparati (see Fig. 2A). Further, this inverted-U dose response does not appear to be due to an increase in either the duration or number of stereotypy bouts (Table 1).

An inverted-U mixture dose response is also observed in the inbred C57Bl/6N (Fig. 1B) and 129S6 strains (Fig. 1C). 3 mg/kg AMPH significantly increases LMA relative to vehicle in both C57Bl/6N ($F_{(6,129)}=21.92$, $p<0.001$; Post hoc, AMPH vs vehicle, $p<0.001$) and 129S6 mice ($F_{(6,127)}=27.75$, $p<0.001$; Post hoc, AMPH vs vehicle, $p<0.001$). As noted above in CD-1 mice, 3 mg/kg CDP in combination with AMPH significantly increases AMPH-induced hyperactivity in both C57Bl/6N (Post hoc, 3 mg/kg CDP + AMPH vs all groups, each $p<0.001$) and 129S6 mice (Post hoc, 3 mg/kg CDP + AMPH vs all groups, each $p<0.001$). In contrast to the CD-1 mice, however, when 1 or 6 mg/kg CDP are combined with AMPH, there is no exacerbation of AMPH hyperlocomotion in either inbred strain. Given that neither 3 nor 6 mg/kg CDP alone reduces LMA in any strain tested, it is unlikely the descending limb of the inverted-U dose response observed in C57Bl6/N and 129S6 mice reflects sedation (Fig. 1B and C). It also does not appear to be due to an increase in either the duration or number of stereotypy bouts (Table 1). These data suggest that the effect of the AMPH–CDP mixture are qualitatively comparable between outbred and inbred strains (inverted-U); however, it appears that the window within which CDP increases AMPH hyperlocomotion is narrower in inbred strains relative to the outbred CD-1 strain.

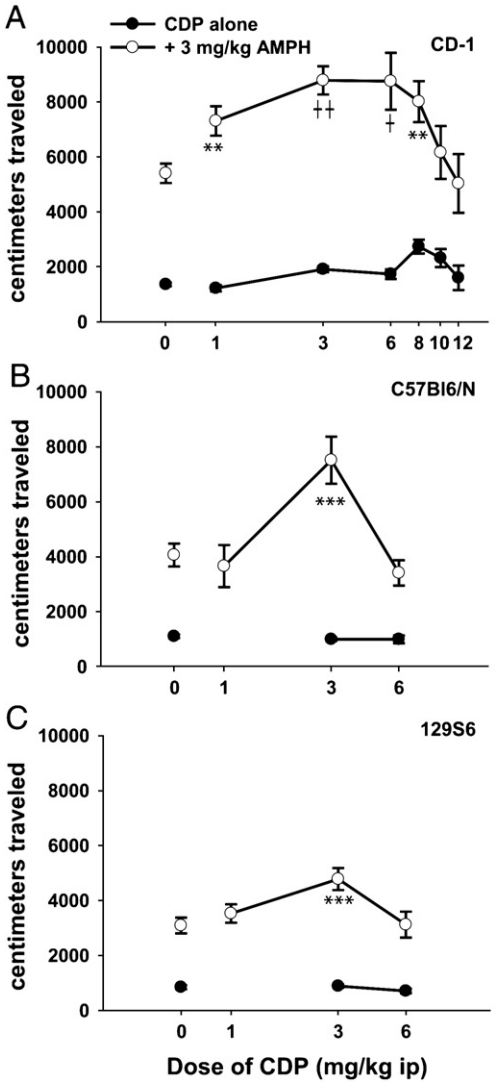


**Fig. 1.** The AMPH–CDP mixture exhibits an inverted-U dose response in both outbred and inbred strains of mice. The locomotor effect of CDP (1–12 mg/kg, ip), AMPH (3 mg/kg, ip), or CDP (1–12 mg/kg, ip) + AMPH (3 mg/kg, ip) was assessed in A) outbred CD-1, B) inbred C57Bl/6N, and C) inbred 129S6 mice placed in a novel locomotor chamber. The number of centimeters traveled was recorded in 2-minute bins for a total of 30 min. In all three strains tested, all groups treated with AMPH, either alone or in conjunction with CDP, exhibit significantly increased locomotor activity relative to vehicle ($p<0.05$–0.001). Further, in all three strains, the AMPH–CDP mixture triggers augmented hyperactivity relative to AMPH alone, but only at select doses. This results in an inverted-U dose response in all three strains. CDP—chlordiazepoxide; AMPH—D-amphetamine. $n=10$–38 per group. Post hoc vs AMPH, ***$p<0.001$; vs AMPH, 10 mg/kg CDP + AMPH, and 12 mg/kg CDP + AMPH, $^{\dagger}p<0.05$ (vs each of the three groups), and $^{\dagger\dagger}p<0.01$ (vs each of the three groups).

### *2.2. Valproic acid, a prototypical mood stabilizer, potentiates the effect of CDP in measures of locomotion and anxiolysis*

The fact that the AMPH–CDP mixture exhibits an inverted-U dose response immediately calls into question the standard interpretation that the ability of a mood stabilizer to normalize AMPH–CDP LMA levels (tested at a single combinatorial dose) reflects an ability to "block" the effects of the mixture (Arban et al., 2005; Aylmer et al., 1987; Cao and Peng, 1993; Foreman et al., 2008; Kozikowski et al., 2007; Lamberty et al., 2001). It is equally possible that mood stabilizers actually potentiate the effect of the mixture components.

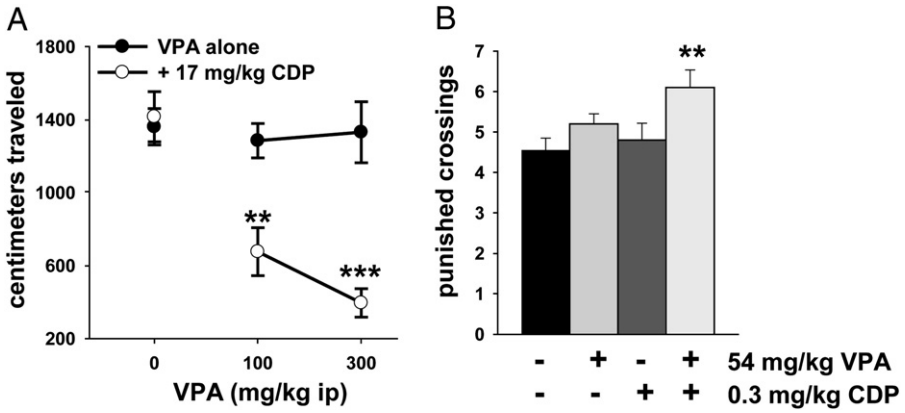


**Fig. 2.** Valproic acid, a prototypical mood stabilizer, potentiates the hypolocomotive and anxiolytic-like effects of chlordiazepoxide. A) CD-1 mice were injected i.p. with vehicle (0 mg/kg valproic acid + 0 mg/kg CDP), valproic acid (100 or 300 mg/kg valproic acid), chlordiazepoxide (17 mg/kg CDP), or a combination of 100 or 300 mg/kg valproic acid + 17 mg/kg CDP. Mice were then placed in a novel locomotor chamber and the number of centimeters traveled was measured over 30 min. Summed data show that valproic acid enhances the hypolocomotive effects of CDP as indicated by a significant dose-dependent decrease in locomotor activity. $n = 8$–23 per group. B) Swiss–Webster mice were injected i.p. with vehicle (0 mg/kg valproic acid + 0 mg/kg CDP), 0.3 mg/kg CDP, 54 mg/kg valproic acid, or a combination of 54 mg/kg valproic acid + 0.3 mg/kg CDP and then tested in the four-plate paradigm. The number of punished crossings was recorded over a 5-minute session. Although these low doses of CDP and valproic acid are without significant effect when administered alone, combining these subeffective doses of CDP and valproic acid produces a synergistic increase in anxiolytic-like behavior, as indicated by a greater number of punished crossings relative to vehicle. $n = 8$–10 per group. Post hoc, vs veh (0 mg/kg valproic acid + 0 mg/kg CDP), $^{**}p<0.01$, $^{***}p<0.001$.

That is, without assessing the effect of a mood stabilizer across the entire ascending and descending limb of the mixture dose response, it is impossible to determine if a mood stabilizer makes the normally supra-additive combination of 3 mg/kg CDP + AMPH act like 0 mg/kg CDP + AMPH *or* 12 mg/kg CDP + AMPH (see Fig. 1A).

Previously, Arban et al. (2005) showed that carbamazepine, a mood stabilizer, exacerbates the hypolocomotor effects of CDP. Decreased activity was also observed in mice treated with the mood stabilizer valproic acid plus CDP, relative to vehicle. It was unclear, however, if this represented true potentiation as valproic acid alone reduced activity in a separate experiment. As such, we sought to clarify whether or not valproic acid, a prototypical mood stabilizer, augments the effects of CDP in mice. To obtain a clear answer to our hypothesis, we tested animals treated with CDP alone or valproic acid alone in the same experiments as those tested with valproic acid plus CDP, and did so across two behavioral paradigms. First, we determined if valproic acid potentiates the hypolocomotive effects of CDP in CD-1 mice exposed to a novel LMA chamber. As shown in Fig. 2A, although CDP (17 mg/kg) or valproic acid (100 and 300 mg/kg) alone do not significantly affect LMA, the combination of valproic acid + CDP synergistically decreases LMA in a dose-dependent manner ($F_{(5,83)} = 10.83$, $p<0.001$; Post hoc, 100 mg/kg valproic acid + CDP and 300 mg/kg valproic acid + CDP vs all other groups, $p<0.005$–$0.001$ for each).

Next, we determined if valproic acid would potentiate the anxiolytic-like effects of CDP. To do so we tested the ability of CDP (0.3–3 mg/kg) and/or valproic acid (54–300 mg/kg) to increase punished crossings of Swiss–Webster mice in the four-plate test, a well-validated model for assessing anxiolytic-like activity (Ripoll et al., 2006). In accordance with its well-established anxiolytic-like profile, CDP increases punished crossings at 1 and 3 mg/kg ($F_{(3,26)} = 3.40$, $p<0.05$; Post hoc, vehicle vs 1 and 3 mg/kg, $p<0.05$ for each; data not shown). Valproic acid also increases punished crossings, but only at 300 mg/kg ($F_{(3,26)} = 15.48$, $p<0.001$; Post hoc, vehicle vs 300 mg/kg valproic, $p<0.001$; data not shown). Notably, combining a subeffective dose of valproic acid (54 mg/kg) with a subeffective dose of CDP (0.3 mg/kg) results in a significant increase in the number of punished crossings observed (Fig. 2B; $F_{(3,35)} = 3.53$, $p<0.025$; Post hoc, vehicle vs valproic acid + CDP, $p<0.01$). Together, these results suggest that valproic acid potentiates not only the hypolocomotor effect but also the anxiolytic-like effect of CDP.

### 2.3. AMPH–CDP potentiation is not due to a pharmacokinetic interaction

To determine if the supra-additive interaction of chlordiazepoxide and amphetamine reflects an effect on pharmacokinetics, we determined if CDP alters the disposition of AMPH (or vice versa) in brain or plasma of CD-1 mice. In this experiment, we employed 3 mg/kg CDP and 3 mg/kg AMPH, as this was the combinatorial dose optimally active across all three strains in the behavioral experiments noted above. Plasma and brain levels of each compound were measured 1, 10, 30, and 60 min following administration. The results show that the brain and plasma exposures of CDP and AMPH were comparable when given alone or in combination resulting in comparable brain-to-plasma exposure ratios as well (Table 2). As such, the potentiative interaction of CDP plus AMPH is unlikely due to a pharmacokinetic interaction.

**Table 1**
As seen with distance traveled, stereotypy triggered by the AMPH–CDP mixture exhibits an inverted-U dose response.

| | Dose of CDP in mg/kg (administered in combination with 3 mg/kg AMPH) | | | | | |
|---|---|---|---|---|---|---|
| Strain | 0 | 1 | 3 | 6 | 10 | 12 |
| CD-1 | 9213 | 10187 | 13502 | 12306 | 6501 | 5186 |
| C57BL/6N | 9311 | 8257 | 12329 | 8580 | | |
| 129S6 | 5539 | 4740 | 5833 | 2346 | | |

AMPH—amphetamine, CDP—chlordiazepoxide.

## 3. Discussion

We show here that the supra-additive effects of an AMPH–CDP mixture are not due to a pharmacokinetic interaction, and that the AMPH–CDP mixture dose response exhibits an inverted-U in both outbred CD-1 and inbred C57Bl/6N and 129S6 mice. We believe it likely that rats would also exhibit such an inverted-U dose response as the original publication on this model (Rushton and Steinberg, 1966) notes in passing (data not actually shown) that the potentiative effects of the mixture were observed over a range of doses "except at the extremes" (page 1313). As discussed below, such an inverted-U dose

**Table 2**
Amphetamine and chlordiazepoxide exhibit similar pharmacokinetic profiles in brain and plasma of male CD-1 mice whether administered alone or in combination.

| Compound | Treatment | Matrix | Dose (mg/kg) | Cmax ng/mL or g | Tmax (min) | AUClast (h*ng/1mL or g) | Ratio (Tissue/fluid) |
|---|---|---|---|---|---|---|---|
| Amphetamine | Alone | Brain | 3 | 1608 | 10 | 1218 | 1.64 |
| | Alone | Plasma | 3 | 1391 | 10 | 743 | |
| Amphetamine | Combo | Brain | 3 | 1802 | 10 | 1185 | 1.73 |
| | Combo | Plasma | 3 | 1237 | 10 | 686 | |
| Chlordiazepoxide | Alone | Brain | 3 | 408 | 10 | 109 | 0.56 |
| | Alone | Plasma | 3 | 610 | 10 | 196 | |
| Chlordiazepoxide | Combo | Brain | 3 | 540 | 10 | 163 | 0.63 |
| | Combo | Plasma | 3 | 970[a] | 1 | 257 | |

$n = 3$ per group.
[a]Concentration at 10 min = 539 mg/mL.

response immediately complicates the interpretation of whether a reduction in mixture-induced hyperactivity caused by a mood stabilizer reflects a "blockade", as holds the standard interpretation, or a "potentiation" of the mixture components. Indeed, we show here that the prototypical mood stabilizer valproic acid potentiates both the hypolocomotive and anxiolytic-like effects of CDP. Together, these data raise practical and theoretical concerns regarding the applicability of the AMPH–CDP mixture model to bipolar disorder research.

### 3.1. Mood stabilizers may potentiate instead of block the effects of chlordiazepoxide

The AMPH–CDP model purportedly identifies mood stabilizing drugs based on the capacity of a compound to "block" the hyperactivating effects of the mixture. Unfortunately, the majority of studies attempting to validate this model have tested mood stabilizers against a single dose of AMPH–CDP and without appropriate controls (e.g., mood stabilizer plus CDP alone). We illustrate here (Fig. 1) that the ability of CDP to potentiate AMPH-induced hyperlocomotion exhibits an inverted-U dose response. Thus, it is impossible to determine if a reduction in hyperactivity caused by 3 mg/kg CDP + AMPH reflects a blockade (i.e., equating to 0 CDP + AMPH) or a potentiation of the mixture components (i.e., equating 12 CDP + AMPH), unless modulation of a full dose response curve is tested.

In fact, there is precedence to suggest that at least some mood stabilizers potentiate the effect of CDP. Arban et al. (2005) clearly showed that carbamazepine increases the hypolocomotive effects of CDP. They also showed that valproic acid plus CDP decreases locomotor activity; however, it was unclear if this reflected true potentiation as valproic acid alone decreased locomotor activity in their hands. We clarify these findings here, showing that valproic acid does, in fact, potentiate the hypolocomotive effects of CDP (Fig. 2A). The fact that valproic acid augments the hypolocomotive effects of CDP underscores concerns presented by Arban et al. (2005) regarding the failure of several studies to test the effects of a given mood stabilizer with CDP alone (in absence of AMPH).

In addition to enhancing CDP-induced hypolocomotion, valproic acid also boosts the anxiolytic-like effects of CDP. In the four-plate test, we show that a subeffective dose of valproic acid potentiates a subeffective dose of CDP, to synergistically increase the number of punished crossings (Fig. 2B). Indeed, it is unclear why one would seek mood stabilizers that 'block' the effect of benzodiazepines, given that over half of bipolar patients suffer from co-morbid anxiety (Boylan et al., 2004; Emilien et al., 2007; Simon et al., 2004). Given the dire consequences of co-morbid anxiety on disease course and functional outcome of patients (Boylan et al., 2004; Keller, 2006; Otto et al., 2006; Simon et al., 2004), including increased risk of suicide (Simon et al., 2007), theoretically it would be beneficial for a mood stabilizer to have no effect or a potentiating effect on benzodiazepines.

Together, these results argue that drastic methodological revisions are required in the design of experiments employing the AMPH–CDP mixture. The effect of a mood stabilizer on LMA must be assessed alone, in combination with CDP, as well as against the full ascending and descending limb of the AMPH–CDP dose response. Only then will it be possible to determine if a mood stabilizer elicits a leftward or rightward shift in the mixture dose response and if that shift is independent of effects on hypolocomotion. To be clear, we do not suggest that all mood stabilizers necessarily potentiate CDP in order to affect behavior in AMPH–CDP treated rodents. We do suggest, however, that the identification of such an interaction for two of the four mood stabilizers used to validate the model is sufficient to call into question the predictive validity of the AMPH–CDP hyperactivity paradigm in the context of mood stabilizer screening.

### 3.2. Additional concerns challenge the use of the AMPH–CDP mixture model in bipolar disorder research

In addition to the concerns regarding the predictive validity of the AMPH–CDP mixture model, there are also practical concerns that hamper our enthusiasm for this model in the context of drug discovery. Throughput is low given the large number of subjects needed within each group, the large number of control groups required, and the fact that others (although not us) appear to observe inconsistent levels of AMPH–CDP hyperactivity from day to day (Arban et al., 2005). In fact, it is unclear what predictive validity is gained in reversing the effects of the AMPH–CDP mixture relative to AMPH alone. Increasing dopaminergic signaling appears sufficient to trigger symptoms of mania in controls and patients with bipolar disorder (Murphy et al., 1971; Peet and Peters, 1995). Further, both acute and chronic administrations of the prototypical mood stabilizer lithium are able to reverse amphetamine-induced hyperactivity in several mouse strains, including multiple C57Bl/6 substrains (e.g., Gould et al., 2007). Together, these results strongly argue that the AMPH–CDP mixture model holds little utility for drug discovery efforts.

### 3.3. A novel approach is needed for modeling bipolar disorder in animals

It is well-accepted that the field of bipolar disorder research suffers from a dearth of well-validated animal models (Cryan and Slattery, 2007; Gould and Einat, 2007; Einat, 2006, 2007). A growing movement in the field of psychiatric research is an endophenotypic approach to developing animal models where a specific aspect or symptom of a given disorder is modeled. We, like others (Cryan and Slattery, 2007; Gould and Einat, 2007; Einat, 2006, 2007), endorse such an approach; however, we believe such an approach may be more problematic for the field of bipolar disorder than it has been for other illnesses such as schizophrenia.

A review of preclinical measures intended to capture aspects of mania are more often than not the very same measures used to indicate anxiolytic-like activity. Patients suffering from mania exhibit increased energy/locomotion, aggression, risk-taking behavior, impulsivity, punished responding, decreased attention, impaired working memory, and shifts in circadian rhythms (c.f., Emilien et al., 2007), and all of these behaviors are induced in rodents when administered an anxiolytic (e.g.; Evenden and Ko, 2005; Biello and Mrosovsky, 1993; Shannon and Love, 2005; Hogan et al., 2005; Millan et al., 2001). As such, any drug reversing these mania-related phenotypes would also be exhibiting an anxiogenic-like profile, which–as noted above–holds severe implications for a disorder characterized by such high levels of co-morbid anxiety. An exception to this rule may be the endophenotype of distractibility (Agmo et al., 1997), which deserves further study.

A key challenge to the field is developing models that may be specific for bipolar depression (as opposed to major depressive disorder) as well as the cyclicity that defines bipolar disorder. Treatment for bipolar

depression remains a high unmet medical need, with patients spending the majority of their illness in a depressive episode (Judd et al., 2002, 2003; Emilien et al., 2007). One of the most debated issues in the field, currently, relates to the efficacy of antidepressants in treating bipolar depression. While a number of small studies have indicated limited efficacy of antidepressants on bipolar depression, (c.f., Dubovsky, 2005), the large STEP-BD study (Systematic Treatment Enhancement Program for Bipolar disorder) has suggested that antidepressants are not efficacious in treating bipolar depression when administered on top of a mood stabilizer (Goldberg et al., 2007; Sachs et al., 2007). That said, the effect of antidepressants on bipolar depression in the absence of a mood stabilizer has not been extensively explored with sufficiently powered studies (c.f., Dubovsky, 2005). Of further concern is the suggestion that traditional antidepressants may be harmful to a subpopulation of patients suffering from bipolar disorder by inducing a 'switch' into mania, worsening manic symptoms, or possibly even—in the case of selective serotonin reuptake inhibitors—increasing suicidality (Goldberg et al., 2007; Marangell et al., 2008; Truman et al., 2007). Finally, there may be reason to believe that bipolar depression differs neurobiologically, from (unipolar) major depressive disorder (MDD; Bielau et al., 2007; Cannon et al., 2006; Hantouche and Akiskal, 2005; Lawrence et al., 2004). With the current controversy regarding efficacy of antidepressants in bipolar patients, the usefulness of models currently used to predict antidepressant-like activity (e.g., porsolt forced swim test, tail suspension test, etc.) remains unclear in the context of bipolar depression. It should be noted, however, that the mood stabilizer lithium shows antidepressant-like activity in the aforementioned models as it does in models of antipsychotic-like efficacy (Gould et al., 2008).

### 3.4. Conclusion

Until more is understood of the pathophysiology of bipolar disorder, the development of valid animal models is likely to be slow-going. Although behavioral measures that show sensitivity to currently used mood stabilizers can be assessed, it remains unclear if those sensitivities reflect mechanisms mediating the therapeutic actions or the side-effect profile of a drug (O'Donnell and Gould, 2007). We hope that this work will improve future efforts in model development by bringing to the forefront methodological and theoretical concerns facing the field today.

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