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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