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Amphetamine-Like Neurochemical and Cardiovascular Effects of α-Ethylphenethylamine Analogs Found in Dietary Supplements

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ABSTRACT

Dietary supplements often contain additives not listed on the label, including α-ethyl homologs of amphetamine such as *N,*α-diethylphenethylamine (DEPEA). Here we examined the neurochemical and cardiovascular effects of α-ethylphenethylamine (AEPEA), *N*-methyl-α-ethylphenethylamine (MEPEA), and DEPEA as compared to the effects of amphetamine. All drugs were tested *in vitro* using uptake inhibition and release assays for monoamine transporters. As expected, amphetamine acted as a potent and efficacious releasing agent at dopamine transporters (DAT) and norepinephrine transporters (NET) *in vitro*. AEPEA and MEPEA were also releasers at catecholamine transporters, with greater potency at NET than DAT. DEPEA displayed fully efficacious release at NET but weak partial release at DAT (i.e., 40% of maximal effect). In freely moving, conscious male rats fitted with biotelemetry transmitters for physiological monitoring, amphetamine (0.1-3.0 mg/kg, sc) produced robust dose-related increases in blood pressure (BP), heart rate (HR), and motor activity. AEPEA (1-10 mg/kg, sc) produced significant increases in BP but not HR or activity, whereas DEPEA and MEPEA (1-10 mg/kg, sc) increased BP, HR, and activity. In general, the phenethylamine analogs were approximately 10-fold less potent than amphetamine. Our results show that α-ethylphenethylamine analogs are biologically active. While less potent than amphetamine, they produce cardiovascular effects that could pose risks to humans. Given that MEPEA and DEPEA increased locomotor activity, these substances may also have significant abuse potential.

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Significance Statement. The α-ethyl homologs of amphetamine have significant cardiovascular, behavioral, and neurochemical effects in rats. Given that these compounds are often not listed on the ingredient labels of dietary supplements, these compounds could pose a risk to humans using these products.

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Introduction

Nutritional supplements often contain ingredients that are not listed on the product labels, including analogs of phenethylamine (PEA) that display structural similarity to amphetamine (Eichner, 2014; Pawar and Grundel, 2017). We recently showed that the supplement additive β-methylphenethylamine (BMPEA), a positional isomer of amphetamine (i.e., α-methylphenethylamine), increases blood pressure (BP) in rats and may therefore produce adverse effects in humans (Schindler et al., 2019). Another PEA analog previously found in dietary supplements is *N,*α-diethylphenethylamine (DEPEA, see Figure 1 for chemical structure). DEPEA has been detected in powdered material confiscated for drug trafficking (Lee et al., 2013) and in supplement products destined for human consumption (ElSohloy and Gul, 2014; ElShohley et al. 2015; Cohen et al., 2014; Walhlstrom et al., 2014).

Urine toxicology testing has also confirmed the presence of DEPEA in users of some dietary supplements (Wojtowicz et al., 2015). For example, in urine samples obtained through routine toxicological testing, DEPEA was detected in samples from individuals who used a dietary supplement suspected of containing added DEPEA (Uralets et al., 2014). In addition to DEPEA, α-ethylphenethylamine (AEPEA) was also found in the some of the same samples, potentially the result of *N*-dealkylation of DEPEA via hepatic metabolism. Both DEPEA and AEPEA have been found in urine samples collected by the World Anti-Doping Agency, demonstrating exposure to these substances among athletes (World Anti-Doping Agency, 2012, 2013). In a survey of German athletes who were asked about their supplement use, some respondents specifically reported seeking products containing DEPEA (Dreher et al., 2018). Since DEPEA and related analogs are not listed as ingredients on the labels of supplement

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products, the FDA considers them adulterants and requires companies selling the products to remove them from the market (Pawar and Grundel, 2017). However, PEA analogs may still be present in those supplements not tested by the FDA.

Despite the apparent widespread use of DEPEA and similar compounds, very little is known about their biological effects. Oberlender and Nichols (1991) reported that AEPEA partially generalizes to the discriminative stimulus effects of amphetamine. In that study, rats were trained to respond on one lever when injected with amphetamine and another lever when injected with saline. When the trained rats received a non-contingent injection of AEPEA, they responded on the amphetamine-associated lever, suggesting that AEPEA and amphetamine might share common effects. Santillo (2014) reported that AEPEA inhibits human monoamine-oxidase type A (MAO-A) *in vitro* in a competitive and reversible manner similar to amphetamine, whereas DEPEA has much weaker effects in this regard. Liu and Santillo (2016) reported that DEPEA inhibits activity of the hepatic cytochrome P-450 enzyme, CYP2D6, which could alter the effects of other drugs taken in combination with DEPEA. Due to the paucity of information about the pharmacology of α-ethyl PEA analogs, we sought to study the effects of AEPEA and its amine-substituted analogs, *N*-methyl-α-ethylphenethylamine (MEPEA) and DEPEA, as compared to the effects of amphetamine (see Figure 1 for chemical structures). Based on their structural similarities to amphetamine, we expected that these PEA analogs would have similar effects to amphetamine, although at potentially different potencies.

Materials and Methods

Drugs and Reagents. α-Ethylphenethylamine (AEPEA), *N*-methyl-α-ethylphenethylamine (MEPEA) and *N*,α-diethylphenethylamine (DEPEA), were synthesized using standard organic

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chemical reactions and techniques as follows. α-Ethylphenethylamine (AEPEA) was prepared by reductive amination of 1-phenyl-2-butanone (TCI America Research Chemicals, Portland OR) using the method of Gonzalez-Sabın et al. (2002). The distilled base was then converted to the HCl salt in acetonitrile-ether. *N*-Methyl-α-ethylphenethylamine (MEPEA) was prepared in two steps by first *N*-formylation of AEPEA with ethyl formate at 135°C for18 hours in a pressure bottle. The resulting *N*-formyl derivative was distilled and then reduced to MEPEA with Vitride in refluxing toluene. The distilled base was then converted to the oxalate salt in acetonitrile. *N*,α–Diethylphenethylamine (DEPEA) was synthesized in two steps by *N*-acetylation of AEPEA with acetic anhydride in a chloroform-saturated sodium bicarbonate two-phase system followed by reduction of the resulting *N*-acetyl derivative with lithium aluminum hydride in tetrahydrofuran. The distilled base was converted to the HCl salt in acetone-ether. Each compound was fully characterized with appropriate high-resolution mass spectral and 400 MHz NMR analyses, and with appropriate combustion analyses for carbon, hydrogen, and nitrogen. Each compound was chromatographically homogenous by thin layer chromatography. Chemical purity for each compound was estimated to be greater than 98%. The corresponding α-methyl comparator compound (*S*)-amphetamine sulfate (amphetamine) was obtained from the Pharmacy at the National Institute on Drug Abuse (NIDA), Intramural Research Program (IRP), in Baltimore, MD. [^{3}H]Methyl-4-phenylpyridinium ([^{3}H]MPP$^+$; 80 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA) while [^{3}H]neurotransmitters (30-50 Ci/mmol) were purchased from Perkin Elmer (Shelton, CT, USA). All other chemicals and reagents were acquired from Sigma-Aldrich (St Louis, MO, USA) unless otherwise noted. Drugs for the *in vivo* experiments were dissolved in sterile saline and doses are expressed as the salts.

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Animals. Male Sprague-Dawley rats were used for all experiments as described in detail below. All procedures were approved by the Animal Care and Use Committee of the NIDA IRP and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. Animals were housed in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

***In Vitro* Transporter Assays.** Twenty-eight male Sprague-Dawley rats (Charles River, Kingston, NY, USA) weighing 250-300 g were used for the synaptosome assays. Rats were group-housed with free access to food and water, under a 12 h light/dark cycle with lights on at 0700 h. Rats were euthanized by CO_2 narcosis, and synaptosomes were prepared from brains using standard procedures (Rothman et al, 2003). Transporter uptake and release assays were performed as described previously (Solis et al., 2017). In brief, synaptosomes were prepared from caudate tissue for dopamine transporter (DAT) assays, and from whole brain minus caudate and cerebellum for norepinephrine transporter (NET) and serotonin (5-HT) transporter (SERT) assays.

For uptake inhibition assays, 5 nM [^{3}H]dopamine, [^{3}H]norepinephrine, or [^{3}H]5-HT was used for DAT, NET, or SERT assays respectively. To optimize uptake for a single transporter, unlabeled blockers were included to prevent the uptake of [^{3}H]transmitter by competing transporters. Uptake inhibition was initiated by incubating synaptosomes with various doses of test compound and [^{3}H]transmitter in Krebs-phosphate buffer. Uptake assays were terminated by rapid vacuum filtration and retained radioactivity was quantified with liquid scintillation counting (Baumann et al, 2013).

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For release assays, 9 nM [^{3}H]MPP+ was used as the radiolabeled substrate for DAT and NET, whereas 5 nM [^{3}H]5-HT was used for SERT. All buffers used in the release assay contained 1 µM reserpine to block vesicular uptake of substrates. The selectivity of release assays was optimized for a single transporter by including unlabeled blockers to prevent the uptake of [^{3}H]MPP+ or [^{3}H]5-HT by competing transporters. Synaptosomes were preloaded with radiolabeled substrate in Krebs-phosphate buffer for 1 h to reach steady state. Release assays were initiated by incubating preloaded synaptosomes with various concentrations of the test drug. Release was terminated by vacuum filtration and retained radioactivity was quantified by liquid scintillation counting. For substrate reversal experiments, the effects of AEPEA and MEPEA on transporter-mediated release were examined as described above, in the presence or absence of 1 nM 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine (GBR12909) for DAT assays or 8 nM desipramine for NET assays. Effects of test drugs on release were expressed as a percent of maximal release, with maximal release (i.e., 100% E_{max}) defined as the release produced by tyramine at doses that evoke the efflux of all 'releasable' tritium by synaptosomes (10 µM tyramine for DAT and NET assay conditions, and 100 µM tyramine for SERT assay conditions).

Effects of test drugs on uptake inhibition and release were analyzed by nonlinear regression using GraphPad Prism 8 (GraphPad Scientific, San Diego, CA, USA). Dose–response values for the uptake inhibition and release were fit to the equation, $Y(x) = Y_{min}+(Y_{max} – Y_{min}) / (1+ 10\exp[(\log P_{50} – \log x)] \times n)$, where x is the concentration of the compound tested, $Y(x)$ is the response measured, Y_{max} is the maximal response, P_{50} is either IC_{50} (the concentration that yields half-maximal uptake inhibition response) or EC_{50} (the concentration that yields half-

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maximal release), and *n* is the Hill slope parameter. We employed the "EC_{50} shift test" in Prism to statistically evaluate the possibility of significant differences in potency across the drugs. Briefly, the EC_{50} shift compares dose-response curves for a series of drugs, and can be used to test the null hypothesis that all drugs are equipotent (i.e., the EC_{50} ratio equals 1). When significant differences in drug potency are observed, the null hypothesis is rejected, and supporting F scores and p values are given.

Receptorome Screening. AEPEA, MEPEA, DEPEA, and amphetamine were submitted to the psychoactive drug screening program (PDSP) program of the National Institute on Mental Health (NIMH) and evaluated for binding affinity at a variety of human G protein-coupled receptors (GPCRs) according to established protocols (Besnard et al., 2012; https://pdsp.unc.edu/pdspweb/content/UNC-CH%20Protocol%20Book.pdf). Compounds were first screened at a fixed concentration of 10 μM to assess inhibition of receptor binding. In those instances where binding was inhibited by more than 50% at 10 μM, full dose-effect functions were obtained, and K_i values were calculated by nonlinear regression using the Cheng-Prusoff equation.

***In Vivo* Biotelemetry.** Five adult male Sprague-Dawley rats (Charles River, Kingston, NY, USA) were used as subjects for the telemetry experiments. Rats were purchased by Data Sciences International (DSI, St. Paul, MN, USA) and received surgically implanted HD-S10 biotelemetry transmitters. For the surgery, the rats were anesthetized with isoflurane and the abdominal cavity was opened. The descending aorta was isolated and the catheter from the transmitter was inserted into the aorta and glued in place. The abdominal muscles and skin were then sutured to close the incision. Rats were treated with subcutaneous (s.c.) meloxicam

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following surgery. After recovery at DSI, the rats were shipped to the NIDA IRP in Baltimore, MD, and underwent a 7-day quarantine period.

Following release from quarantine, the rats were individually housed in a temperature (22.2 ± 1.1 ^{o}C) and humidity (45 ± 10%) controlled vivarium on a 12 h reverse light-dark cycle (lights off at 0700) with free access to water. Food was restricted to maintain a constant or slowly increasing weight of approximately 400-500 g over the course of the experiment. The rats were subsequently adapted to the experimental chambers and injection procedure over a period of 3-4 weeks. Each weekday, rats were transported from the vivarium to a testing room where the food and water were removed from the home cage, and the entire home cage was placed on top of a telemetry receiver (RPC-001, Data Sciences) inside a small acoustical chamber (BRS/LVE, Laurel, MD, USA). Transmitters were turned on by placing a magnet near the abdomen of the rat. The chambers were then closed, and experimental parameters were monitored for 3 h. At the end of the session, the transmitters were turned off by again placing a magnet near the abdomen of the rats, water and food were returned to the home cages, and the rats were returned to the vivarium housing room. Once experimental parameters were stable from day to day, injections of saline were given s.c. twice per week (typically on Tuesdays and Fridays) 5 min prior to the rats being placed in the experimental chamber. Once experimental parameters were again stable following saline injections, drug or saline injection procedures began.

Dose-effect determinations for AEPEA (1 – 10 mg/kg), MEPEA (1 – 10 mg/kg), DEPEA (1 – 10 mg/kg) and amphetamine (0.1 – 3 mg/kg) were determined first. Drugs were tested no more frequently than twice per week, typically Tuesday and Friday. Order for the dose-effect

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testing was non-systematic, although all rats were typically tested with the same drug and dose on any given test day to simplify drug preparation and administration. All drugs were administered s.c. 5 min prior to placement of the cages in the experimental chambers. Saline was tested every 2-3 weeks and responses following saline were stable over the testing period. Rats were 2 to 9 months of age over the course of the experiment. Rats for this study had been previously exposed to BMPEA, prazosin and chlorisondamine, but were allowed at least 3 weeks washout prior to testing (Schindler et al., 2019).

Data from the transmitters was polled for 10 sec every min and these 1-min readings were used to construct time-course profiles and calculate mean effects over the 3 h session for statistical analysis. The transmitters supplied readings for BP, heart rate (HR, derived from the BP signal), core body temperature and motor activity. Activity was measured continuously by tracking the strength of the transmitter radio signal as the rat moved about its home cage, but this measure does not have any units. Data for AEPEA time course data were subject to a mixed-effects analysis, whereas the data for each drug and measurement in the dose-effect study were subject to analysis-of-variance (ANOVA) with follow-up tests using the Dunnett's Multiple Comparison Test that can compare drug effects to control (GraphPad Prism, Version 8). ED_{50} values for the effects of drugs on BP were calculated using nonlinear regression, and these potency values were compared using the EC_{50} shift test in Prism.

Results

Transporter Uptake and Release Assays. Figure 2 depicts the dose-response curves for inhibition of [^{3}H]neurotransmitter uptake and stimulation of [^{3}H]MPP^+ efflux (i.e., release) at

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DAT and NET. None of the compounds showed measurable ability to inhibit uptake or stimulate release at SERT, for doses up to 10 μM (data not shown). Table 1 summarizes the IC_{50} values for uptake inhibition, and the EC_{50} and $\%E_{max}$ values for release, at DAT and NET. Amphetamine was the most potent uptake inhibitor at DAT (IC_{50}=122 nM) and NET (IC_{50}=69 nM). DEPEA was 5-fold less potent than amphetamine at DAT, whereas the other compounds were even weaker at DAT, with IC_{50} values > 1 μM. EC_{50} shift analysis showed that amphetamine was significantly more potent at DAT inhibition than all PEA analogs ($F_{3,89}$ = 578, $p < 0.0001$), whereas DEPEA was more potent than AEPEA and MEPEA in this regard ($F_{2,66}$ = 256, $p < 0.0001$). All of the PEA analogs had IC_{50} values for NET inhibition that were 6- to 8-fold less potent than amphetamine. EC_{50} shift analysis demonstrated that amphetamine was significantly more potent at NET inhibition than all PEA analogs ($F_{3,89}$ = 62.7, $p < 0.0001$), but potencies for AEPEA, MEPEA, and DEPEA to inhibit NET did not differ from each other.

As expected, amphetamine was a fully efficacious substrate-type releasing agent with high potency at DAT (EC_{50}=5 nM) and NET (EC_{50}=8 nM). AEPEA and MEPEA were much less potent than amphetamine as releasers at DAT, but were approximately equipotent with each other at this transporter. It is noteworthy that DEPEA was a weak partial releaser at DAT, achieving only 40% of the maximal release response (i.e., partial agonist effect). EC_{50} shift analysis showed that amphetamine was significantly more potent as a releaser at DAT when compared to PEA compounds ($F_{3,89}$ = 374, $p < 0.0001$), while DEPEA was significantly less potent than AEPEA and MEPEA in this regard ($F_{2,57}$ = 158, $p < 0.0001$). All of the PEA compounds were efficacious substrate-type releasers at NET. EC_{50} shift analysis demonstrated that amphetamine was a more potent releaser at NET when compared to PEA compounds ($F_{3,89}$ = 96.2, $p < 0.0001$),

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and DEPEA was less potent than AEPEA and MEPEA ($F_{2,66}$ = 29.6, p < 0.0001). In general, AEPEA and MEPEA displayed greater potency as releasers at NET (EC_{50}= 80 nM and 58 nM) when compared to DAT (EC_{50}= 273 and 179 nM).

In order to explore the mechanism of [^{3}H]neurotransmitter efflux produced by the efficacious releasers AEPEA and MEPEA, we carried out "substrate reversal" studies. These experiments involved testing the ability of selective uptake blockers to antagonize the releasing actions of the drugs. Figure 3 illustrates the effects of the selective DAT uptake inhibitor GBR12909 (1 nM) or the selective NET uptake inhibitor desipramine (8 nM) on releasing activity produced by AEPEA and MEPEA. The results show that GBR12909 produced a parallel rightward shift in the DAT release curves for AEPEA and MEPEA, suggesting that both drugs interact at the orthosteric site on DAT to elicit their releasing actions. An EC_{50} shift analysis confirmed that GBR12909 significantly reduced the potency of AEPEA ($F_{1,43}$ = 1875, p < 0.0001) and MEPEA ($F_{1,43}$ = 504, p<0.0001) at DAT. For the NET substrate reversal experiments, desipramine shifted the NET release curves for AEPEA and MEPEA to the right, demonstrating that these drugs interact at NET sites. The EC_{50} shift results confirmed that desipramine significantly reduced the potency of AEPEA ($F_{1,43}$ = 64, p<0.0001) and MEPEA ($F_{1,43}$ = 109, p < 0.0001) at NET.

Receptorome Screening. Table 2 presents the results for AEPEA, MEPEA and DEPEA in the human GPCR screening in comparison to amphetamine. In general, the PEA analogs had little activity at GPCRs when tested at a 10 μM concentration (see receptors listed in footnote of Table 2). Specific exceptions included the 5-HT_{1A} receptor where MEPEA and DEPEA had low μM affinities (1,966-1,588 nM) and the alpha2 receptor subtypes where all three PEA analogs

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showed affinities in the range of their transporter releasing potency (411-2,320 nM). DEPEA showed activity at DAT and NET (108-124 nM), whereas AEPEA and MEPEA did not. AEPEA and DEPEA had low μM affinities to inhibit binding to the Sigma-2 site, while AEPEA and MEPEA had low to mid μM affinities to inhibit binding at the histamine H1 site. Amphetamine showed activity at the alpha receptor subtypes and also at the NET, but interestingly, not at the DAT. While this latter finding seems counterintuitive, previous studies demonstrate that amphetamine displays weak ability to displace high-affinity phenyltropane analogs at monoamine transporters (Eshleman et al., 1999, 2017; Rothman et al., 1999).

Biotelemetry in Rats. Rats quickly adapted to the experimental injection procedure, and results for the control conditions remained relatively stable throughout testing. Figure 4 depicts representative time-course effects of saline or AEPEA injection on BP, HR, activity, and body temperature. In saline-treated rats, BP rapidly decreased and stabilized over the 3 h session, whereas AEPEA produced time- ($F_{17,338} = 14.9$, $p < 0.0001$) and dose-related ($F_{4,20} = 18.0$, $p < 0.0001$) elevations in BP that were maintained throughout the session. The hypertensive effect of AEPEA was significantly greater than saline at all doses (Dunnett's $p < 0.05$). The HR response to AEPEA displayed significant effects of time ($F_{17,338} = 7.3$, $p < 0.0001$) but not dose ($F_{4,20} = 2.3$, $p = 0.098$), even though the higher doses of AEPEA caused modest tachycardia towards the end of the session. AEPEA produced small increases in motor activity at the beginning of the session which were significant with respect to time ($F_{17,338} = 17.1$, $p<0.0001$) but not dose ($F_{4,20} = 1.7$, $p = 0.1905$). Core body temperature was not significantly altered by AEPEA administration ($F_{4,20} = 1.7$, $p = 0.1922$), despite a hypothermic response at the highest dose administered.

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The time-course data from Figure 4 demonstrated that BP effects of AEPEA lasted for most of the 3 h session, and the effects of MEPEA and DEPEA followed similar time courses (data not shown). Given this information, we examined dose-response relationships for the averaged responses over the 3 h sessions. Figure 5 shows the effects of amphetamine and the PEA analogs on BP, HR, locomotor activity, and body temperature averaged over the full 3 h session. As expected, amphetamine produced dose-dependent increases in BP ($F_{4,24} = 27.7$, $p < 0.0001$) and HR ($F_{4,24} = 9.4$, $p = 0.0002$), with the highest 3 doses (0.3 – 3.0 mg/kg) producing increases in BP and HR significantly above saline control. Amphetamine also produced increases in locomotor activity ($F_{4,24} = 5.4$, $p = 0.0041$), although those effects were not dose-dependent. Activity increased as dose increased up to 1 mg/kg, which was significantly above saline levels. However, at the 3 mg/kg dose, activity decreased when compared to the 1 mg/kg effect. This apparent suppression of activity most likely reflects an increase in stereotypy that is not measured by the telemetry devices. At the doses tested, amphetamine did not significantly affect body temperature.

Similar to amphetamine, all three PEA analogs increased BP in a dose-dependent manner (AEPEA $F_{4,24} = 28.4$, $p < 0.0001$; MEPEA $F_{3,19} = 17.2$, $p < 0.0001$; DEPEA $F_{3,19} = 10.6$, $p = 0.0004$), with higher doses of each drug producing increases in BP significantly above saline control. The maximal effects of each drug on BP were similar. When only the doses inducing maximal effects were compared by one-way ANOVA, the overall effect was significant ($F_{3,16} = 3.5$, $p = 0.04$), however comparisons between the drugs (Tukey) failed to show any significant differences between any of the PEA analogs and amphetamine. MEPEA and DEPEA also produced dose-dependent increases in HR (MEPEA $F_{3,19} = 6.6$, $p < 0.01$; DEPEA $F_{3,19} = 8.1$, $p =$

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0.0017), with the effects of each drug at the highest dose tested being significantly above saline control. The effects of AEPEA ($F_{4,24}$ = 8.6, p = .0003) were more complicated, with HR increasing following treatment doses up to 3 mg/kg, but then returning toward saline levels at 10 mg/kg. For both their effects on BP and HR, the PEA analogs appeared to be approximately 10 times less potent than amphetamine. Like with BP, the maximal effects of each drug were similar ($F_{3,16}$ = 2.1, p = 0.14).

Because all of the test drugs produced dose-dependent increases in BP, ED_{50} values were calculated based on the maximal effect of amphetamine compared to saline. The calculated ED_{50} for amphetamine was 0.31 mg/kg (95% CI = 0.17 – 0.48 mg/kg). The ED_{50}'s for the PEA analogs were from 8- to 14-fold greater than that of amphetamine, and the 95% CI's for the PEA analogs did not overlap with those of amphetamine (AEPEA ED_{50} = 2.54 mg/kg, 95% CI = 1.89 – 3.53 mg/kg; DEPEA ED_{50} = 3.74 mg/kg, CI = 2.33 – 6.71 mg/kg; MEPEA ED_{50} = 4.47 mg/kg, CI = 2.80-9.02 mg/kg). Moreover, an EC_{50} shift analysis confirmed that the potency of amphetamine to induce increases in BP was significantly greater than the potencies of the PEA compounds ($F_{3,56}$ = 13.3, p < 0.0001), though the effects of AEPEA, MEPEA, and DEPEA on BP did not differ from each other. Taken together, these results support the conclusion that the PEA analogs are generally 10 times less potent than amphetamine at increasing BP.

Similar to amphetamine, MEPEA and DEPEA produced significant increases in locomotor activity (MEPEA $F_{3,19}$ = 7.4, p = 0.0025; DEPEA $F_{3,19}$ = 10.9, p = 0.0004), with the increases at 10 mg/kg being significantly above saline controls. In contrast to amphetamine, there was no downturn in activity at the highest doses of MEPEA or DEPEA, though doses above 10 mg/kg were not tested. For their effects on locomotor activity, MEPEA and DEPEA were at least 10

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times less potent than amphetamine. There was a trend for AEPEA to also produce small dose-related increases in activity ($F_{4,24}$ = 3.1, p = 0.0376), however, follow-up tests failed to reveal any significant changes from saline control at any dose tested. Unlike with BP and HR, the maximal effects for the drugs on locomotor activity were different for the dose range tested. When the maximal effects were compared ($F_{3,16}$ = 4.1, p = 0.02), the effects of AEPEA were significantly different from amphetamine. None of the PEA analogs produced significant effects on body temperature.

Discussion

PEA analogs continue to be found in dietary supplements, despite being banned by the FDA (Cohen et al., 2014; Elsohly et al., 2015; Zhao et al., 2018). Here, we studied compounds that possess an ethyl group at the α-position carbon, in contrast to amphetamine, which has a methyl group at this position. Our results show that compounds found in nutritional supplements can have effects that mimic those produced by the abused psychostimulant amphetamine. Like amphetamine, all three of the structurally related PEAs produced statistically significant elevations in BP, while MEPEA and DEPEA also increased HR. MEPEA and DEPEA produced increases in locomotor activity similar to amphetamine. In general, the PEAs were about 10 times less potent than amphetamine. If supplement products are taken orally according the manufactures recommendations, the amount of DEPEA ingested in these nutritional supplements would be 35–45 mg/serving (Cohen et al., 2014; ElSohly et al., 2015). In humans, the threshold oral dose of amphetamine that can be discriminated is 5 mg (Chait et al., 1985) whereas amphetamine increases in BP and HR at oral doses as low as 7.5 – 10 mg

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(Brauer and de Wit, 1996; Harvanko et al., 2016; Kelly et al., 2006). If the relative potency estimates for PEA analogs observed here for s.c. administration in rats hold for oral consumption in humans, a single serving of the supplement containing one of the compounds may be sufficient to produce similar subjective effects to amphetamine, and as little as two servings may produce cardiovascular effects. Thus, it is possible for an individual to use these supplements in amounts that could produce amphetamine-like effects, including mood stimulation and cardiovascular complications.

While the PEAs all produced similar effects on BP with comparable potencies, there were substantial differences in their other *in vivo* effects. DEPEA appeared to produce larger and more potent effects on HR. MEPEA may produce larger HR effects at higher doses, but the effect of AEPEA appeared to peak at 3 mg/kg. Unlike DEPEA and MEPEA, AEPEA had minimal effects on locomotor activity at the dose range tested. These differences in effects on activity may translate into differences for other behavioral effects such as drug self-administration, although additional research would be needed to confirm this. None of the drugs, including amphetamine, had significant effects on body temperature at the doses tested.

As expected, in the release assays, amphetamine was a potent efficacious releaser at both DAT and NET in rat brain synaptosomes (Rothman and Baumann, 2003). AEPEA and MEPEA were also releasers, but they were less potent than amphetamine and displayed selectivity for the NET over the DAT. The releasing abilities of AEPEA and MEPEA were antagonized by inhibitors of DAT and NET, implicating monoamine transporters in their effects. However, any definitive conclusions about the molecular mechanism of action for PEA compounds will require the replication of our findings using transfected cells expressing pure

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populations of DAT and NET. Despite the similarity of effects produced by AEPEA and MEPEA in the *in vitro* assays, MEPEA significantly increased locomotor activity *in vivo* while AEPEA did not. DEPEA displayed fully efficacious release at NET but only weak partial releasing activity at DAT (i.e., 40% of E_{max}). The precise molecular underpinnings of DEPEA's partial releasing activity at DAT is not known, but we speculate that uptake inhibition at DAT is the predominant effect of DEPEA at this transporter. Like the other PEA analogs, DEPEA was slightly more potent at NET than DAT. In general, DEPEA was less potent than AEPEA or MEPEA at both DAT and NET *in vitro*, but was at least equipotent to both MEPEA and AEPEA on BP and HR *in vivo*, and clearly more potent than AEPEA on locomotor activity. This pattern of results suggests that *in vivo* effects of these PEA analogs are influenced by factors other than uptake inhibition or release properties at DAT and NET, such as pharmacokinetic factors or actions at other binding sites.

The α-ethyl substituted PEA analogs failed to interact (i.e., K_i > 10 μM) with most of the GPCRs studied (footnote of Table 2). Further, for the few receptors they bind to, the compounds do not show potency relationships that mirrored any of the *in vivo* effects observed. For example, at the 5-HT1A receptor, amphetamine was equipotent to MEPEA and DEPEA, but amphetamine was much more potent than both of these compounds in all measures of *in vivo* activity. At the Alpha2B receptor, MEPEA appears to have twice the potency of DEPEA, however MEPEA displayed equivalent or less potency than DEPEA for the *in vivo* tests. Therefore, it does not appear as though activity at these other receptors is responsible for the observed *in vivo* effects. Given the fact that amphetamine showed clearly greater potency than the PEA analogs on the DAT and NET release assays, and the same potency relationship held for the *in vivo* tests, actions at DAT and NET seem most likely to

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mediate the *in vivo* effects, although we cannot rule out that pharmacokinetic effects may be responsible for some of the observed differences.

In a previous study (Schindler et al., 2019), we examined the pharmacological effects of a PEA analog in which the α-methyl group of amphetamine was moved to the β-position to form BMPEA. This change reduced the potency of BMPEA to increase BP about 30-fold when compared to amphetamine, and at the doses tested, BMPEA did not increase HR or activity. BMPEA maintained substrate activity at DAT and NET, but was more potent at NET than DAT. By comparison, in the experiments reported here, replacing the α-methyl group of amphetamine and methamphetamine with an α-ethyl group to yield AEPEA and MEPEA led to a reduction in potency of only about 10-fold for the *in vivo* effects, and also maintained releaser efficacy. Importantly, the α-ethyl compounds seem to show a preference for the NET over the DAT, similar to the profile of BMPEA. Replacing the amine methyl group of MEPEA with an ethyl group to produce DEPEA markedly reduced the efficacy of release at DAT, and this observation agrees with findings reported for amphetamine analogs with extended *N*-alkyl chain length (see, Solis et al., 2017). Overall, replacing the α-methyl group of amphetamine with an ethyl group maintains the amphetamine-like effects of PEA analogs more effectively than moving the methyl group to the β-position for BMPEA. Thus, α-ethyl-substituted analogs may also have abuse potential similar to amphetamine.

While not directly studied here, it is likely that locomotor activating effects of α-ethyl compounds are related to their effects at the DAT. Activity at DAT is known to be primarily responsible for the locomotor activating effects of a variety of stimulants, including cocaine amphetamine, and related analogs (Rothman and Baumann, 2003; Uhl et al., 2002; Zolkowska

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et al., 2009). Activity at DAT is also known to be primarily responsible for maintaining drug self-administration (Howell and Kimmel, 2008; Uhl et al., 2002; Wise and Bozarth, 1985), and our present findings suggest that at least some of the α-ethyl-substituted analogs will be self-administered and have abuse potential in humans. Likewise, previous work with other psychomotor stimulants has shown that central or peripheral noradrenergic systems are primarily responsible for their cardiovascular effects. For example, increases in BP produced by BMPEA were blocked by the alpha-adrenergic antagonist prazosin (Schindler et al., 2019). Beta-adrenergic antagonists can block the HR increasing effects of psychomotor stimulants, including some amphetamines (Schindler et al. 1992b, Schindler et al., 2014), cathinones (Alsufyani and Docherty, 2015; Schindler et al., 2016; Varner et al., 2013) and cocaine (Schindler et al., 1992a; Branch and Knuepfer, 1992). With respect to cocaine, while pretreatment with beta antagonists can antagonize tachycardia, some studies show that such antagonists can exacerbate the hypertensive effects (Branch and Knuepfer, 1992; Schindler et al., 1992a).

Limitations of the current study include the measurement of only BP and HR as indices of cardiac function. While increases in both BP and HR would be expected to increase the workload on the heart and lead to potential adverse effects, the assessment of other relevant endpoints such as cardiac output, cardiac electrical activity, and contractility (Mladenka et al., 2018) would produce a more complete picture of the effects of α-ethyl-substituted PEAs on cardiac function. Nevertheless, the effects shown here do raise concern for the presence of these substances in dietary supplements, particularly if they are not listed on the ingredients label. In addition, the *in vivo* effects reported here were observed following passive administration of the compounds. In humans, these substances would be self-administered,

which could influence the effects of the compounds on cardiac function, as well as other measures (Graziella De Montis et al., 1998; Hemby et al., 1997).

In summary, similar to amphetamine, AEPEA and MEPEA function as efficacious neurotransmitter releasers at DAT and NET, but with greater potency at NET relative to DAT. By contrast, DEPEA displays efficacious release at NET but low-efficacy partial release at DAT. Despite these minor differences from amphetamine in neurochemical mechanism, all three α-ethyl PEA analogs increased BP similar to amphetamine, although at reduced potency. MEPEA and DEPEA also increased HR. As a result, these compounds could produce toxic effects if taken in large enough amounts. Like amphetamine, MEPEA and DEPEA increased locomotor activity, suggesting these agents could have central effects, including abuse potential in humans.

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Authorship Contributions

Participated in research design: Schindler, Baumann.

Conducted Experiments: Schindler, Thorndike, Partilla, Baumann

Contributed new reagents or analytic tools: Rice.

Performed data analysis: Schindler, Thorndike, Baumann.

Wrote or contributed to writing of the manuscript: Schindler, Partilla, Rice, Baumann

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Footnote

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Legends for Figures

Figure 1. Chemical structures of α-ethylphenethylamine (AEPEA), *N*-methyl-α-ethylphenethylamine (MEPEA) and *N*,α-diethylphenethylamine (DEPEA) as compared to amphetamine and methamphetamine.

Figure 2. Effects of amphetamine and PEA analogs on inhibition of uptake and stimulation of efflux (i.e., release) at DAT (upper panels) or NET (lower panels) in rat brain synaptosomes. For uptake assays, synaptosomes were incubated with different concentrations of test drugs in the presence of 5 nM [^{3}H]dopamine ([^{3}H]DA)or [^{3}H]norepinephrine ([^{3}H]NE). Data are mean ± SD, expressed as a percentage of transmitter uptake for N=3 experiments performed in triplicate. For release assays, synaptosomes were preloaded with 9 nM [^{3}H]MPP$^+$ then incubated with different concentrations of test drugs to evoke release via reverse transport. Data are mean ± SD, expressed as a percentage of [^{3}H]MPP$^+$ release for 3 experiments performed in triplicate.

Figure 3. Effects of GBR12909 or desipramine on drug-induced release of [^{3}H]MPP$^+$ at DAT (upper panels) or NET (lower panels). For substrate reversal assays, synaptosomes were preloaded with 9 nM [^{3}H]MPP$^+$, then test drugs were incubated with or without GBR12909 (1 nM) for DAT assays or desipramine (8 nM) for the NET assays. Data are mean ± SD expressed as a percentage of [^{3}H]MPP$^+$ release for 3 experiments performed in triplicate.

Figure 4. Time-course effects of AEPEA administration on blood pressure (BP), heart rate (HR), motor activity, and core body temperature. Male rats bearing biotelemetry transponders received s.c. injection of 1, 3, or 10 mg/kg AEPEA or its saline vehicle and were returned to their home cages. Five min later, cages were placed atop telemetric receivers. Data

were collected in 10-min epochs for 3 h. Data are expressed as mean ± SEM for 5 rats per group.

Figure 5. Dose-effect functions for amphetamine and PEA analogs on blood pressure (BP), heart rate (HR), motor activity, and core body temperature. Data represent mean values across the full 3 h session. Solid symbols indicate significant differences from the respective saline group. Data are mean ± SEM for 5 rats per group.

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TABLE 1

Effects of amphetamine and PEA analogs on the uptake of [^{3}H]neurotransmitters and on the release of [^{3}H]MPP+ at DAT or NET in rat brain synaptosomes

Data are mean ± SD for N=3 experiments performed in triplicate. % Emax is defined as % of maximal releasing response induced by 10 µM tyramine. DAT/NET ratio = (DAT IC_{50}^{-1})/(NET IC_{50}^{-1}); higher value indicates greater DAT selectivity.

Drug	Uptake inhibition at DAT [IC_{50} (nM]	Uptake inhibition at NET [IC_{50} (nM]	DAT/NET ratio
Amphetamine	122 ± 12	69 ± 9	0.56
AEPEA	3,366 ± 333	573 ± 78	0.16
MEPEA	2,248 ± 245	503 ± 95	0.22
DEPEA	510 ± 38	427 ± 60	0.84
	Release at DAT EC_{50} (nM) [%Emax)	Release at NET EC_{50} (nM) [%Emax)	
Amphetamine	5 ± 1 [102]	8 ± 1 [96]	1.6
AEPEA	273 ± 36 [101]	80 ± 17 [100]	0.29
MEPEA	179 ± 25 [101]	58 ± 12 [96]	0.32
DEPEA	604 ± 159 [43]	209 ± 35 [82]	0.35

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TABLE 2

G-Protein coupled receptorome screening for amphetamine and PEA analogs*

Data represent Ki (nM) values obtained from non-linear regression using the Cheng-Prusoff equation when inhibition was > 50%.

	AMPH	AEPEA	MEPEA	DEPEA
Serotonin				
5-HT1A	2625	>10,000	1,966	1,588
5-HT2B	971	>10,000	>10,000	>10,000
Norepinephrine				
Alpha2A	420	>10,000	>10,000	>10,000
Alpha2B	192	1362	411	1013
Alpha2C	171	1404	1805	2320
NET	31	>10,000	>10,000	108
Dopamine				
DAT	>10,000	>10,000	>10,000	124
Sigma				
Sigma 2	>10,000	>10,000	806	1352
Histamine				
H1	>10,000	2541	5800	>10,000

*Ki values for the following receptors were >10,000 nM for all 4 drugs tested: Serotonin receptors 5-HT1B, 5-HT1D, 5-HT1E, 5-HT2A, 5-HT2C, 5-HT3, 5-HT5A, 5-HT6, 5HT7 and SERT; Norepinephrine receptors Alpha1A, Alpha1B, Alpha1D, Beta1, Beta2 and Beta3; Dopamine D1, D2, D3, D4 and D5; Histamine receptors H2, H3, H4; Opioid receptors Mu, Kappa, Delta; Muscarinic receptors M1, M2, M3, M4, M5; and Sigma 1, PBR, BZP. The results for amphetamine (AMPH) were reported previously (Schindler et al., 2019).

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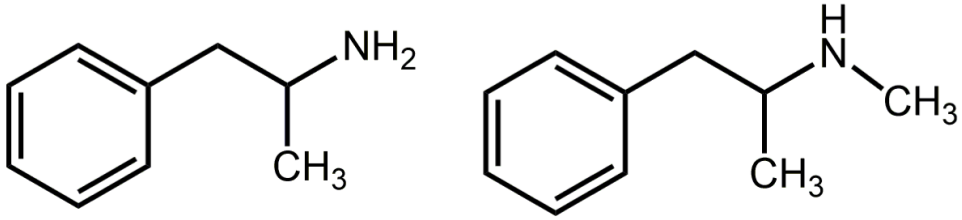
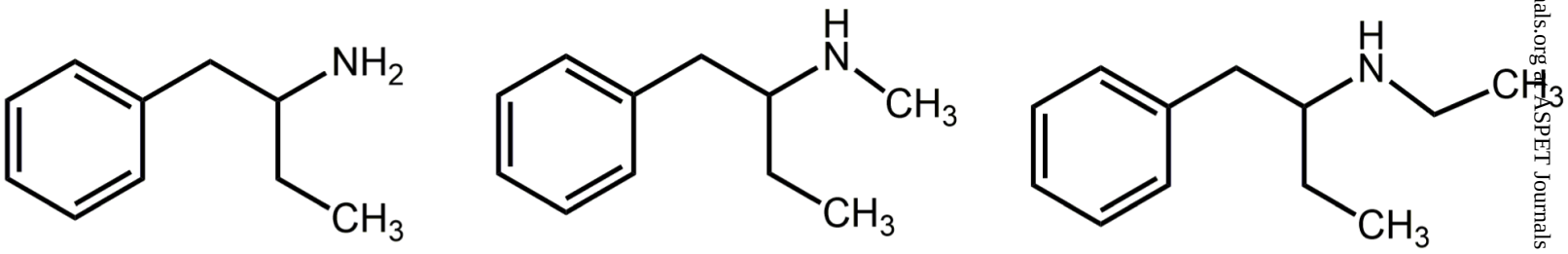


amphetamine **methamphetamine**



α-ethyl-PEA (AEPEA) ***N*-methyl-α-ethyl-PEA (MEPEA)** ***N*,α-diethyl-PEA (DEPEA)**

Figure 1

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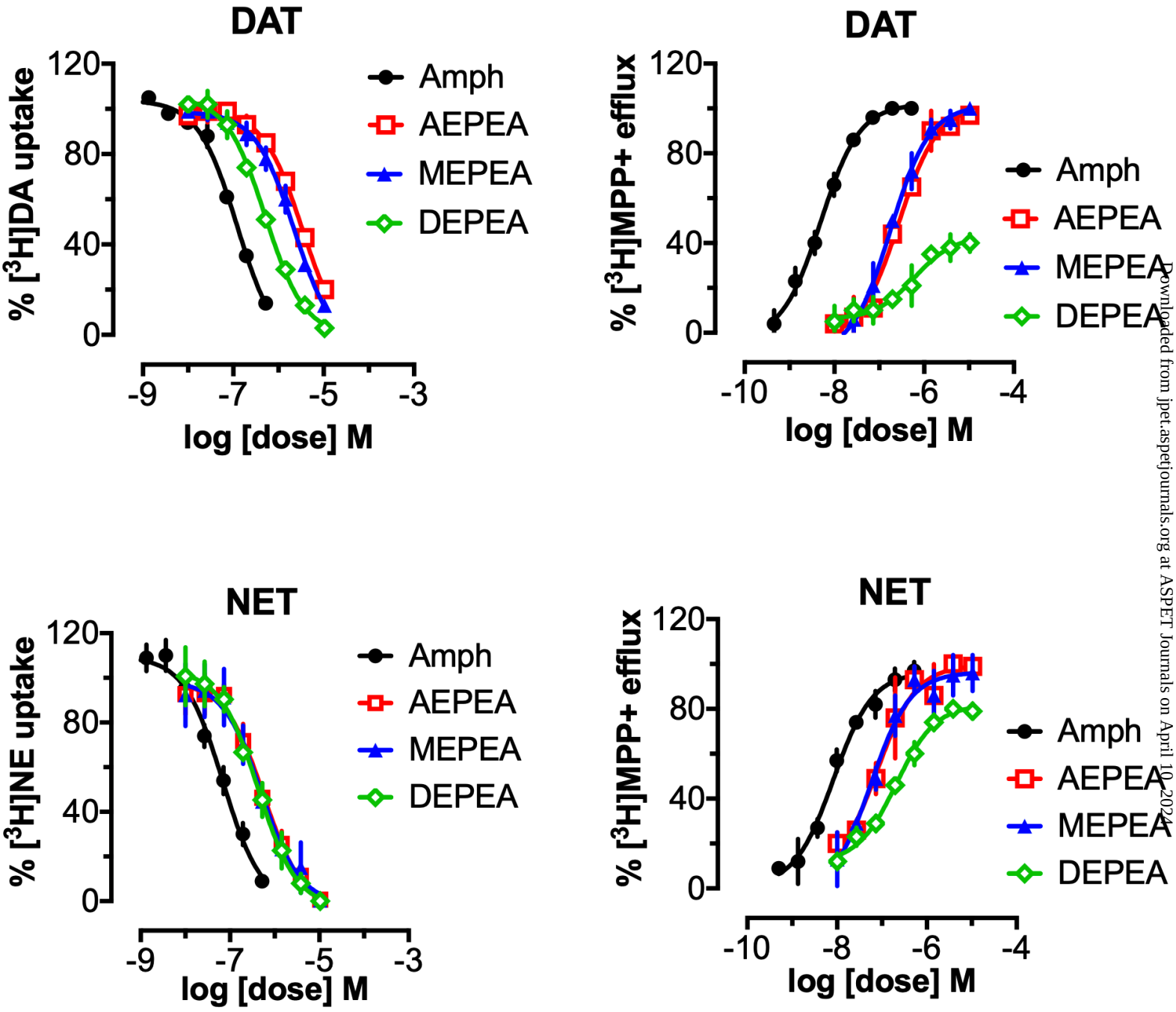

Figure 2

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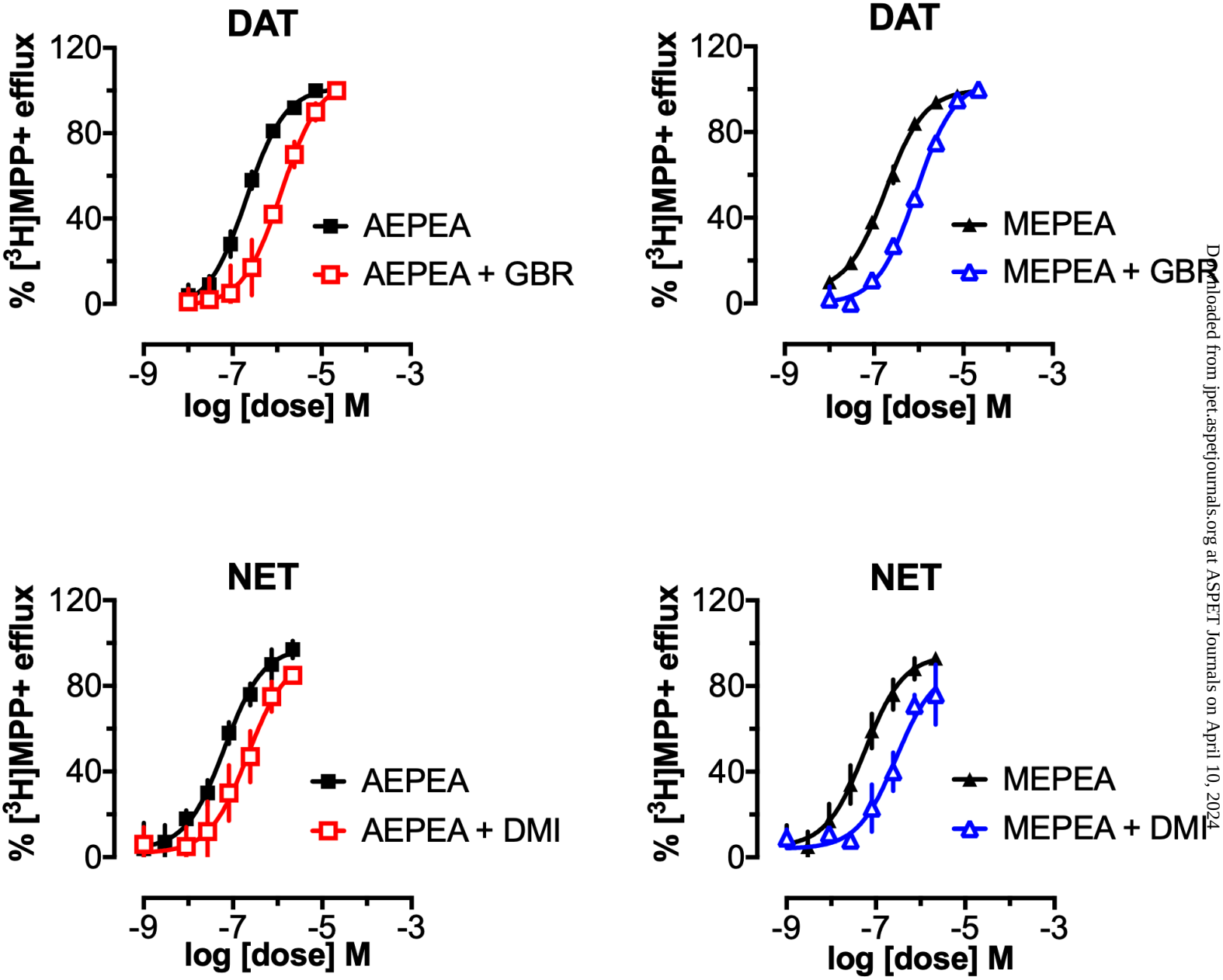

Figure 3

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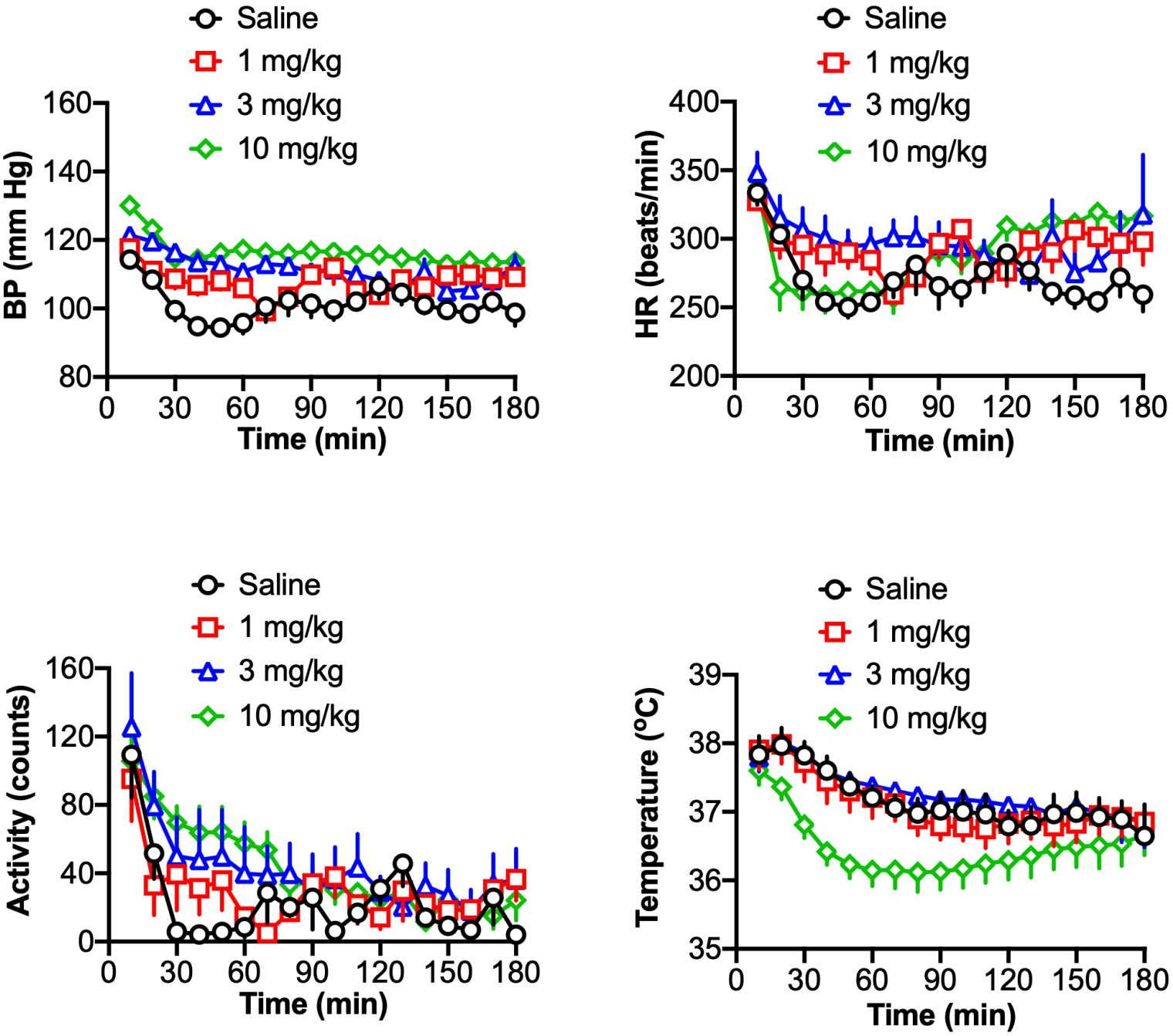

Figure 4

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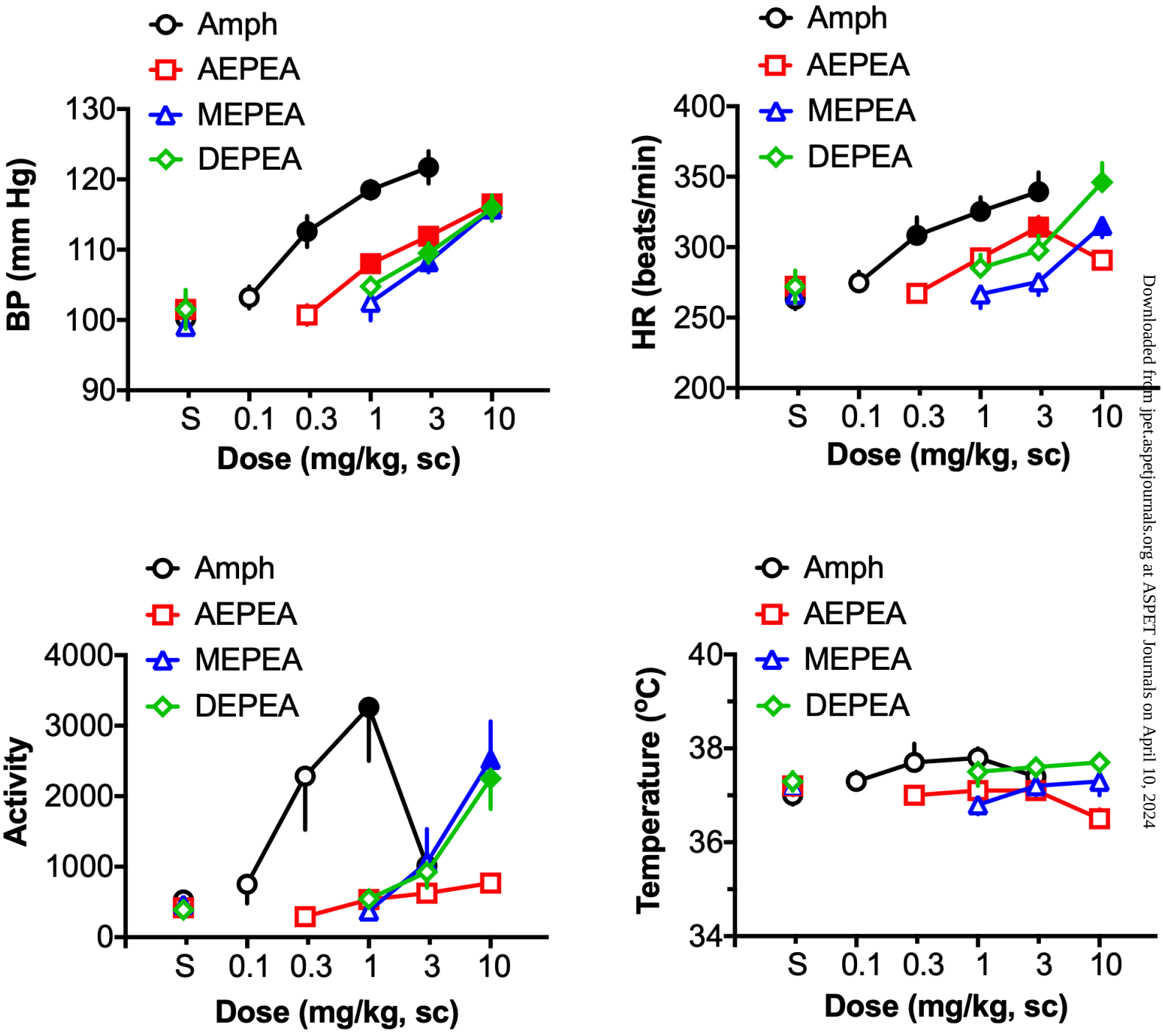

Figure 5