Vaccine-driven pharmacodynamic dissection and mitigation of fenethylline psychoactivity

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Fenethylline, also known by the trade name Captagon, is a synthetic psychoactive stimulant that has recently been linked to a substance-use disorder and ‘pharmacoterrorism’ in the Middle East1–4. Although fenethylline shares a common phenylethylamine core with other amphetamine-type stimulants, it also incorporates a covalently linked xanthine moiety into its parent structure5,6. These independently active pharmacophores are liberated during metabolism, resulting in the release of a structurally diverse chemical mixture into the central nervous system7–9. Although the psychoactive properties of fenethylline have been reported to differ from those of other synthetic stimulants, the in vivo chemical complexity it manifests upon ingestion has impeded efforts to unambiguously identify the specific species responsible for these effects10,11. Here we develop a ‘dissection through vaccination’ approach, called DISSECTIV, to mitigate the psychoactive effects of fenethylline and show that its rapid-onset and distinct psychoactive properties are facilitated by functional synergy between theophylline and amphetamine. Our results demonstrate that incremental vaccination against a single chemical species within a multi-component mixture can be used to uncover emergent properties arising from polypharmacological activity. We anticipate that DISSECTIV will be used to expose unidentified active chemical species and resolve pharmacodynamic interactions within other chemically complex systems, such as those found in counterfeit or illegal drug preparations, post-metabolic tissue samples and natural product extracts.

In the last decade, an increasing number of substance-use problems have been caused by synthetic psychoactive drugs, including amphetamine and methamphetamine12,15. Despite their predominance in the United States, these two drugs are not the most widely abused amphetamine analogues in every country14,15. In Saudi Arabia, it has been estimated that 40% of drug users between the age of 12 and 22 are addicted to fenethylline1. While fenethylline addiction has historically been regionally confined, increased production and global trafficking of counterfeit tablets from Syria have been implicated as a source of revenue for militant groups to fund operations16–18. Furthermore, as with the use of amphetamine and methamphetamine in prior conflicts, fenethylline has been identified as a source of pharmacological morale in battle5,12,18–20. The ultimate source of the psychoactive effects of fenethylline has historically been a debated subject, with some results supporting the position that the parent compound itself exhibits psychoactive effects distinct from those of other central stimulants, whereas others posit that it acts as merely an amphetamine prodrug19–21. Considering the additional effort that is required to synthesize fenethylline, its continued manufacture and use in resource-strained conflict areas provides support for the hypothesis that this drug exhibits exploitable psychostimulant effects that differ from amphetamine alone.

Therefore, we supposed that any platform developed to block the effects of fenethylline would be most useful if it could also unambiguously identify the chemical sources of the psychoactivity of this drug. A large body of literature supports the overall concept of vaccination for the treatment of drug abuse, where a small-molecule hapten–protein conjugate is delivered with the intention of generating antibodies that sequester the target drug in the periphery, preventing induction of central nervous system (CNS)-mediated effects22. A ‘dynamic’ variant of this general approach was recently developed to blunt heroin self-administration, for which a single chemically labile hapten was used to induce antibodies directed against multiple opioid metabolites23. These foundational studies inspired us to pursue a generalizable ‘dissection through vaccination’ method that could isolate the effects of single compounds within a structurally and pharmacologically diverse chemical system, such as that generated by fenethylline7–9. We termed the resulting approach ‘Determining the Identities of Species Supporting Expression of CNS-activity Through Incremental Vaccination’ (DISSECTIV) (Extended Data Fig. 1).

Our chemical strategy for synthesizing the parent fenethylline hapten was informed by the knowledge that fenethylline (1) generates metabolites 2–5, including amphetamine (2) and substituted-theophylline metabolites, which are further processed to theophylline (6) following oxidative metabolism by cytochrome P450 (CYP450) enzymes (Fig. 1a). We therefore installed our linker on the amphetamine-associated nitrogen that separates the two carbons where oxidation produces metabolites 2–5. Starting from 6, we first performed an N-alkylation with 1-chloro-2-iodoethane to generate 7, which was subjected to an in situ Finkelstein reaction and a second N-alkylation with 2 to generate 1. The final hapten species (FEN, 8) was generated from the condensation of 1 with glutaric anhydride with a 24% overall yield (Fig. 1b and Supplementary Methods). This hapten was conjugated to keyhole limpet haemocyanin (KLH) using sulfo-N-hydroxysuccinimide in phosphate buffered saline (PBS) to generate the immunogenic species for vaccination, termed FEN–KLH. A separate conjugation to bovine serum albumin (BSA) was carried out to generate FEN–BSA for in vitro analyses (Extended Data Fig. 2).

To prepare the vaccine formulation, FEN–KLH was combined with two adjuvants, alum and CpG 1826. Intraperitoneal administration of the vaccine to Swiss Webster mice on days 0, 14 and 28 generated robust antibody midpoint titres (Fig. 1c). Competitive surface plasmon resonance was then used to measure the relative binding strength of antibodies generated from FEN–KLH vaccination against fenethylline and its active metabolites. In these assay conditions, the binding of fenethylline was strongest, followed by theophylline, then amphetamine (Fig. 1d). Complementary use of an enzyme-linked immunosorbent assay to assess antibody specificity confirmed that antibodies within FEN–KLH serum were able to recognize the general structure of all three compounds (Fig. 1e). To further explore the functional antibody-binding profile of FEN–KLH in an immediately relevant model, we measured whether vaccination could alter the pharmacokinetics of fenethylline, theophylline and amphetamine in vivo, using liquid chromatography–mass spectrometry analysis to quantify drug concentrations (Extended Data Fig. 3). Notably, when the effect of vaccination on drug disposition was assessed over time after intraperitoneal
administration, FEN–KLH binding was indeed found to be sufficient to increase peak fenethylline, theophylline and amphetamine sequestration in the periphery due to the binding of drug-specific antibodies (Fig. 1f). As anticipated, this peripheral binding ultimately led to reductions in the rapid distribution of these compounds into the CNS (Fig. 1g).

We also assessed whether endophenotypes relevant to fenethylline abuse could be blocked by FEN–KLH in a number of animal models. When control animals were given fenethylline in an open-field, hyperlocomotor activity assay, a clear dose-dependent increase in locomotor activity was observed, and this effect was significantly diminished in FEN–KLH-vaccinated animals (Fig. 2a–c). Furthermore, we used an elevated plus maze (EPM) assay to measure anxiety-related behaviours following drug administration, as recent reports of fenethylline use in Syria have noted its apparent ability to induce hypervigilance, a behaviour that is associated with persistent anxiety states in humans.23,24. In this experiment, fenethylline reduced the time that control animals spent in the open arms of the apparatus, whereas no such change was found in animals that had been vaccinated with FEN–KLH (Fig. 2d, e).

In addition, because fenethylline demonstrates rewarding effects in human users, we assessed whether it could induce a conditioned place preference (CPP). We found that training with fenethylline could indeed induce CPP and observed that FEN–KLH showed a trend towards blockade of this effect, although efficacy was variable (Fig. 2f).

Importantly, vaccination with FEN–KLH was able to decrease the concentration in the periphery due to the binding of drug-specific antibodies (Fig. 1f). As anticipated, this peripheral binding ultimately led to reductions in the rapid distribution of these compounds into the CNS (Fig. 1g).

Because FEN–KLH demonstrated binding to fenethylline and its active metabolites, its blockade of stimulant effects could not be attributed to perturbation of one specific chemical species. Therefore, we used our DISSECTIV approach to identify the compound(s) supporting the overall psychoactivity of fenethylline. The presence of the
distinct psychoactive effects of fenethylline, as compared to amphetamine alone, imply that either the parent drug has unique activity or that the combined activities of the metabolites act together to produce an altered response\(^9,10\). Notably, the fenethylline structure itself demonstrated little binding at 10\(\mu\)M across a suite of 31 CNS targets, so we prioritized exploration of how active metabolites of fenethylline contribute to its unique psychoactive profile (Extended Data Table 1). Therefore, a theophylline hapten (THEO, \(9\)) and two amphetamine haptens (AMPH, \(10\); 1-A\(_1\), \(11\)) were generated\(^{25}\) (Fig. 3a and Supplementary Methods). Following formulation and immunization with these haptens, robust titres were observed for THEO–KLH and 1-A\(_1\)–KLH (Fig. 3b). While THEO–KLH serum readily bound theophylline and fenethylline, but not amphetamine, 1-A\(_1\)–KLH bound amphetamine and fenethylline, but not theophylline (Fig. 3c, d). The same pattern of binding activity was observed for \(in vivo\) serum measurements (Fig. 3e–g). By contrast, AMPH–KLH generated ineffective antibodies, despite its structural similarity to the previously reported hapten SMA–KLH\(^{26}\) (Extended Data Fig. 5). While THEO–KLH had a relatively minor impact on total distance travelled in the hyperlocomotor assay, 1-A\(_1\)–KLH vaccination substantially decreased fenethylline-induced activity (Fig. 3h, i). This indicated that amphetamine was a major component of the stimulant behaviour of fenethylline. Similarly, the activity of fenethylline in the EPM assays was found to be substantially blunted by 1-A\(_1\)–KLH, but not by THEO–KLH (Fig. 3j, k). The CPP data again showed a trend towards 1-A\(_1\)–KLH being slightly more effective than THEO–KLH, although the efficacy was variable, similar to FEN–KLH (Fig. 3l). Although the impact of vaccination with 1-A\(_1\)–KLH was more obvious across the entire set of behavioural testing, vaccination with THEO–KLH did appear to weakly blunt the behavioural effects of fenethylline overall, implying that theophylline has a supportive, rather than antagonistic, role in modulating these amphetamine-driven effects of fenethylline.

Notably, during further analysis of the hyperlocomotor behaviour induced by fenethylline, the onset of its stimulant activity was found to be more rapid than that of amphetamine alone (Fig. 4a). However, the penetration of amphetamine into brain tissue following release from fenethylline lags behind that of directly administered amphetamine (Fig. 4b). This disparity further implicated a potential polypharmacological basis for stimulant activity, and indeed, when otherwise ineffective doses of theophylline and amphetamine were administered together, the total locomotor response was potentiated (Fig. 4c). Furthermore, this synergistic stimulant effect was observed for other xanthine/phenethylamine combinations, opposing the earlier concept that coliboration of these two classes of drugs would uniformly improve the risk/benefit profile of fenethylline (Fig. 4d). These findings instead support the emergence of an altered subjective stimulant experience generated by coincident alteration of adenosinergic and dopaminergic signalling, thus providing a plausible explanation for why militant groups are motivated to expend additional effort to incorporate xanthines into counterfeit products, instead of amphetamine-type stimulants alone\(^{27,28}\).

Overall, by using FEN–KLH, THEO–KLH, and 1-A\(_1\)–KLH as chemical neuroscience tools in our DISSECTIV approach, we have reconciled previously conflicting data regarding the underlying pharmacology
driving the psychoactive profile of fenethylline. Its prominent stimulant features can be attributed to amphetamine, with synergistic support from theophylline, and no direct contributions from the parent drug molecule (Extended Data Table 2). These results have direct relevance to theophylline-binding haptens, including FEN and 1-A1, as efficacious molecule (Extended Data Table 2). These results have direct relevance to theophylline-binding haptens, including FEN and 1-A1, as efficacious

**Figure 4** Synergistic stimulant effects of theophylline and fenethylline support activity of fenethylline. a. Hyperlocomotor activity for fenethylline (50 mg kg\(^{-1}\)) or amphetamine (5 mg kg\(^{-1}\)) n = 10, repeated-measures two-way ANOVA (\(P_{\text{interaction}} = 0.2281; F_{1,32} = 1.49\)). b. Time-course of brain concentrations for amphetamine release due to fenethylline (50 mg kg\(^{-1}\)) or amphetamine (5 mg kg\(^{-1}\)) administration. n = 5, two-way ANOVA (\(P_{\text{interaction}} < 0.0001; F_{4,10} = 41.77; *P < 0.001\) versus amphetamine, Bonferroni correction). c. Total hyperlocomotion (90 min) for saline (n = 10, amphetamine (A, 1 mg kg\(^{-1}\), n = 10), theophylline (T, 8 mg kg\(^{-1}\), n = 11), or amphetamine + theophylline (A + T, 1 mg kg\(^{-1}\) + 8 mg kg\(^{-1}\), n = 11) Kruskal–Wallis test (\(P = 0.0014; \ast\ast\ast *P < 0.001\) versus A + T, \*P < 0.05 versus A + T, \*\*P < 0.01 versus A + T). Dunns correction). d. Total hyperlocomotion (90 min) due to saline (n = 10), methamphetamine (M, 1 mg kg\(^{-1}\), n = 5), caffeine (C, 8 mg kg\(^{-1}\), n = 5), methamphetamine + theophylline (M + T, 1 mg kg\(^{-1}\) + 8 mg kg\(^{-1}\), n = 5) or methamphetamine + caffeine (M + C1, mg kg\(^{-1}\) + 8 mg kg\(^{-1}\), n = 5). One-way ANOVA (\(P = 0.1177; F_{1,32} = 2.051; *P < 0.05\) versus M + T, Bonferroni correction). a, b, Data are shown as mean ± s.e.m. c, d, Data are shown as median with quartiles ±10–90% CI; \(+, \ast\ast\ast\) mean.

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**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** C.J.W. designed and carried out the chemistry, enzymelinked immunosorbent assays and in vivo behavioural experiments, analysed and interpreted the data and prepared the manuscript. B.Z. carried out and analysed the surface plasmon resonance experiments. K.D.J. oversaw design of the experiments, interpretation of the data and preparation of the manuscript.

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**Methods**

**Animals.** Animal studies were approved by TSRI's Institutional Care and Use Committee and carried out according to NIH guidelines. Male Swiss Webster mice (Taconic Biosciences, 6–8 weeks old) were used for all studies, and housed four per cage in a temperature-controlled (22 °C) vivarium on a reversed 12-h light cycle (21:00–09:00) with *ad libitum* access to food and water. Animals were randomly assigned to their experimental groups with stratification to use litters as controls. The experimenter was blinded to group identity during data processing, and behavioural results were automatically scored using AnyMAZE v4.99 (Stoelting Co.).

**Drugs.** Theophylline, amphetamine hemisulfate and methamphetamine hydrochloride (>95%, Sigma–Aldrich) were obtained commercially. Fenethylline (>95%) was synthesized and purified at TSRI. Amphetamine-d11, fenethylline-d3 (Lipomed) and theophylline-d9 (CDN Isotopes) were obtained commercially for use as liquid chromatography–mass spectrometry (LC–MS) internal standards. For in vivo studies, all drugs were dosed intraperitoneally at a volume load of 10 ml kg<sup>−1</sup> in bacteriostatic saline (0.9% w/v).

**Chemical analysis.** Details of chemical synthesis, characterization and relevant spectra are included in the Supplementary Methods.

**Hapten conjugation and vaccination.** Conjugation of haptons 8–11 to carrier proteins was performed as previously reported<sup>30</sup>. Each hapten–KLH conjugate was combined with alum (Invivogen) and CpG 1826 (Eurofins Genomics) and shaken for 20 min before injection. Animals were immunized by intraperitoneal injection on study days 0, 14 and 28 with a vaccine formulation containing 50 μg of hapten, 1 mg of alum and 50 μg of CpG 1826 in 125 μl of sterile-filtered PBS (pH 7.4).

**Antibody binding.** ELISA analyses were carried out as previously described<sup>15,30</sup>. Serum was pre-incubated with compound at room temperature across samples. Serum was pre-incubated with compound at room temperature for 30 min, the mixture was injected over the flow cell for 5 min, and dissociated chemically and dilution or anti-sera was performed to normalize baseline binding values. Groups were analysed for similarity of variance using Bartlett's test, and analysed using Prism 5.02 (GraphPad software), setting P < 0.05 as the critical value.

**Amphotericin brain tissue homogenization.** Brain tissue was homogenized in four volumes of H2O, and 1 ml was then placed into 1 ml dichloromethane (DCM) + 200 ng internal standard. This was centrifuged for 10 min at 10,000 r.p.m., and 60 μl were transferred to a LC–MS vial. Fenethylline brain samples were diluted into four volumes of H2O, homogenized and 1 ml was placed into 1 ml dichloromethane (DCM) + 200 ng internal standard. This was centrifuged for 2 h, the DCM was collected, centrifuged as above, dried under vacuum and then resuspended in 60 μl methanol. Theophylline brain tissue was homogenized in four volumes of H2O, and 1 ml + 200 ng internal standard was stirred for 2 h, centrifuged, dried and resuspended as above. Amphetamine brain tissue was homogenized in four volumes of 10 M NaOH, 1 ml was then placed into 1 ml hexane + 200 ng internal standard, stirred for 2 h, and the hexane fraction was counter-extracted into 200 μl of 0.1 M HCl, dried and resuspended as above. Analysis was carried out on an Agilent 1100 LC–MS system with a Poroshell 120 SB-C8 column using H2O/ACN (with 0.1% formic acid) as the mobile phase (5–95% ACN, 10 min gradient). Using the ratio of drug to internal standard integration values, the unknown tissue concentrations were determined using a standard curve for the drug in question.

**Statistical analysis.** Sample sizes were calculated to give >80% power using means and standard deviations from our previous results for small molecule vaccines. Where ‘n’ is listed, it represents the number of animals used for analysis. Where ‘x replicates’ are listed, it represents the number of times the experiment was repeated in the laboratory. Outliers were detected at a predefined level of P < 0.05 using Grubbs’ outlier test and excluded from analysis. Data were graphed and analysed using Prism 5.02 (GraphPad software), setting P < 0.05 as the critical value. Groups were analysed for similarity of variance using Bartlett’s test, and non-parametric tests were used where the data was found to be non-normally distributed. All statistical tests were performed using two-tailed analysis.

**Data availability.** Source Data supporting the findings of this study can be found with the respective figures in the online version of the paper. Additional information can be supplied by the corresponding author upon reasonable request.

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Extended Data Figure 1 | General schematic demonstrating the concept behind using DISSECTIV to isolate CNS activity of single chemical species within a complex chemical mixture that elicits psychoactive effects. Mixtures of known (solid) and unknown (dashed) chemical species are frequently observed due to active metabolite generation from a parent compound, within natural products, toxins or their extracts, and in counterfeit or clandestine production of synthetic drugs. In DISSECTIV, incremental vaccination against individual known chemical species can identify effects due to the actions of those species (A/B), effects emerging from the interaction of multiple known species (C), effects due to actions of unknown species (D) or effects emerging from the interaction of known and unknown species (E). Furthermore, while five discrete ‘all or nothing’ effects are listed here for clarity, the approach can also be used to determine the impact of each compound in altering the magnitude of each discrete effect.
Extended Data Figure 2 | Conjugation of FEN, THEO, 1-A1 and AMPH hapten to BSA. a, BSA (m/z 66,134–66,284; ESI + m/z 66,431). b, FEN–BSA (m/z 70,031–70,106). c, THEO–BSA (m/z 69,424–71,275). d, 1-A1–BSA (m/z 70,727–71,058). e, AMPH–BSA (m/z 69,563).
Representative data shown for BSA, FEN–BSA, THEO–BSA and 1-A1–BSA; two replicates.
Extended Data Figure 3 | Standard curves used to quantify the concentrations of drugs in biological tissue samples. a, b, Standard curves for fenethylline (solid circles) at low (a, slope = 44,941 ± 1,026; \( r^2 = 0.9806 \)) and high (b, slope = 45,929 ± 143.1; \( r^2 = 0.9998 \)) concentrations in the presence of fenethylline-\( \text{d}_3 \) (open circles) as an internal standard. c, d, Standard curve for theophylline (solid triangles) at low (c, slope = 93,380 ± 844.4; \( r^2 = 0.9970 \)) and high (d, slope = 83,746 ± 1,406; \( r^2 = 0.9954 \)) concentrations in the presence of theophylline-\( \text{d}_6 \) (open triangles) as an internal standard. e, f, Standard curve for amphetamine (solid squares) at low (e, slope = 299,394 ± 1,980; \( r^2 = 0.9995 \)) and high (f, slope = 254,747 ± 16,197; \( r^2 = 0.9898 \)) concentrations in the presence of amphetamine-\( \text{d}_{11} \) (open squares) as an internal standard. All lines fit through the origin.
Extended Data Figure 4 | Vaccination with FEN–KLH blunts the behavioural effects of amphetamine and methamphetamine.

a, Amphetamine (2 mg kg$^{-1}$) locomotor behaviour in animals vaccinated with KLH or FEN–KLH ($P_{\text{interaction}} < 0.0001; F_{30,240} = 3.70$; *$P < 0.01$ versus KLH, Bonferroni correction). $n = 5$, repeated-measures two-way ANOVA.

b, Methamphetamine (2 mg kg$^{-1}$) locomotor behaviour in animals vaccinated with KLH ($n = 9$) or FEN–KLH ($n = 11$) ($P_{\text{interaction}} = 0.0385; F_{30,540} = 1.52$; *$P < 0.01$ versus KLH, Bonferroni correction). Repeated-measures two-way ANOVA.

c, EPM in KLH-vaccinated animals following saline ($n = 9$), amphetamine (A., 2 mg kg$^{-1}$, $n = 10$) or methamphetamine (M., 2 mg kg$^{-1}$, $n = 10$) administration. One-way ANOVA ($P = 0.1325; F_{2,26} = 2.187$).

d, EPM in FEN–KLH-vaccinated animals following saline ($n = 9$), amphetamine (2 mg kg$^{-1}$, $n = 10$) or methamphetamine (2 mg kg$^{-1}$, $n = 10$) administration. One-way ANOVA ($P = 0.9588; F_{2,26} = 0.042$).

a, b, Data are presented as mean ± s.e.m.

c, d, Data are shown as median with quartiles ± 10–90% CI; +, mean.
Extended Data Figure 5 | Vaccination with AMPH–KLH generates ineffective antibodies. 

**a**, Midpoint titres, day 35. Dotted line is mean 1-A1–KLH titre (n = 6). **b**, AMPH–KLH serum binding to AMPH–BSA with competing fenethylline or amphetamine. Data are pooled from n = 6, two replicates. Dotted line is mean 1-A1–KLH + amphetamine binding. **c**, Total hyperlocomotion (90 min) due to fenethylline (20 mg kg\(^{-1}\)) in KLH-vaccinated (n = 6) and AMPH–KLH-vaccinated (n = 6) animals. Dotted line is mean 1-A1–KLH + fenethylline 20 mg kg\(^{-1}\) locomotor response, t-test (P = 0.3584, d.f. = 9). **a, c**, Data are shown as median with quartiles ± 10–90% CI; +, mean. **b**, Data are presented as mean ± s.e.m.
## Extended Data Table 1 | Fenethylline binding to CNS targets

| Target                  | Inhib. (%) * | Target                  | Inhib. (%) * |
|-------------------------|--------------|-------------------------|--------------|
| Adenosine $A_{1}$       | -5           | Muscarinic M$_2$        | -6           |
| Adenosine $A_{2}$       | 0            | Muscarinic M$_3$        | 2            |
| Adrenergic $\alpha_{1A}$ | 16           | Nicotinic Ach           | 2            |
| Adrenergic $\alpha_{2A}$ | 27           | Nicotinic Ach $\alpha_2$ | 9            |
| Adrenergic $\alpha_{2B}$ | 16           | Mu Opiate               | 7            |
| Adrenergic $\beta_1$    | 3            | Phorbol Ester           | 9            |
| Adrenergic $\beta_2$    | 9            | K ATP Channel           | 21           |
| L-Type Ca Channel       | 6            | hERG K Channel          | -4           |
| Cannabinoid CB$_1$      | 16           | Prostanoid EP$_1$       | 3            |
| Dopamine D$_3$S         | 1            | Rolipram                | 9            |
| Dopamine D$_3$          | 8            | Serotonin 5-HT$_{2A}$   | 23           |
| GABA A – Site 1         | 0            | Sigma $\sigma_1$       | 7            |
| GABA A – Site 2         | 5            | Na Channel – Site 2     | -4           |
| Glutamate – NMDA        | 10           | Norepi. Transporter     | 10           |
| Histamine H$_3$         | 12           | Dop. Transporter        | 9            |
| Imidazoline I$_2$       | 4            |                         |              |

Data are shown as the average of two replicates. Dop., dopamine; Inhib., inhibition; Norepi., norepinephrine.

*Radioligand displacement measured in the presence of 10 $\mu$M fenethylline using the method of Eurofins Cerep Panlabs.
## Extended Data Table 2 | Binding and behavioural activity summary for DISSECTIV results

| Measurement                        | KLH        | FEN-KLH    | THEO-KLH   | 1-A1-KLH   |
|------------------------------------|------------|------------|------------|------------|
| Fenethylline pIC₅₀                | >3.00      | 5.912 ± 0.079 | 5.727 ± 0.021 | 5.000 ± 0.022 |
| Theophylline pIC₅₀                | >3.00      | 5.378 ± 0.079 | 5.385 ± 0.022 | >3.00      |
| Amphetamine pIC₅₀                 | >3.00      | >3.00      | >3.00      | 4.707 ± 0.012 |
| Methamphetamine pIC₅₀             | >3.00      | >3.00      | >3.00      | 3.682 ± 0.031 |
| Cocaine pIC₅₀                     | >3.00      | >3.00      | >3.00      | >3.00      |
| FEN-BSA Binding(OD)               | 0.076 ± 0.013 | 0.539 ± 0.112 | 0.321 ± 0.107 | 0.064 ± 0.054* |
| THEO-BSA Binding(OD)              | 0.066 ± 0.003 | 0.372 ± 0.033 | 1.226 ± 0.192 | 0.062 ± 0.056 |
| 1-A1-BSA Binding(OD)              | 0.070 ± 0.051 | 0.306 ± 0.005 | 0.071 ± 0.067 | 2.590 ± 0.023 |
| Serum Fenethylline (µM)*          | 15.76 ± 6.21 | 43.40 ± 10.90 | 36.70 ± 3.81 | 33.53 ± 8.25 |
| Serum Theophylline (µM)*          | 90.80 ± 12.68 | 127.20 ± 22.10 | 151.20 ± 16.87 | 51.50 ± 12.12 |
| Serum Amphetamine (µM)*           | 18.79 ± 2.51 | 30.07 ± 4.26 | 20.40 ± 1.08 | 31.73 ± 6.18 |
| Locomotor Activity†               | 326.1 ± 62.88 | 95.86 ± 24.73 | 270.70 ± 73.89 | 128.30 ± 27.64 |
| Plus Maze Activity†               | 51.31 ± 17.32 | 108.80 ± 22.99 | 62.46 ± 19.00 | 106.20 ± 16.17 |
| Place Preference Activity†        | 100.04 ± 22.60 | 53.60 ± 48.29 | 57.79 ± 31.84 | 62.00 ± 44.84 |

Data shown as mean ± s.e.m.

*Binding verified in alternative assay.
†Concentration measurements taken at 15 min after injection.
‡Percentage of baseline (20 mg kg⁻¹ fenethylline).
Experimental design

1. Sample size
   Describe how sample size was determined.
   Sample sizes were calculated to give > 80% power using means and standard deviations from our previous results for small molecule vaccines.

2. Data exclusions
   Describe any data exclusions.
   One outlier removed each for Figure 2b and 2d, mentioned in legend.
   Outliers were detected at a predefined level of p <0.05 using Grubb’s Outlier test and excluded from analysis.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   See individual figure legends for the number of biological and technical replicates, listed for each experiment.
   Where ‘n’ is listed, it represents the number of biological replicates used for analysis. Where ‘x replicates’ is listed, it represents the number of times the experiment was repeated in the laboratory.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Animals were randomly assigned to their experimental groups with stratification to use littermates as controls.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   The experimenter was blinded to group identity during data processing, and behavioral results were automatically scored using AnyMAZE v.4.99 (Stoelting Co).

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters
For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☑️ | ☑️ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| ☑️ | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☑️ | A statement indicating how many times each experiment was replicated |
| ☑️ | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
| ☑️ | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ☑️ | The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted |
| ☑️ | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
| ☑️ | Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.

7. Software
Describe the software used to analyze the data in this study.

- Behavioral results were automatically scored using AnyMAZE v.4.99 (Stoelting Co)
- Data were graphed and analyzed using Prism 5.02 (GraphPad Software)

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability
Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

- Theophylline, amphetamine hemisulfate, and methamphetamine hydrochloride (>95%, Sigma Aldrich) were obtained commercially. Fenethylline (>95%) was synthesized and purified at TSRI. Amphetamine-d11, Fenethylline-d3 (Lipomed) and Theophylline-d6 (CDN Isotopes) were obtained commercially for use as LC/MS internal standards (IS).

9. Antibodies
Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

- ELISA analyses were carried out as previously described using horseradish peroxidase- donkey-anti-mouse IgG (Jackson Immunoresearch Catalog # 715-035-151).
- Antibody activity was validated against control mouse serum containing IgG using a TMB substrate for visualization of HRP activity

10. Eukaryotic cell lines
a. State the source of each eukaryotic cell line used.
b. Describe the method of cell line authentication used.
c. Report whether the cell lines were tested for mycoplasma contamination.
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Male Swiss Webster mice (Taconic Biosciences, 6-8 weeks) were used for all 34 studies, and housed four per cage in a temperature-controlled (22 °C) vivarium on a reversed 12-h light cycle (9 35 PM – 9 AM) with ad libitum access to food and water.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants.