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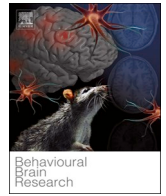
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Dopamine and norepinephrine transporter inhibition for long-term fear memory enhancement

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ABSTRACT

Psychostimulants are highly effective cognitive-enhancing therapeutics yet have a significant potential for abuse and addiction. While psychostimulants likely exert their rewarding and addictive properties through dopamine transporter (DAT) inhibition, the mechanisms of their procognitive effects are less certain. By one prevalent view, psychostimulants exert their procognitive effects exclusively through norepinephrine transporter (NET) inhibition, however increasing evidence suggests that DAT also plays a critical role in their cognitive-enhancing properties, including long-term memory enhancement. The present experiments test the hypothesis that combined strong NET and weak DAT inhibition will mimic the fear memory-enhancing but not the addiction-related effects of psychostimulants in mice. We examined the effects of the high affinity NET inhibitors atomoxetine or nisoxetine and the low affinity DAT inhibitor bupropion, either alone or in combination, on short- and long-term memory of Pavlovian fear conditioning. We also examined the addiction-related effects of combined strong NET and weak DAT inhibition using conditioned place preference and a locomotor activity test. While atomoxetine or nisoxetine alone enhanced short-term fear memory, the addition of bupropion was required to significantly enhance long-term fear memory. Additionally, combined atomoxetine and bupropion did not produce substantial motor stimulation or place preference. These findings suggest that combining strong NET and weak DAT inhibition could lead to the development of a highly effective cognitive enhancer that lacks the potential for addiction.

1. Introduction

Classical psychostimulants (e.g., methylphenidate, amphetamine, and cocaine) all target the dopamine and norepinephrine transporters (DAT and NET) with high affinity—methylphenidate and cocaine are “reuptake inhibitors” and amphetamine is a “releaser” resulting in large increases in extracellular dopamine and norepinephrine levels [1,2]. The behavioral effects of psychostimulants are highly dose-dependent—low doses enhance cognition and rarely produce addiction, while high doses impair cognition and are closely associated with addiction [3]. Although amphetamine and methylphenidate have proven highly effective at enhancing cognition in patients with attention-deficit hyperactivity disorder (ADHD) and other disorders [4,5], these patients face a major public health deficit due to poor access to psychiatrists and other health providers [6–9], as well as complex and expensive

procedures for obtaining refills [10]. Given that dose markedly dissociates the cognitive-enhancing and abuse-related effects of psychostimulants [3], it is likely possible to develop a drug that retains the therapeutic effects of psychostimulants but lacks abuse potential.

Our previous work explored if psychostimulant-induced memory enhancement is dependent on dose, and if efficacy for long-term memory (LTM) enhancement could be predicted based on DAT and/or NET affinity [3,11–16]. If LTM enhancement is due to exclusive action at one of these transporters, then a selective inhibitor of DAT or NET should also enhance LTM. However, given individually, bupropion (a low affinity DAT inhibitor) or atomoxetine (a high affinity NET inhibitor) did not enhance LTM [13] (see also Fig. 1), indicating that psychostimulant-induced LTM enhancement likely requires some combination of DAT and NET activity.

Although DAT inhibition appears to be required for LTM

Abbreviations: ADHD, attention-deficit hyperactivity disorder; ATX, atomoxetine; BUP, bupropion; CS, conditioned stimulus; DAT, dopamine transporter; LTM, long-term memory; NET, norepinephrine transporter; NIS, nisoxetine; PFC, prefrontal cortex; STM, short-term memory; US, unconditioned stimulus

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enhancement, increased extracellular dopamine levels are also responsible for the addictive potential of drugs, including psychostimulants [17–19]. However, drugs with weak activity at DAT (*i.e.*, low binding affinity, slow kinetics, and/or low doses) are not likely to produce addiction. For instance, the atypical antidepressant bupropion, a cathinone derivative, binds to DAT with low affinity, has slow kinetics, and has little abuse liability [1,20,21]. This suggests that weak DAT inhibition may be sufficient for LTM enhancement but insufficient for producing addiction-related behaviors.

While our previous work suggested that affinity for DAT and NET may be required for LTM enhancement and considered that it may be possible to develop a drug that retains the procognitive effects of psychostimulants but that lacks the potential for addiction [13], the present study aims to directly test these predictions. We hypothesized that combined strong NET and weak DAT inhibition will mimic the memory-enhancing but not the addiction-related effects of psychostimulants. Here, we use combinations of existing drugs—the high affinity NET inhibitors atomoxetine (ATX) or nisoxetine (NIS) and the low affinity DAT inhibitor bupropion (BUP). ATX is a non-stimulant ADHD medication that is non-controlled and lacks abuse potential but remains clinically inferior to psychostimulants [22–24]. NIS has a similar binding profile to ATX but has not been pursued clinically [1]. BUP is an atypical antidepressant that is occasionally used as a non-stimulant ADHD adjunct [21,25].

We examined the effects of these drugs alone and in combination on short-term memory (STM) and LTM using Pavlovian fear conditioning, a simple and efficient tool for modeling the effects of drugs on memory in rodents [13,26]. In Pavlovian fear conditioning, a discrete conditioned stimulus (CS) is paired with an aversive footshock unconditioned stimulus (US) in a novel context. After training, mice will exhibit freezing behavior to both the discrete CS as well as the context (*i.e.*, the conditioning chamber); both cued and contextual fear memory depend on the amygdala, whereas contextual fear memory further depends on the hippocampus [26–30]. When administered pre-training, we have found that clinically-relevant doses of several psychostimulants enhance short- and long-term fear memory [13–16]. In the present study, we found evidence that NET inhibition alone enhances short-term fear memory, but the addition of some DAT inhibition seems to be required to enhance long-term fear memory. We also examined the addiction-related effects of combined strong NET and weak DAT inhibition using conditioned place preference (a model of drug-seeking) and a locomotor activity test and found no substantial evidence of reward or motor stimulation.

2. Materials and methods

2.1. Subjects

480 hybrid C57BL6/Jx129T2/SvEmsJ (129B6) (Jackson Laboratory, West Sacramento, CA, USA) male ($n = 255$) and female ($n = 225$) mice were used. Separate cohorts of mice were used for the fear conditioning, locomotor activity, and conditioned place preference experiments. Mice were weaned at 3 weeks of age and group-housed (2–5 mice per same sex cage) with continuous access to food and water. The animal colony was maintained on a 14:10-h light/dark schedule and all testing occurred during the light phase of the cycle. Mice were at least 10 weeks old and handled for 3 days (1 min/day) prior to testing. All animal care and testing procedures were approved by the UCSD IACUC and compliant with the 8th NRC Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

Atomoxetine HCl (Sigma-Aldrich, TCI America), Nisoxetine HCl (Abcam, Tocris Bioscience), and Bupropion HCl (Sigma-Aldrich, Spectrum Chemical, TCI America) were dissolved in 0.9% physiological

saline, either alone or in combination (ATX + BUP, NIS + BUP). A range of doses were selected (0.1, 0.5, 1, and 10 mg/kg ATX; 0.1, 0.5, 1, 5, and 10 mg/kg NIS; 0.5, 2.5, 5, 10, and 20 mg/kg BUP; salt weights). Only clinically-relevant doses were given in combination, because previous experiments indicated that higher doses would produce deficits [13]. All injections were given intraperitoneally in a volume of 10 mL/kg. As further described, “on-drug” sessions were performed immediately or up to 30 min following drug injections (and necessarily includes all STM tests) and “off-drug” sessions were performed in a drug-naïve state.

2.3. Fear conditioning

The VideoFreeze system (Med-Associates Inc., St. Albans, VT, USA) and fear conditioning protocol were used as described previously [13,26,28,31,32]. Up to eight mice were trained/tested concurrently in individual conditioning chambers that contained stainless-steel rod floors, white acrylic sidewalls, and clear polycarbonate front walls. Training and context testing took place in the ‘training context’ in which the chambers were illuminated with moderate (80 lx) white light and were cleaned and scented with 7% isopropanol. Tone testing took place in the ‘alternate context’, as the chambers were transformed across multiple sensory dimensions to create a distinct context—a black plastic, triangular teepee was inserted into the chamber, white acrylic sheets were placed over the floors, only near-infrared light (980 nm) was used to create a dark environment, and the chambers were cleaned and scented with 5% vinegar. During all trials, the VideoFreeze system continuously scored locomotor activity (in arbitrary units [au], see [26] for a full description) and freezing behavior of each mouse.

425 mice were randomly assigned to drug dose groups as presented in Table 1. Groups were completely counterbalanced by sex and assigned chamber for training/testing.

2.3.1. Training

Mice were given an injection of drug or saline 15–30 min before being placed into one of eight identical chambers for training. Training began with a 3-min baseline period, followed by a single tone-shock pairing. The tone-shock consisted of a 30-s tone (2.8 kHz, 85 dBA) presented through a speaker in the chamber sidewall, which co-terminated with a 2-s scrambled footshock (0.75 mA, AC, RMS constant current) delivered through the rod floor. 1.5 min following the tone-shock pairing, mice underwent a 5-min STM test. Locomotor activity

Table 1

Drug dose groups and sample sizes for fear conditioning experiments. 425 mice were randomly assigned to groups by dose of atomoxetine (ATX), nisoxetine (NIS), or bupropion (BUP), or dose combination of atomoxetine and bupropion (ATX + BUP) or nisoxetine and bupropion (NIS + BUP). ^aThe NIS and NIS + BUP experiments were performed together and used the same saline control animals.

Drug	Dose (mg/kg)	N	Drug Combination	Dose (mg/kg)	N
ATX	0.0	20	ATX + BUP	0 + 0	43
	0.1	13		0.1 + 2.5	12
	0.5	13		0.5 + 2.5	12
	1.0	18		1 + 2.5	15
	10.0	19		0.1 + 5	22
NIS	0.0	16 ^a	NIS + BUP	0.5 + 5	22
	1.0	8		1 + 5	19
	5.0	8		0.1 + 10	24
	10.0	8		0.5 + 10	21
BUP	0.0	14	NIS + BUP	1 + 10	16
	0.5	11		0 + 0	16 ^a
	5.0	11		0.1 + 10	8
	10.0	10		0.5 + 10	8
	20.0	10		1 + 10	8
				5 + 10	8
				10 + 10	8

and freezing behavior were continuously scored to measure on-drug baseline locomotion, shock reactivity, and STM.

2.3.2. Context test

Seven to nine days after training, mice were returned to the training context, off-drug, for one 5-min context test. Freezing behavior was scored for all 5 min to measure contextual LTM.

2.3.3. Tone test

One to three days after context testing, mice were placed in the alternate context, off-drug, for one 5-min tone test. Tone testing consisted of a 2-min baseline period, followed by the presentation of 3, 30-s tones identical to the training tone (2.8 kHz, 85 dBA), each separated by 30 s. The difference in freezing behavior during the 3 tone presentations and the 2-min baseline period (tone minus baseline freezing) was used to measure tone LTM.

2.4. Locomotor activity

Eight mice were tested concurrently in individual chambers (one side of the two-compartment conditioned place preference chambers) (Med-Associates Inc.). Each chamber measured $21.6 \times 43.2 \times 30.5$ cm, contained stainless steel rod flooring and polycarbonate walls (three white and one black), and was cleaned with glass cleaner between trials. Activity Monitor software (Med-Associates Inc.) used the interruption of infrared beams to identify mouse position and measure locomotor activity (ambulatory distance in cm).

Testing was conducted over 5 alternating days in a within-subjects design, such that 24 mice (not used in other experiments) were tested once at each of the five doses in a pseudorandom order: 0 + 0, 0.1 + 5, 0.5 + 5, 0.1 + 10, and 0.5 + 10 mg/kg ATX + BUP (all n's = 24). On each testing day, mice were given an injection and immediately placed in the testing chamber. Ambulatory distance was scored for a total of 60 min to measure acute drug effects on locomotor activity.

2.5. Conditioned place preference

Seven or eight mice were tested concurrently in individual chambers (Med-Associates Inc.) as described previously [13,31]. Each chamber ($43.2 \times 43.2 \times 30.5$ cm) consisted of two sides separated by a black wall with a removable insert (that was removed only for place preference testing). The two sides provided distinct tactile and visual cues, as they differed by flooring (stainless steel rods or wire-mesh) and walls (decorated white or undecorated clear polycarbonate). The chambers were counterbalanced by the combination of flooring/walls and were cleaned with glass cleaner between trials. Activity Monitor software (Med-Associates Inc.) used the interruption of infrared beams to identify mouse position and measure percent time spent on each side during testing.

31 mice were randomly assigned to drug dose groups: 0 + 0 (n = 11), 0.1 + 5 (n = 10), or 1 + 10 (n = 10) mg/kg ATX + BUP. Testing chamber and paired/unpaired side assignments were completely counterbalanced across groups.

2.5.1. Habituation

Mice were habituated to the testing chamber for two consecutive days prior to training. On each habituation day, mice were introduced to both sides for 30 min each, off-drug. The sequence of habituation to the paired/unpaired sides was counterbalanced across groups and day.

2.5.2. Training

The day following habituation, mice were trained for seven consecutive days. On each training day, mice were injected with saline and immediately placed into the unpaired side for 15 min, and then injected with drug and immediately placed into the paired side for 15 min.

2.5.3. Place preference test

24 h following training, mice were tested off-drug for place preference. The inserts that previously separated the two sides of the chambers were removed. Mice were placed into the center of the chamber (direction of entry was counterbalanced) and allowed access to both sides for 15 min. Time spent on each side was scored to evaluate place preference (percent time spent on the paired minus the unpaired side).

2.6. Statistical analyses

Univariate or multivariate analysis of variance (ANOVA) were used to identify overall group differences; these were followed by Fisher's Least Significant Difference (LSD) post-hoc tests against the saline control groups. Data from male and female mice were merged as we found no statistically significant sex differences that meaningfully influenced our findings (p values > 0.05).

3. Results

3.1. Effects of ATX, NIS, and BUP on fear learning and memory

The effects of ATX (0–10 mg/kg i.p.), NIS (0–10 mg/kg i.p.), and BUP (0–20 mg/kg i.p.) on fear learning and memory were examined alone¹ and in combination using Pavlovian fear conditioning. Mice were trained on-drug with a single tone-shock pairing, immediately tested for STM, and then tested off-drug at least one week later for contextual and tone LTM.

3.1.1. ATX alone

During the baseline period, a dose of 10 mg/kg ATX significantly reduced locomotor activity relative to saline controls ($p = 0.044$). All other doses had no effect on baseline locomotion (p values > 0.35). The shock elicited a large activity burst that did not significantly differ between groups ($F(4,78) = 0.883$, $p = 0.478$) (Fig. 1A). ATX dose-dependently modulated freezing during the STM test ($F(4,78) = 6.16$, $p < 0.001$). Doses of 0.5, 1, and 10 mg/kg ATX significantly enhanced STM relative to saline controls (p values < 0.04). A dose of 0.1 mg/kg ATX had no effect on STM ($p = 0.97$) (Fig. 1B). Freezing did not significantly differ between groups during the contextual ($F(4,78) = 0.59$, $p = 0.668$) (Fig. 1C) nor the tone ($F(4,78) = 0.94$, $p = 0.446$) LTM tests (Fig. 1D).

Low locomotor activity during training could be directly related to enhanced freezing, as seen in mice given 10 mg/kg ATX (*i.e.*, reduced baseline locomotion and enhanced STM freezing). However, such an effect could also reflect improved executive function, which could appear as both enhanced inhibition (*e.g.*, a 'calming' effect) and enhanced STM. Although one can never really completely separate these two views because STM tests are necessarily on-drug, we approached this problem by subtracting freezing behavior during baseline from that during the STM test. This eliminates the portion of post-shock freezing that may be due to the drug directly reducing activity and thereby enhancing freezing. Using this measure, a dose of 10 mg/kg ATX significantly enhanced STM relative to saline controls (data not shown; 0 mg/kg, $23.45 \pm 4.67\%$; 10 mg/kg, $42.47 \pm 3.62\%$; $p = 0.004$). Therefore, the significant reduction in baseline locomotion produced by 10 mg/kg ATX is not responsible for the significant enhancement in freezing during the STM test. This is typical of drug or lesion effects that produce small changes in locomotor activity – they are unlikely to affect freezing [29,30].

¹ Incomplete portions of this data (ATX alone and BUP alone) appear in the Supplemental Figures of Carmack et al. [13].

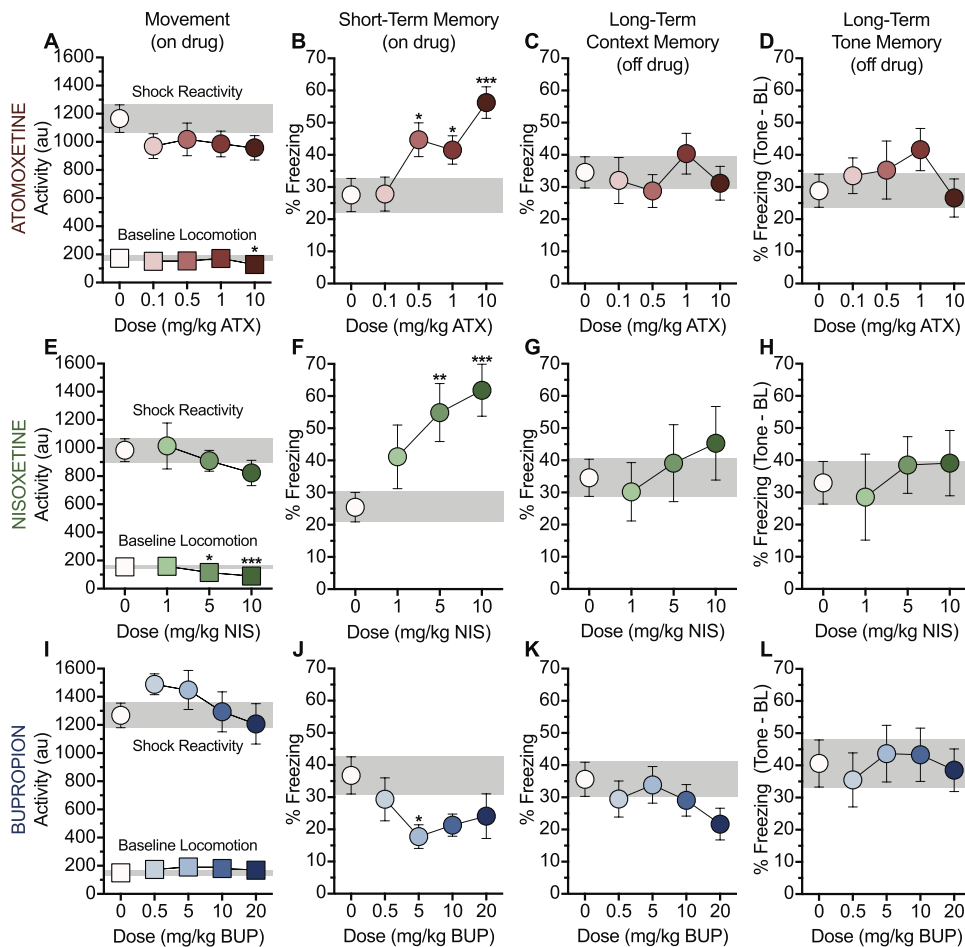


Fig. 1. The effects of atomoxetine (ATX; a–d), nisoxetine (NIS; e–h), and bupropion (BUP; i–l) on fear learning and memory. On-drug activity during the 3-min training baseline period and the 2-s footshock (a, e and i), short-term memory as measured by percent freezing during the 5-min post-shock period (b, f and j), and long-term context (c, g and k) and tone (d, h and l) memory as measured by percent freezing during off-drug testing, 1 week or more after training. (a) A dose of 10 mg/kg ATX significantly reduced baseline locomotion relative to saline controls. ATX had no effect on shock reactivity. (b) Doses of 0.5, 1, and 10 mg/kg ATX significantly enhanced short-term memory relative to saline controls. (c and d) ATX had no effect on long-term context or tone memory. (e) Doses of 5 and 10 mg/kg NIS significantly reduced baseline locomotion relative to saline controls. (f) Doses of 5 and 10 mg/kg NIS significantly enhanced short-term memory relative to saline controls. (g and h) NIS had no effect on long-term context or tone memory. (i) BUP had no effect on baseline locomotion or shock reactivity. (j) A dose of 5 mg/kg BUP significantly impaired short-term memory relative to saline controls. (k and l) BUP had no effect on long-term context or tone memory. Each point represents the mean \pm 1 standard error. The grey bar indicates standard error range for the comparison saline control group. Starred data points identify significant comparisons against the saline control group using Fisher's LSD (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). Incomplete portions of this data (ATX alone and BUP alone) appear in the Supplemental Figures of Carmack et al. [13].

3.1.2. NIS alone

NIS dose-dependently modulated locomotor activity during the baseline period ($F(3,36) = 7.06$, $p < 0.001$). Doses of 5 and 10 mg/kg NIS significantly reduced baseline locomotion relative to saline controls (p values < 0.02). A dose of 1 mg/kg NIS had no effect on baseline locomotion ($p = 0.793$). The shock elicited a large activity burst that did not significantly differ between groups ($F(3,36) = 0.60$, $p = 0.619$) (Fig. 1E). NIS dose-dependently modulated freezing during the STM test ($F(3,36) = 5.67$, $p = 0.003$). Doses of 5 and 10 mg/kg NIS significantly enhanced STM relative to saline controls (p values ≤ 0.005). A dose of 1 mg/kg NIS had no effect on STM ($p = 0.122$) (Fig. 1F). Freezing did not significantly differ between groups during the contextual ($F(3,36) = 0.45$, $p = 0.719$) (Fig. 1G) nor the tone ($F(3,36) = 0.24$, $p = 0.866$) LTM tests (Fig. 1H).

Similar to a dose of 10 mg/kg ATX, doses of 5 and 10 mg/kg NIS significantly reduced baseline locomotion and significantly enhanced freezing during the STM test. Again, we subtracted freezing behavior during baseline from that during the STM test and found that doses of 5 and 10 mg/kg NIS significantly enhanced STM relative to saline controls (data not shown; 0 mg/kg, $23.63 \pm 4.57\%$; 5 mg/kg, $45.58 \pm 6.90\%$; 10 mg/kg, $50.1 \pm 8.3\%$; p values ≤ 0.026). Therefore, the significant reductions in baseline locomotion produced by 5 and 10 mg/kg NIS again are not responsible for the significant enhancements in freezing during the STM test.

3.1.3. BUP alone

Locomotor activity during the baseline period did not significantly differ between groups ($F(4,51) = 0.58$, $p = 0.679$). The shock elicited a large activity burst that also did not significantly differ between groups

($F(4,51) = 1.05$, $p = 0.389$) (Fig. 1I). During the STM test, a dose of 5 mg/kg BUP significantly reduced freezing relative to saline controls ($p = 0.015$). All other doses had no effect on STM (p values > 0.05) (Fig. 1J). Freezing did not significantly differ between groups during the contextual ($F(4,51) = 1.00$, $p = 0.416$) (Fig. 1K) nor the tone ($F(4,51) = 0.18$, $p = 0.949$) LTM tests (Fig. 1L).

3.1.4. Combined ATX and BUP

ATX + BUP dose-dependently modulated locomotor activity during the baseline period ($F(9,196) = 2.93$, $p = 0.003$). A dose of 0.5 + 2.5 mg/kg ATX + BUP significantly reduced baseline locomotion ($p = 0.021$) and a dose of 0.1 + 10 mg/kg ATX + BUP significantly enhanced baseline locomotion ($p = 0.024$) relative to saline controls. All other doses had no effect on baseline locomotion (p values > 0.06). The shock elicited a large activity burst that did not significantly differ between groups ($F(9,196) = 1.63$, $p = 0.11$). A dose of 0.1 + 2.5 mg/kg ATX + BUP did produce a statistically significant decrease in shock reactivity relative to saline controls ($p = 0.008$) (Fig. 2A). However, this was unlikely related to any effects seen in fear conditioning, as no memory effects were observed at this dose. ATX + BUP dose-dependently modulated freezing during the STM test ($F(9,196) = 3.48$, $p < 0.001$). Doses of 1 + 2.5, 0.5 + 5, and 1 + 5 mg/kg ATX + BUP significantly enhanced STM relative to saline controls (p values < 0.015). All other doses had no effect on STM (p values > 0.25) (Fig. 2B).

During the contextual LTM test, mice given 0.5 + 10 mg/kg ATX + BUP exhibited significantly enhanced freezing relative to saline controls ($p = 0.044$). Mice given 1 + 2.5 mg/kg ATX + BUP exhibited a trend towards significantly enhanced freezing relative to saline

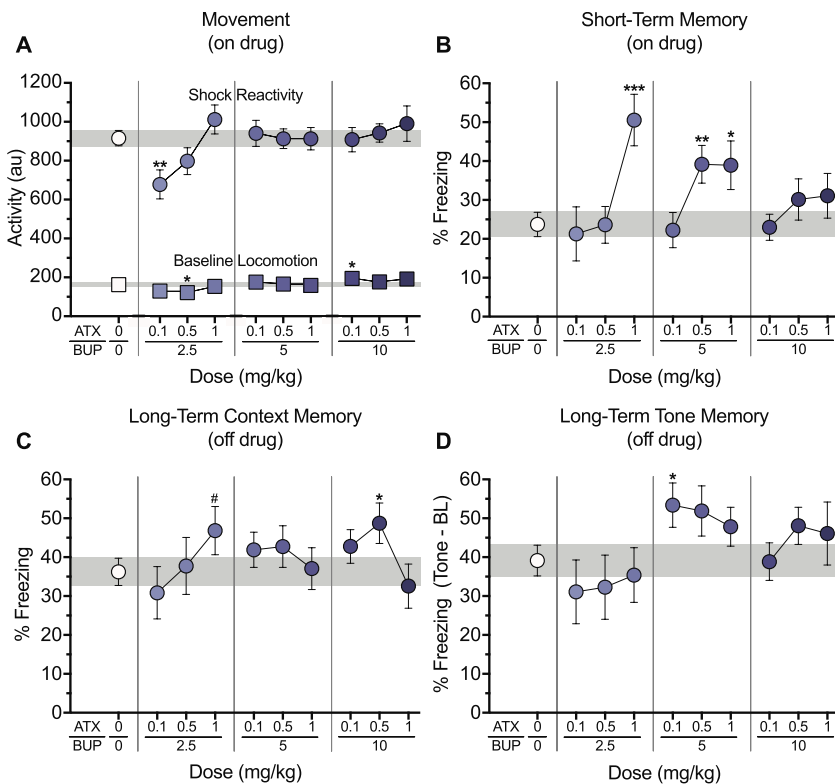


Fig. 2. The effects of combined atomoxetine (ATX) and bupropion (BUP) on fear learning and memory. (a) On-drug activity during the 3-min training baseline period and the 2-s footshock. A dose of 0.5 + 2.5 mg/kg ATX + BUP significantly reduced baseline locomotion relative to saline controls and a dose of 0.1 + 10 mg/kg ATX + BUP significantly enhanced baseline locomotion relative to saline controls. A dose of 0.1 + 2.5 mg/kg ATX + BUP significantly reduced shock reactivity relative to saline controls. (b) Short-term memory as measured by percent freezing during the 5-min post-shock period. Doses of 1 + 2.5, 0.5 + 5, and 1 + 5 mg/kg ATX + BUP significantly enhanced short-term memory relative to saline controls. (c) Long-term context memory as measured by percent freezing during off-drug context testing, 7–9 days after training. A pre-training dose of 0.5 + 10 mg/kg ATX + BUP significantly enhanced long-term context memory relative to saline controls. A pre-training dose of 1 + 2.5 mg/kg ATX + BUP significantly enhanced long-term context memory relative to saline controls during only the first minute of context testing. (d) Long-term tone memory as measured by percent freezing during off-drug tone testing (difference between tone presentations and tone baseline period), 1–3 days after context testing. A pre-training dose of 0.1 + 5 mg/kg ATX + BUP significantly enhanced long-term tone memory relative to saline controls. Hash-tagged data points identify significant comparisons against the saline control group during a certain portion of testing ($^{\#}P < 0.05$).

controls ($p = 0.129$), which was driven by a significant enhancement during the first minute of testing (data not shown; $p = 0.017$). During the tone LTM test, mice given 0.1 + 5 mg/kg ATX + BUP exhibited significantly enhanced freezing relative to saline controls ($p = 0.041$). All other doses had no effect on contextual (p values > 0.25) or tone (p values > 0.06) LTM (Figs. 2C and D).

3.1.5. Combined NIS and BUP

Locomotor activity during the baseline period did not significantly differ between groups ($F(5,50) = 1.25$, $p = 0.302$). The shock elicited a large activity burst that also did not significantly differ between groups ($F(5,50) = 0.84$, $p = 0.526$) (Fig. 3A). NIS + BUP dose-dependently modulated freezing during the STM test ($F(5,50) = 3.05$, $p = 0.018$). Doses of 0.5 + 10 and 5 + 10 mg/kg NIS + BUP significantly enhanced STM relative to saline controls (p values ≤ 0.045). All other doses had no effect on STM (p values > 0.1) (Fig. 3B).

During the contextual LTM test, mice given 0.1 + 10 and 0.5 + 10 mg/kg NIS + BUP exhibited a trend towards significantly enhanced freezing relative to saline controls (p values = 0.073 and 0.131), which were driven by significant enhancements during the fourth (data not shown, 0.1 + 10 mg/kg NIS + BUP, $p = 0.021$) or the second and third (data not shown, 0.5 + 10 mg/kg NIS + BUP, p values < 0.04) minutes of testing. During the tone LTM test, mice given 0.5 + 10 mg/kg NIS + BUP exhibited significantly enhanced freezing relative to saline controls ($p = 0.01$). All other doses had no effect on contextual (p values > 0.25) or tone (p values > 0.3) LTM (Fig. 3C and D).

3.2. Addictive potential of combined ATX and BUP

3.2.1. Locomotor activity

We selected a range of fear memory-enhancing dose combinations of ATX + BUP (0.1 + 5, 0.5 + 5, 0.1 + 10, and 0.5 + 10 mg/kg) and assessed their effects on locomotion over a 60-min period. There was no main effect of group on locomotor activity ($F(4,115) = 1.66$, $p = 0.165$). Doses of 0.1 + 10 and 0.5 + 10 mg/kg ATX + BUP

significantly enhanced locomotor activity relative to saline during the first 10-min block (p values < 0.015) but not during any other blocks (p values > 0.2). Because increased locomotion was only observed during the first 10 min post-injection (before the peak of the drug), this effect may be a physical reaction to receiving a higher concentration of drug rather than an actual drug effect. All other doses of ATX + BUP had no effect on locomotion relative to saline during any time block (p values > 0.1) (Fig. 4A).

3.2.2. Conditioned place preference

We assessed the rewarding effects of ATX + BUP at two clinically-relevant dose combinations selected from the fear conditioning studies—a lower fear memory-enhancing dose (0.1 + 5 mg/kg) and the highest dose tested (1 + 10 mg/kg). Mice were trained for seven consecutive days to associate saline with one side and drug treatment with the other side of a two-compartment chamber. 24 h later, mice were returned off-drug with free access to both compartments. Place preference to the drug-paired side was scored as the difference in percent time spent on the paired side versus the unpaired side. None of the groups exhibited a significant preference for either side (one sample two-tailed t -test against hypothesized $\mu = 0$, 0 + 0 mg/kg: $t(10) = 0.305$, $p = 0.766$, 0.1 + 5 mg/kg: $t(9) = 1.946$, $p = 0.084$, 1 + 10 mg/kg: $t(9) = 0.808$, $p = 0.44$). Place preference did not significantly differ between groups ($F(2,28) = 1.80$, $p = 0.183$). Mice given either ATX + BUP dose combination did not differ in place preference relative to saline controls (p values > 0.2) (Fig. 4B).

4. Discussion

We tested the effects of ATX, NIS, and BUP, alone and in combination, across a range of doses on Pavlovian fear conditioning. While ATX and NIS enhanced STM and BUP impaired STM, these drugs given alone failed to enhance LTM across a wide range of doses. However, BUP in combination with ATX or NIS produced enhancements in STM and LTM at certain dose combinations. On the locomotor activity and place preference tests, combined ATX and BUP did not produce

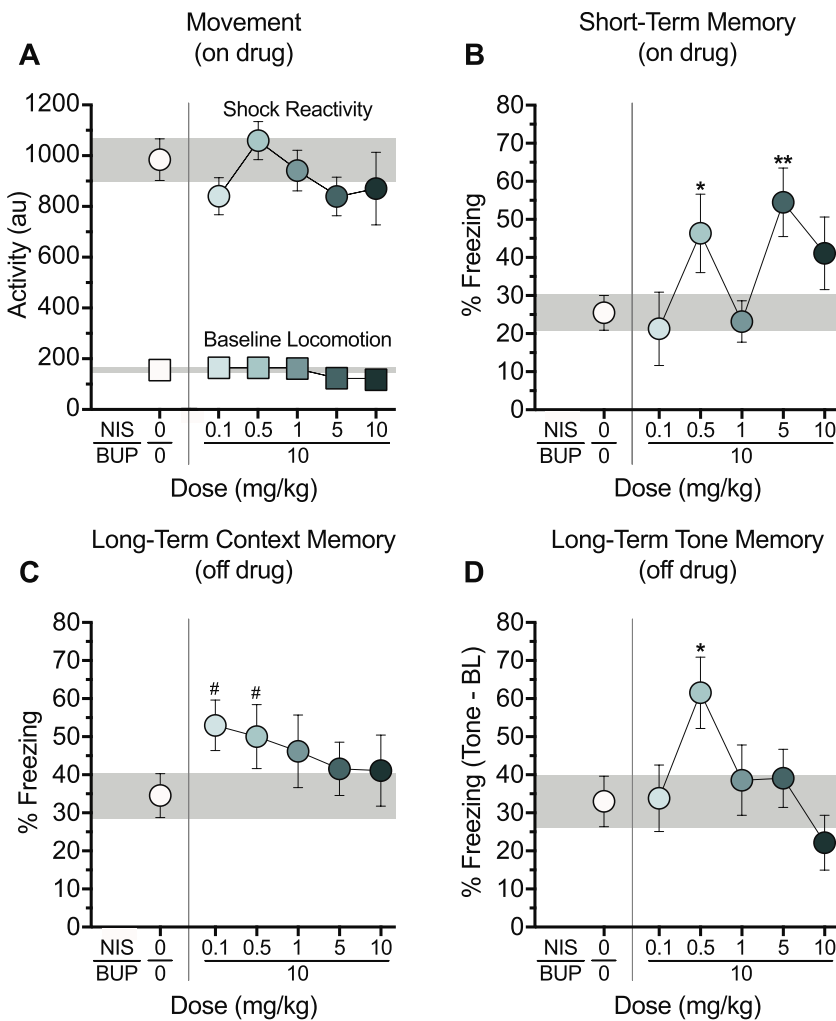


Fig. 3. The effects of combined nisoxetine (NIS) and bupropion (BUP) on fear learning and memory. (a) On-drug activity during the 3-min training baseline period and the 2-s foot-shock. NIS + BUP had no effect on baseline locomotion or shock reactivity. (b) Short-term memory as measured by percent freezing during the 5-min post-shock period. Doses of 0.5 + 10 and 5 + 10 mg/kg NIS + BUP significantly enhanced short-term memory relative to saline controls. (c) Long-term context memory as measured by percent freezing during off-drug context testing, 7–9 days after training. Pre-training doses of 0.1 + 10 and 0.5 + 10 mg/kg NIS + BUP significantly enhanced long-term context memory relative to saline controls during only the fourth minute (0.1 + 10 mg/kg) or the second and third minutes (0.5 + 10 mg/kg) of context testing. (d) Long-term tone memory as measured by percent freezing during off-drug tone testing (difference between tone presentations and tone baseline period), 1–3 days after context testing. A pre-training dose of 0.5 + 10 mg/kg NIS + BUP significantly enhanced long-term tone memory relative to saline controls.

substantial motor stimulation or reward. These findings indicate that NET inhibition alone is sufficient for short-term fear memory enhancement, but both DAT and NET inhibition seems to be needed for long-term fear memory enhancement. It also appears that weak DAT inhibition, when combined with strong NET inhibition, is sufficient for long-term fear memory enhancement but insufficient for producing addiction-related behaviors, at least in terms of motor stimulation or place preference.

In many previous experiments [14–16], LTM has been much more resistant than STM to enhancement or impairment by stimulant-like drugs (e.g., modafinil, amphetamine, cocaine). Here, the STM and LTM

tests differed in that STM was measured (unavoidably) on-drug and LTM was measured off-drug. Freezing behavior during the STM test could have been influenced by other drug effects, such as those on locomotor activity or fear. Only a few doses of ATX and NIS alone significantly reduced baseline locomotion and also enhanced freezing during the STM test. While reduced locomotor activity could reflect a ‘calming’ effect from improved executive function, we accounted for baseline drug effects on activity and found that these doses still enhanced STM (see Results section). It is unlikely that memory enhancements were confounded by drug-induced increases in fear or anxiety, as ATX, NIS, and BUP are typically *not* anxiogenic and both

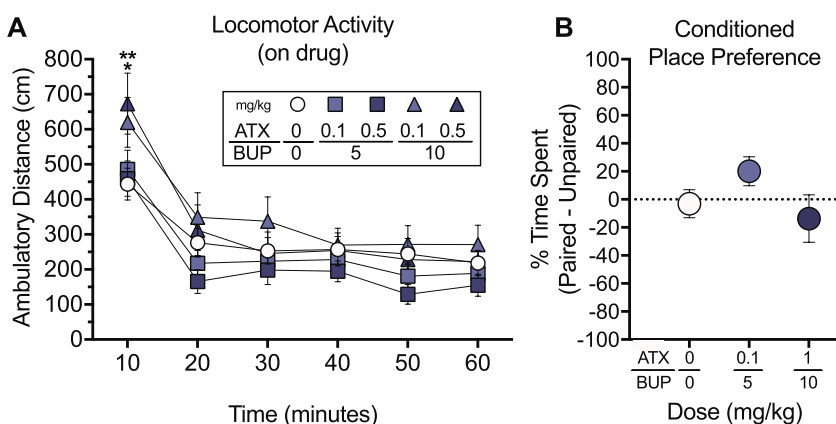


Fig. 4. The effects of combined atomoxetine (ATX) and bupropion (BUP) on addiction-related behaviors. (a) On-drug locomotor activity as measured by ambulatory distance during the 60 min (six 10-min blocks) immediately following drug administration. There was no main effect of dose on locomotor activity (total ambulatory distance in 60-min period). Doses of 0.1 + 10 and 0.5 + 10 mg/kg ATX + BUP significantly enhanced locomotor activity relative to saline during the first 10-min block only. (b) Conditioned place preference as measured by the difference in percent of time spent on the drug-paired side versus the unpaired side following seven days of training. None of the groups exhibited a significant preference for either side. Treatment with ATX + BUP had no significant effect on place preference relative to saline controls.

ATX and BUP are even prescribed for comorbid anxiety disorders [21,22,33,34]. It is also unlikely that memory enhancements were confounded by drug-induced increases in pain sensitivity, as we found no drug effects on nociception as measured by shock reactivity (except for the lowest dose of ATX + BUP, which had no effect on memory). If such confounds were present, we would expect nonspecific increases in freezing behavior across all tests; instead, we found that no doses significantly increased freezing across all three memory tests. Because the LTM tests were conducted in the absence of drug, we can conclude that certain dose combinations of DAT and NET inhibitors enhance fear memory acquisition and retention and the presence of drug is not required for retrieval.

Pavlovian fear conditioning is an efficient way to screen potential cognitive enhancers in rodents and is especially useful when testing many drugs at many doses [13,26]. Specifically, contextual fear memory is hippocampus-dependent and thus directly relevant to many conditions wherein memory is impaired [27,29,30]. Our previous work demonstrated that psychostimulants enhance both short- and long-term fear memory in mice at doses that are prescribed to treat ADHD and other cognitive disorders in humans [13–16], and these enhancements are also seen in other forms of learning and memory such as spatial memory [12,14]. Given this, we hypothesize that the drug combinations tested here may also be highly effective cognitive-enhancing therapeutics that target several forms of learning and memory.

LTM enhancement should be a critical therapeutic target of cognitive enhancers, as significant deficits in LTM are implicated in a wide range of disorders such as ADHD, dementia, Alzheimer's disease, schizophrenia, aphasia, and learning disabilities [35–40]. Despite this, clinical efficacy studies of cognitive enhancers often neglect LTM and focus primarily on attention, working memory, and response inhibition, conceivably because clinical assessment of these factors is far less laborious than long-term effects [41–45]. When left untreated, LTM deficits can lead to academic underachievement, poor job performance and retention, and limitations in major life activities [46]. LTM enhancement may be necessary to reverse deficits in academic and occupational achievement [44]. In particular, working and STM improvements are unlikely to improve school test performance unless LTM is also improved. We believe that an increased focus on LTM is crucial to develop novel, highly effective cognitive enhancers.

Existing theories suggest that psychostimulants and atomoxetine exert influence on “frontal” executive functions (e.g., working memory, STM, attention, response inhibition) exclusively through NET inhibition in the prefrontal cortex (PFC) and all other procognitive effects, including LTM enhancement, are incidental to improvements in those functions [47–50]. It is believed that inhibiting NET in the PFC increases extracellular levels of both dopamine and norepinephrine, as there is a low density of DAT and a high density of NET in the PFC, and NET is non-selective in transporting either catecholamine [51–53]. In the present study, NET inhibition alone enhanced STM but did *not* enhance LTM unless combined with DAT inhibition. Thus, while increasing extracellular levels of dopamine and norepinephrine in the PFC may be responsible for enhancing STM and other executive functions, this mechanism is insufficient for enhancing LTM. We speculate that increasing extracellular dopamine levels in areas outside the PFC may also be necessary to enhance LTM. According to one view, the corelease of dopamine along with norepinephrine from the locus coeruleus to the dorsal hippocampus is key to successful learning and memory [54], which may explain our findings that the *combination* of DAT and NET affinity is necessary for LTM enhancement. Another possible mechanism by which the NET inhibitors enhanced STM may be increased brain-derived neurotrophic factor (BDNF) mRNA expression in the hippocampus, which atomoxetine has been shown to increase [55] and previous reports suggest is associated with improved STM [56–58].

There is much additional evidence implicating the critical role of DAT in learning and memory. DAT dysfunction is associated with age-

related cognitive decline and several conditions wherein memory is impaired such as ADHD, dementia with Lewy bodies, Parkinson's disease, and chronic schizophrenia [59–62]. The 10-repeat VNTR allele of the dopamine transporter gene (DAT1) also correlates with ADHD as well as the combined inattentive/hyperactive-impulsive diagnostic subtype, higher levels of symptom severity, and an enhanced response to methylphenidate [63–65]. Taken together, some activity at DAT may be essential to treating learning and memory impairments.

We found that combinations of strong NET and weak DAT inhibitors mimic the short- and long-term fear memory-enhancing effects but lack the addiction-related effects of psychostimulants. Given that only certain dose combinations enhanced long-term fear memory, there is likely an ideal ratio of NET/DAT activity for maximal memory enhancement yet no addictive potential, and our future work will be aimed at exploring this. We propose that these drug combinations may be an effective alternative to psychostimulants in the treatment of cognitive dysfunction that may have decreased health risks and increased patient access.

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Declaration of Competing Interest

None.

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