The uncompetitive N-methyl-D-aspartate antagonist memantine reduces binge-like eating, food seeking behavior and compulsive eating: role of the nucleus accumbens shell

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SUPPLEMENTARY MATERIALS AND METHODS

Subjects

Male Wistar rats, 45 days old upon arrival (Charles River, Wilmington, MA), were grouped housed (unless otherwise stated) on a 12:12 hour reverse light cycle (lights off at 11am daily), in a humidity- (30-70%) and temperature-controlled (20-26^{o}C) vivarium. Rats were given access to corn-based chow (Harlan Teklad LM-485 Diet 7012 (65% (kcal) carbohydrate, 13% fat, 21% protein, 341 cal/100 g); Harlan, Indianapolis, IN) and water *ad libitum*. Procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Boston University Institutional Animal Care and Use Committee (IACUC).

Drugs

Memantine hydrochloride (1,3-dimethyl-5-aminoadamantane hydrochloride) was purchased from Acros Organics (Geel, Belgium). For both systemic and site-specific studies memantine was dissolved in isotonic filtered saline immediately before administration. For within-subject experiments, treatment days were separated by 1 to 3 intervening days until the variables returned to baseline. Doses, injection volume, suitability of the vehicle, and pretreatment times were based on previously published reports (Cottone *et al*, 2013; Sabino *et al*, 2013).

Apparatus for self-administration procedures

The individual operant test chambers (30×24×29 cm) (Med Associates Inc., St. Albans, VT) had a grid floor and were located in ventilated, sound-attenuating enclosures (66×56×36 cm) (Blasio *et al*, 2014; Cottone *et al*, 2012a). Food reinforcers were delivered by a pellet

dispenser; water reinforcers were delivered by a solenoid into a liquid cup nosepoke receptacle. Two retractable levers were placed on the opposite wall of the chamber. 28-V stimulus cue-lights were located above each lever and above the food magazine. All responses were recorded automatically by a microcomputer with 10 ms resolution.

Operant binge-like eating procedure in *ad libitum* fed rats

Training: Rats were habituated to the home-cage AIN-76A-based diet, hereafter referred to as 'Chow A/I' (5TUM diet formulated as 4–5 g extruded pellets, 65.5% (kcal) carbohydrate, 10.4% fat, 24.1% protein, 330 cal/100 g; TestDiet, Richmond, IN). As previously described (Blasio *et al*, 2014; Cottone *et al*, 2012a; Velazquez-Sanchez *et al*, 2014), animals were trained to self-administer food pellets (45-mg precision food pellets (Chow A/I)) and water (100 µl) under a fixed ratio 1 (FR1) schedule of reinforcement in the operant chambers. During instrumental training, food pellets were 45-mg precision pellets, identical in composition to the diet that rats received in the home cage as ~5 g extruded pellets. Therefore, in the operant chambers, rats were provided with a diet identical to the one received in the home cage during the remaining 23 hours, to ensure that food intake during operant sessions was not influenced by any hedonic factor, but only by homeostatic needs (Blasio *et al*, 2014; Cottone *et al*, 2012a; Velazquez-Sanchez *et al*, 2014). Daily 1h sessions were performed before dark cycle onset. Pellet delivery was paired with a light-cue located above the nose-poke hole.

Testing: After stable baseline performance was achieved in the 1h FR1 schedule self-administration sessions, rats were matched for body weight, daily food intake, feed efficiency, water and food responding in the self-administration sessions. Half of the rats were assigned to a

"*Chow*" control group, in which the operant boxes dispensed the same 45 mg chow pellets offered in the training phase. The remaining rats were assigned to a "*Palatable*" group, which received a nutritionally complete, chocolate-flavored, high-sucrose (50% kcal) AIN-76A-based diet, comparable in macronutrient composition and energy density to the chow diet (chocolate-flavored Formula 5TUL: 66.7% [kcal] carbohydrate, 12.7% fat, 20.6% protein, metabolizable energy 344 cal/100g; formulated as 45 mg precision food pellets; TestDiet, Richmond, IN). It was previously determined that this chocolate-flavored diet is strongly preferred by all rats (Cottone *et al*, 2008, 2009).

Experiment 1: Effects of systemic administration of memantine on operant binge-like eating. Chow and *Palatable* rats (n=17), trained in the binge-like eating procedure, were injected with memantine (0, 1.25, 2.5, 5, and 10 mg/kg, *i.p.*), 30 min prior to the operant sessions, using a within-subject Latin square design.

High rate of responding for Chow A/I induced by food restriction

This task was performed to determine whether the effects of drug treatment on the binge-like eating procedure were palatability-dependent or only dependent on the rate of responding (Cottone *et al*, 2012a). A separate cohort of rats was trained to acquire operant self-administration for Chow A/I diet. To reach a higher rate of responding for Chow A/I during the operant self-administration sessions, rats were food restricted in their home cages. For this purpose, a specific amount of Chow A/I food was provided in the home cages at the end of the operant self-administration sessions so that the total daily intake, including the food consumed during the self-administration session, equaled the 70% of a rat daily intake. This expedient was used to: i) ensure that the rats consumed the entire home cage food before the beginning of the

following self-administration session, ii) induce overeating in the operant self-administration sessions which was driven by energy homeostatic factors and, therefore, iii) increase the rate of responding for the Chow A/I diet in the operant self-administration sessions (Cottone *et al*, 2012b). Under these experimental conditions, the rate of responding for the Chow A/I diet of food-restricted rats was comparable to the rate of responding for the highly palatable sugary diet of ad libitum-fed *Palatable* rats. Rats were food restricted for 10 days before the drug treatment was initiated.

Experiment 2: Effects of systemic administration of memantine on high rate of responding for Chow A/I induced by food restriction. Food-restricted rats (*n*=10), trained in FR1 schedule for Chow A/I, were injected with memantine (0, 10 mg/kg, *i.p.*) 30 min prior to their operant sessions, using a within-subject Latin square design.

Food seeking behavior in *ad libitum* fed rats: second-order schedule of reinforcement

Food-seeking behavior under a second-order schedule of reinforcement is a procedure in which responding is maintained by the contingent presentation of food-paired stimuli that serve as conditioned reinforcers of instrumental behavior (Everitt and Robbins, 2000). The task was adapted from a previously published procedure (Giuliano *et al*, 2012) and modified to be used in *ad libitum* fed rats. A cohort of *Chow* and *Palatable* rats, trained in the *ad libitum* binge-like eating procedure, was used. In the second-order schedule of reinforcement (FI5(FR10:S)) every 10^{th} active lever press (Fixed Ratio 10, FR10) resulted in a brief illumination of lights above both the active lever and the food magazine for 1 s. Responses on the inactive lever had no programmed consequences but were recorded to assess discriminated responding and general levels of motor activity. Following the tenth active lever press, after a Fixed Interval of 5 min

(FI5 min) had elapsed (Gonzalez and Goldberg, 1977; Kantak *et al*, 2013; Kelleher and Goldberg, 1977; Thornton-Jones *et al*, 2005), 20 pellets (45-mg chow pellets for *Chow* rats or 45-mg chocolate pellets for *Palatable* rats) were delivered in the food magazine, both the active and inactive levers retracted, and the lights above both the active lever and the food magazine were presented for 20 s time out. During the FI interval animals who pressed the active lever did not receive any pellets. After the time out, the lights above both the active lever and the food magazine turned off, and the two levers were again extended into the chamber. The second-order schedule of reinforcement session lasted 40 min. The number of responses on the active lever in this task in *ad libitum* fed rats was comparable or higher to previously published second order seeking procedures in food restricted rats (Thornton-Jones *et al*, 2005).

Experiment 3: Effects of systemic administration of memantine on food-seeking behavior using a second-order schedule of reinforcement.

Chow and *Palatable* rats (n=15), trained in the second-order schedule of reinforcement, were injected with memantine (0, 2.5, 5, and 10 mg/kg, *i.p.*) 30 min prior to their operant sessions, using a within-subject Latin square design.

Compulsive eating of palatable food: light/dark conflict test

In this test, a light/dark rectangular box (50×100×35 cm) was used, in which the aversive, bright compartment (50×70×35 cm) was illuminated by a 60 lux light. The dark compartment (50×30×35 cm) had an opaque cover and ~0 lux of light. The two compartments were connected by an open doorway which allowed the subjects to move freely between the two (Cottone *et al*, 2012a; Dore *et al*, 2014; Teegarden and Bale, 2007). A shallow, metal cup containing a pre-weighed amount of the same food received during self-administration (45-mg chow pellets for

Chow rats or 45-mg chocolate pellets for *Palatable* rats) was positioned in the center of the light compartment. Rats were habituated to an ante-room 2 h prior to testing. White noise was present during both habituation and testing. On the test day, rats were placed into the light compartment, facing both the food cup and the doorway. Under normal, control conditions, eating behavior is typically suppressed when a rat is in the aversive, bright compartment; a significant increase in food intake in spite of the adverse conditions, as compared to control conditions, was operationalized as a construct of "compulsive-like eating" (Cottone *et al*, 2012a; Davis *et al*, 2011; Dore *et al*, 2014; Heyne *et al*, 2009; Johnson and Kenny, 2010; Velazquez-Sanchez *et al*, 2014). The apparatus was cleaned with a water-dampened cloth after each subject.

Experiment 4: Effects of systemic administration of memantine on compulsive-like eating. *Chow* and *Palatable* rats (total n=39) were injected with memantine (0, 2.5 mg/kg, *i.p.* the lowest dose effective in reducing binge-like eating) 30 min prior to the 10-min light/dark conflict test, using a between-subjects design.

Intracranial surgeries, microinfusion procedure and cannula placement

Intracranial surgeries. Rats were stereotaxically implanted with bilateral, intracranial cannulas as described previously (Dore *et al*, 2013; Iemolo *et al*, 2013; Sabino *et al*, 2007). Briefly, 24 gauge stainless steel guide cannulas (Plastics One, Inc., Roanoke, VA) were lowered bilaterally 1.5 mm above the NAcc shell or core. Four stainless steel jeweler's screws were fastened to the rat's skull around the cannula. Dental restorative filled resin (Henry Schein Inc., Melville, NY) and acrylic cement were applied, forming a pedestal firmly anchoring the cannula. The cannula coordinates from bregma used for the NAcc shell were: A/P +1.06 mm, M/L ± 0.75 mm, D/V -5.5 mm, flat skull. The cannula coordinates from bregma used for the NAcc core

were: A/P +1.4 mm, M/L ± 2.5 mm (6° angle), D/V -5.5 mm, flat skull. Cannula coordinates were calculated according to the Paxinos atlas (Paxinos, 1986). Stainless steel dummy stylets (Plastics One, Inc., Roanoke, VA) maintained patency of the cannula. After surgery, the rats were allowed a recovery period, during which they were handled daily.

Microinfusion procedure. For intracranial microinfusion, the dummy stylet was removed from the guide cannula, and was replaced with a 33-gauge stainless steel injector projecting 1.5 mm beyond the tip of the guide cannula; the injector was connected via PE 20 tubing to a Hamilton microsyringe (Hamilton Company, Reno, NV) driven by a multi-syringe microinfusion pump (Kd Scientifics, Holliston, MA). Microinfusions were performed in 0.5 µl volume per side delivered over 2 minutes; injectors were left in place for 1 additional minute to minimize backflow.

Cannula placement. Cannula placement was verified at the conclusion of all testing. Subjects were anaesthetized (isoflurane, 2–3% in oxygen) and microinfused with India Ink (0.5 µl/side). Brains were then flash-frozen and stored at -80°C. Coronal sections of 30 µm were collected using a cryostat and placements were verified under a microscope. 7 subjects (n=2 for NAcc shell, and n=5 for NAcc core) were excluded from analysis because of incorrect cannula placement.

Experiment 5: Effects of administration of memantine into the NAcc shell on operant binge-like eating. Intra-NAcc shell cannulated rats (n=11), trained in the binge-like eating procedure, were injected with memantine (0, 2.5, 10 and 20 µg/side), immediately before their operant sessions, using a within-subject Latin square design.

Experiment 6: Effects of administration of memantine into the NAcc core on operant binge-like eating.

Intra-NAcc core cannulated rats (*n*=14), trained in the binge-like eating procedure, were injected with memantine (0, 2.5, 10 and 20 μg/side), immediately before their operant sessions, using a within-subject Latin square design.

Statistical Analysis

Data from binge-like eating, PR, second-order schedule of reinforcement tests were analyzed using two-way ANOVAs, with Dose as a within-subject factor, and Diet as a between-subjects factor. Data from the food restriction experiment were analyzed using 2-tailed paired Student's *t*-test (Dose as a within-subject factor). Data from the light/dark conflict test were analyzed using a two-way between-subjects ANOVA (Dose as a between-subjects factor, and Diet as a between-subjects factor). Follow up pair-wise comparisons were analyzed using the Newman-Keuls post-hoc test where appropriate.

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