High trait impulsivity predicts food addiction-like behavior in the rat

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Running title: High impulsivity predicts food addiction

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SUPPLEMENTARY MATERIALS AND METHODS

Subjects

Male Wistar rats (*n*=47), 45 days old on arrival (Charles River, Wilmington, MA), were triple-housed in wire-topped plastic cages in a 12-h reverse light cycle (lights off at 11:00 am) AAALAC-approved humidity-(60%) and temperature-controlled (22°C) vivarium. Animals were allowed a four-day habituation period to the research facility. Corn-based chow (Harlan Teklad LM-485 Diet 7012) and water were available in the home cage. Procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Principles of Laboratory Animal Care, and were approved by Boston University Medical Campus Institutional Animal Care and Use Committee. No experimental procedures involved food or water restriction/deprivation.

Apparatus

Operant chambers (30×24×29 cm) were identical to those previously described (1, 2). Briefly, the chambers (Med Associates, St Albans, VT) had wire- mesh floors and were located in ventilated, sound-attenuating enclosures (66×56×36 cm). A syringe pump delivered the solution into a stainless steel receptacle located 2 cm above the floor in the middle of the left wall of the chamber. Levers were placed 3.2 cm next to the drinking cup. Food pellets were delivered by a dispenser into a nosepoke aperture on the opposite wall of the chamber. 28-V stimulus cue-lights were located above each lever and above the food magazine. Water was also delivered by a solenoid into a liquid cup nosepoke receptacle placed in the center of the right wall. All responses were recorded automatically by a microcomputer with 10 ms resolution.

Differential reinforcement of low rates of responding (DRL) task in *ad libitum* fed rats

Performance on the DRL task has been used to provide a measure of impulsive action, defined as the inability to withhold a response (3-5). The DRL procedure was adapted from previous reports (6-8) in *ad libitum* fed rats. Rats were trained to lever press for a "supersaccharin" solution in an overnight fixed ratio 1 (FR1) self-administration session (9, 10), consisting of 1.5% w/v glucose and 0.4% w/v saccharin (9, 10). Rats were then trained on a DRL-5 s schedule for four sessions, during which a lever press resulted in the delivery of the supersaccharin solution if at least 5 s had elapsed since the previous lever press. If the rat made a premature lever press, the 5 s time period was reset; thus, rats were only reinforced if they withheld a response for longer than 5 s. Rats were then trained for four sessions on a DRL-10 s schedule, during which the response had to be withheld for 10 s in order to obtain reinforcement. Finally, rats were given 15 sessions on a DRL-15 s schedule. Impulsive action was assessed on the last four sessions of the DRL-15 s schedule, and was defined as efficiency [ratio between the rewarded responses and the total (rewarded + incorrect) responses], with higher efficiency reflecting more accurate performance, indicative of less impulsive action. During the last four sessions of DRL-15 s schedule, responding was highly stable and an intraclass correlation analysis of the efficiency showed very high internal consistency (Intraclass Correlation, Efficiency: $ICC[2,4]=0.93$, $F(28,84)=15.61$ $p<0.0001$) across the 4 days. The high intraclass correlation coefficient suggests very stable individual differences. Rats were ranked according to their efficiency scores and subjects falling above the 60^{th} percentile were assigned to the *Low-impulsive* group, while subjects falling below the 40^{th} percentile were assigned to the *High-impulsive* group.

Spontaneous locomotor activity

The analysis of locomotor reactivity to a novel environment was carried out during the first two hours of the dark phase as previously shown (11). The motor activity was measured in novel Plexiglas chambers (27×48×20 cm) using an Opto –M3 activity system (Columbus Instruments, Columbus, OH). The Opto-M3 system consisted of a series of 16 sensor beams spaced 2.54 cm apart and able to measure horizontal activity. Sensor beams were located along the longest side of the horizontal plane of the cage. White noise was present throughout testing. Rats were ranked according to their photocell beam breaks and subjects falling below the 40th percentile were assigned to the *Low-responder* group, while subjects falling above the 60th percentile were assigned to the *High-responder* group.

Elevated Plus-Maze test

The elevated plus-maze (12-14) apparatus was made of black Plexiglas and consisted of four arms (50 cm long 10 cm wide). Two arms had 40-cm-high dark walls (enclosed arms), and two arms had 0.5-cm-high ledges (open arms). The maze was elevated to a height of 50 cm. Open arms received 1.5–2.0 lux of illumination. Animals were habituated to the anteroom the day before testing. On the day of testing, rats were kept in the quiet, dark anteroom for at least 2 h before testing. White noise was present throughout habituation and testing. For testing, rats were placed individually onto the center of the maze facing an open arm and removed after a 5-min period. The apparatus was cleaned with water and dried between subjects. The primary measures were the percentage of total arm time directed toward the open arms [i.e., 100*open arm/(open arm+closed arm)], a validated index of anxiety-related behavior and the number of closed arm entries, a specific index of locomotor activity. Rats were ranked according to their percentage of open arm time and subjects falling above the 60th percentile were assigned to the *Low-anxiety* group, while subjects falling below the 40th percentile were assigned to the *High-anxiety* group.

Binge-like eating procedure in *ad libitum* fed rats

Following the DRL procedure and separation of *Low*- and *High-impulsive* rats, *ad libitum* fed rats were trained to acquire a nosepoke response for food and water in individual test cages (30×24×29 cm) on a fixed ratio 1 (FR1) continuous schedule of reinforcement in 1 h sessions, as described previously (1, 2). The operant boxes had grid floors and were located in ventilated, sound-attenuating enclosures (66×56×36 cm). Food reinforcers were delivered by a pellet dispenser (Med Associates Inc., St. Albans, VT). 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 (5TUM diet formulated as 4-5g extruded pellets, 65.5% [kcal] carbohydrate, 10.4% fat, 24.1% protein, metabolizable energy 330 cal/100 g; TestDiet, Richmond, IN). Therefore, in the operant chambers, rats were provided with a diet identical to the one received in the home cage to ensure that Chow rats' food intake during operant sessions was not influenced by any hedonic factor, but only by homeostatic needs (1, 2, 15, 16). Pellet delivery was paired with a light-cue located above the nosepoke hole. Water reinforcers were 100 µl in volume, delivered by a solenoid into a liquid cup nosepoke receptacle. The sessions were performed daily before dark cycle onset. After attaining stable baseline performance in the 1-hr self-administration sessions, the testing procedure was initiated. Rats were assigned to either a "Chow" control group (*Low-impulsive/Chow* and *High-impulsive/Chow*) and continued to receive the same 45-mg chow pellets offered in the training phase, or a "Palatable" group (*Low-impulsive/Palatable*, and *High-impulsive/Palatable*), which instead 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/100 g;

formulated as 45 mg precision food pellets; TestDiet, Richmond, IN). This chocolate-flavored diet is strongly preferred by all rats (16, 17). The rats were tested daily for 1 h sessions. After acquiring stable responding under the FR1 schedule of reinforcement, the number of responses required to obtain one pellet was increased from FR1 to FR3 during 4 consecutive sessions, and then from FR3 to FR5 for 4 additional sessions.

Progressive ratio schedule of reinforcement for food

Following testing under the fixed ratio schedules, rats were moved to a progressive ratio schedule of reinforcement, where the number of responses required to obtain a food pellet increased with successive food reinforcers based on the following shallow exponential progression: response ratio = $[4\cdot(e^{\#\text{ of reinforcer}*0.075}) - 3.8]$, rounded to the nearest integer. To avoid unintended session starts (e.g., due to exploratory, rather than food-directed activity), the first reinforcement required three responses. Thus, the progressive ratio schedule was 3, 1, 1, 2, 2, 2, 3, 3, 4, 5, 5, 6, 7, 8, 9, 9, 11, 12, 13, 14, 16, 17, 19, 20, 22, 24, 27, 29, etc. responses. Sessions ended when subjects had not completed a ratio for 14 min, with the last completed ratio defined as the breakpoint. This criterion was used because male Wistar rats do not voluntarily wait longer than 14 min between meals without eating a pellet (12, 18). Thus, sessions involved rats initiating a meal but with the meal ending prematurely (prior to full satiation); when escalating, response requirements surpassed the rats' breakpoints. The latency was set to a maximum of 1 h. At the end of each session, subjects were returned to their home cage, where the regular chow was always available *ad libitum*.

Light/Dark conflict test

The day after the last PR session, rats were tested in a light/dark conflict test. The same rats used for the development of the binge-like eating procedure were tested in a light/dark rectangular box (50×100×35 cm) 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 (2, 19, 20). 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 environment; a significant increase in food intake in spite of these adverse conditions, as compared to control conditions, was operationalized as a construct of "compulsive-like eating" (2, 11, 20-24). The apparatus was cleaned with a water-dampened cloth after each subject. Rats had access to food *ad libitum* at all times; water was not available during the 10-min test.

ΔFosB immunohistochemistry

Perfusions and immunohistochemistry

At the end of the behavioral procedures, animals were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1 phosphate buffer (PB) under deep isoflurane anesthesia. Brains were removed, postfixed in 4% PFA for 24 h and then stored in 30% sucrose in PB buffer at 4°C until saturation. Brains were sectioned coronally at 30μm using a cryostat and stored in a cryoprotectant solution at -20°C. For technical reasons

related to the section quality four subjects were excluded. Every sixth section from 2.16 mm of the nucleus accumbens and dorsal striatum were selected in a systematic manner and processed for immunohistochemistry. Briefly, for ΔFosB immunohistochemistry, mounted sections were washed in a phosphate saline (PBS) buffer. After the initial washing, sections were incubated in 1% hydrogen peroxide PBS solution for 10 min to block endogenous peroxidases. Sections were washed again and placed in blocking solution (5% normal goat serum and 0.4% Triton X100) for 1 h respectively. Slides were transferred into primary antibodies (ΔFosB, 1:500, sc-48 Santa Cruz Biotechnology) (25, 26) in blocking solution and incubated for 24 h at 4°C. Following an additional wash, sections were incubated in the secondary antibody (biotinylated goat anti-rabbit 1:500 in PBS, Vector Laboratories) for 1 h at room temperature. Sections were washed and incubated in an avidin–biotin horseradish peroxidase ABC solution (1:1000 in PBS, Vector Laboratories) for 1 h. Slides were then incubated using diaminobenzidine substrate kit (Vector Laboratories) according to the manufacturer's instructions and once the reaction was complete, sections were rinsed in PBS. For the reaction, nickel sulfate was also used. Slides were dehydrated using graded alcohol solutions and coverslipped using DPX mountant (Electron Microscopy Sciences, Hatfield, PA, USA).

Quantification of ΔFosB+ cells

The quantification was performed using an Olympus (Center Valley, PA, USA) BX-51 microscope equipped with a Rotiga 2000R live video camera (QImaging, Surrey, BC, Canada), a three-axis MAC6000 XYZ motorized stage (Ludl Electronics, Hawthorne, NY, USA), and a personal computer workstation. The investigators were blind to the treatment conditions. Contours of the core and shell for the nucleus accumbens and for the dorsal striatum were drawn at 2X magnification using Stereo Investigator software

(MicroBrightField, Williston, VT, USA). The grid frame and the counting frame were set to 360 x 360 μm and 75 x 75 μm, respectively. The thickness was measured several times in a random manner in each animal and an average was used to estimate the total volume of the sample region and the total number of ΔFosB cells. Two subjects were excluded because outliers. The results were expressed as the number of ΔFosB-positive cells (cell/volume) for each of the region studied.

Data and Statistical analyses

Establishment of food addiction-like criteria, food addiction-like score and subpopulations of rats.

The approach used to establish the food addiction-like criteria and the food addiction-like score was analogous those previously used to establish addiction-like behaviors in cocaine self-administering outbred rats (11, 27). Animals were ranked for each criterion independently. The five-day average of the plateaued intake under FR1 responding, the two-day average of the breakpoint under the progressive ratio schedule of reinforcement and the intake in the light/dark conflict test, were used to operationalize the three food addiction-like behaviors [*i*) excessive intake, *ii*) motivation for food, and *iii*) compulsive-like eating]. The three food addiction-like criteria were operationally defined based on the following inclusion/exclusion condition: a subject was considered positive for a given criterion, if it fell above the 67th percentile of that distribution. If a rat was considered positive for a given food addiction-like criterion, it was given an arbitrary criterion score of 1. Then the arbitrary criteria scores were added so that four different groups were identified depending on the number of positive criteria they met (from 0 to 3). The food addiction-like score was calculated as follows (11, 27):

Food Addiction-like score $= \sum_1^n z_n$

where $z_n = (x_i - \bar{x}_n)/s_n$

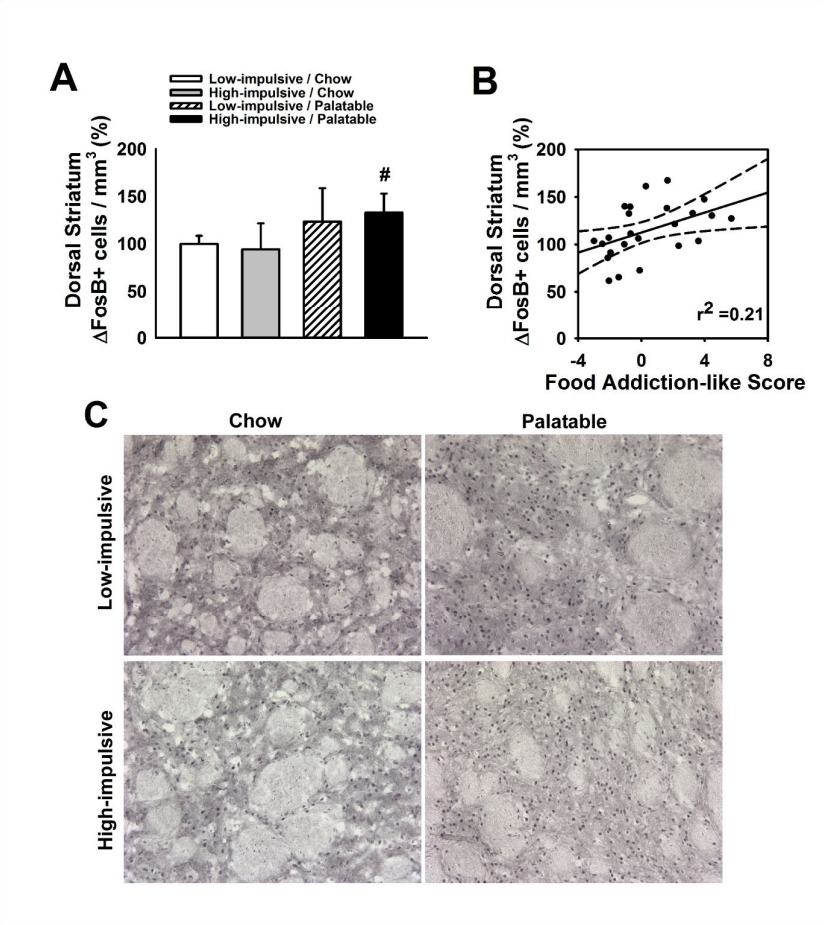
The food addiction-like score was calculated as the algebraic sum of the individual standardized scores (z_n), of each of the three food addiction-like behaviors (n, excessive intake, motivation for food, or compulsive-like eating). Each individual standardized score (z_n) for a given distribution (n) was calculated by subtracting each behavioral measure (x_i) of each individual subject (i) from the mean of that distribution $(\bar{x}_n)$ and this difference was then divided by the standard deviation of that distribution (s_n). Individual standardized scores had a mean of 0 and a standard deviation of 1.

Data analysis

Two-way analyses of variance (ANOVAs) with Impulsivity and Food as between-subjects factors were used to analyze efficiency in the DRL test, Day 1 food intake, food intake under FR3 and FR5 schedules of reinforcement, breakpoint under a progressive ratio schedule of reinforcement, intake in the light/dark conflict test, food addiction-like score, and ΔFosB-positive cell counts in the nucleus accumbens core and shell. Changes in daily food intake over the first 14 days of testing were analyzed using a three-way mixed-design analysis of variance with Impulsivity and Food as between-subjects factors and Day as a within-subject factor. To evaluate the internal consistency of measurements and to determine whether rats stably differed in their individual performances, two-way, random effect intraclass correlations (*ICC*) of absolute agreement (9, 13, 17, 28) were performed on the efficiency during the last 4 days of training in the DRL. Differences in food intake in FR1 schedule of reinforcement, breakpoint under progressive ratio schedule of reinforcement, and intake in the light/dark conflict test across the 4 criteria were analyzed using one-way

ANOVAs with Criteria as a between-subjects factor. Differences in the food addiction-like score across the 4 criteria were analyzed using one-way ANOVA with Criteria as a between-subjects factor. Regression analyses were used to test the relationship between the criteria and food intake in FR1 schedule of reinforcement, breakpoint under progressive ratio schedule of reinforcement, and intake in the light/dark conflict test. Pairwise comparisons were interpreted using Fisher's LSD tests. The software/graphic packages were Systat 12.0 and SigmaPlot 12.0 (Systat Software Inc., Chicago, IL), SPSS Statistics 18 (SPSS Inc., Chicago, IL), InStat 3.0 (GraphPad, San Diego, CA).

SUPPLEMENTARY FIGURES



Supplementary Figure 1. ΔFosB expression in the dorsal striatum. (*A* and *B*) ΔFosB expression in the dorsal striatum expressed as density change (%) in relation to the *Low-impulsive/Chow* group. Statistical analysis revealed a main effect of Food (Food: $F_{1,20}$=9.5, $p \leq 0.05$). (*B*) A small correlation between the food addiction-like behavior and ΔFosB expression in the dorsal striatum was found (r^2=0.21, $p \leq 0.05$). (*C*) Representative micrographs (20X) of ΔFosB expression in dorsal striatum of the different groups are shown. *n=24*. Data show *M*±SEM.

Supplementary References

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