Cognitive and neural determinants of response strategy in the dual-solution plus-maze task

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Supplementary Methods

Experimental groups

Animals were randomly assigned to four groups, according to the pharmacological treatment and the extension of training: 26 mice were injected with saline (Sham) into the DMS and trained for 26 days; 21 mice were injected with saline (Sham) into the DLS and trained for 61 days; 22 mice were injected with BoNT/A into the DMS (DMS) and trained for 26 days; 13 mice were injected with BoNT/A into the DLS (DLS) and trained for 61 days. The same two vehicle-injected groups are used to describe the effect of devaluation on the RS as well as to be compared to striatal lesions. The number of animals using the response strategy submitted to the devaluation of the outcome procedure was: Sham trained for 26 days =22; Sham trained for 61 days =15; DMS trained for 26 days =15; DLS trained for 61 days =9. In the DMS experiment the study was run once and then replicated with another squad of animals (no differences were revealed between the different runs). Other 8 animals were focally injected in the DMS and used for immunostaining for cleaved SNAP-25.

Surgery

Mice were anesthetized by intraperitoneal injection of chloral hydrate (500 mg/kg; Fluka) and placed in a stereotaxic apparatus (David Kopf Instruments) with mouse adapter and lateral ear bars. The mice were bilaterally implanted with 7-mm-long bilateral stainless steel cannulae dorsal to the dorsomedial or dorsolateral striatum at the following coordinates: AP = +0.3 mm; L = ± 2.0 mm; DV = -1.6 mm; and AP = +0.4 mm; L = ± 2.5 mm; DV = -1.7 mm
relative to bregma, respectively for DMS and DLS according to the mouse brain atlas (Franklin and Paxinos, 1997) according to a previously described procedure (De Leonibus et al. 2005). Guide cannulae were fixed on the skull surface with dental acrylic cement. After cannulae implantation, mice were injected into the DMS or DLS either with saline (0.9%) or with Botulinum neurotoxin A (BoNT/A) (25 pgtox/0.25 µl saline) solution. BoNT/A was a kind gift from Prof. C. Montecucco (Padua University, Italy). The toxin was frozen in liquid nitrogen and stored at -80°C in 10 mM NaHepes, 150 mM NaCl, pH 7.2. Stock solutions of 150-kDa di-chain molecule of purified BoNT/A were tested for activity in the ex vivo mouse hemidiaghram model and in the in vitro cleavage of SNAP-25 and VAMP/synaptobrevin. Injectable solutions of BoNT/A were freshly made in saline (0.9% NaCl). An 8-mm injection needle connected with polyethylene tubing to a 2.0-µl Hamilton syringe was inserted into the guide cannula. A volume of 0.25 µl/side of BoNT/A or saline solution was infused. Each infusion lasted for 1 minute, followed by 3 additional minutes with the needle left in place to allow diffusion. After infusion, animals were allowed to recover for approximately 4-5 days. At the end of the behavioral training the injection needle was again inserted into the cannula to trace the site of drug injection. Immediately after the animals were sacrificed, the brains removed and fixed in a 4% formaldehyde solution. The brains were cut into 40 µm-sections using a freezer microtome. Injector placements were determined by examining serial coronal sections stained with Cresyl Violet.

**Outcome devaluation procedure**

After the first probe test, the maze was rotated by 180° again, and the training took place as usual, only for animals using the RS. Mice were also submitted to an outcome devaluation procedure based on conditioning taste aversion (CTA), which lasted 5 consecutive days (Fig. 1A – 3rd panel). Immediately after training the mice were placed in a waiting cage placed in the experimental room, out of the gazebo were the maze was located. There they received 50 grains of chocolate and were allowed to eat them in a time limit of 15 minutes. After this period, animals were injected i.p. with either saline (valued group) or lithium chloride 0.6 M (devalued group) and left in their waiting cages for 2 hours. Development of taste aversion for chocolate was assessed by measuring the percentage of chocolate consumed both in the waiting cages [(number of chocolate grain consumed/50)*100] and in the maze during training [number of chocolate grain retrieved/15)*100]. A new probe test from the novel arm was performed only when food consumption in the maze was significantly reduced (after 5 days for all groups).

Effects of devaluation of the outcome on RS were analyzed during the last conditioning day in the maze calculating the percentage of correct trials [(number of consistent turn/15)*100]. The day after a further probe test (Fig. 1A – 4th panel) was given to test the effects of outcome devaluation from the novel arm.
**Immunofluorescence and confocal analysis**

To test the effectiveness and diffusion of BoNT/A in the mediodorsal striatum a group of mice (n=8) was unilaterally injected into the DMS with BoNT/A and vehicle on the contralateral side. 7 (n=4) or 46 (n=4) days after surgery BoNT/A injected animals were deeply anesthetized with avertin and perfused through the aorta with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 and equilibrated with 30% sucrose overnight. Coronal sections (30 µm thick) were cut with a freezing microtome. Serial sections (one of five) were pre-incubated with a 10% Normal Donkey serum solution (NDS) for 1 h at room temperature and then incubated overnight at 4°C a polyclonal antibody raised against the BoNT/A-truncated C-terminal peptide of SNAP-25 (1:500 dilution). The antibody recognizes specifically the cleaved form of SNAP-25 and not the whole protein, and has been also correlated to the block of neuronal activity in the injected area. On the following day, sections were incubated with Cy3-conjugated secondary antibody (Jackson ImmunoResearch) 1:200 for 2h at RT and rinsed in PBS 1x. Some sections were incubated with DAPI (4′,6-diamidino-2-phenylindole, Molecular Probes) solution (300nM DAPI in 0.1M PBS) for 15 min RT. Sections were mounted on poly-L-lysine coated slides, dried on air and coverslipped. We used a LSM5 Zeiss confocal laser-scanning microscope (Zeiss, Germany). A 5x, 10x and 63x oil-immersion lens were used. An initial analysis on stained sections from BoNT/A and saline hemispheres was performed to establish settings for laser intensity, gain, offset, and pinhole size. Care was taken to avoid saturation at either end of the pixel intensity range (0 –255). Confocal settings were then held constant through the study. We measured the spread of staining around the injection site to test the diffusion of the BoNT/A. The spread analysis of staining around the injection site was performed using a 10x objective with an additional digital zoom factor of 0.7x (field). The placement of injection was verified examining the serial sections in bright field and phase contrast. The image with the highest average pixel intensity was selected for the spread analysis (MetaMorph; Crisel Instruments, srl). The spread area of the staining was defined as the area in which the fluorescence signal was 15% higher than background. For each brain, background was determined by measuring the signal intensity in the hemisphere injected with saline. This value was used to calculate the threshold.

**Statistical analyses**

Percentage of correct trials during training was analyzed by two-way ANOVA for repeated measures, with Treatment (2 levels: sham and BoNT/A) as between factor, and Days (26 or 61 levels) as repeated measure. Effects of outcome devaluation were analyzed by means of three-way ANOVA with Treatment (2 levels: sham and BoNT/A) and Outcome (2 levels: valued and devalued) as between factors, and days (2 levels: pre-dev, post-dev) as repeated measure. Tukey Honestly Significant Difference (HSD) post hoc analysis was used when appropriate.
Binomial test was used to analyze the frequency of response and place strategy on the first probe test, while a simple *Chi square* test was used for comparisons within group to evaluate any strategy change from 100% after outcome devaluation, and the *Yates Chi square* test was applied for comparisons between groups (Sham vs DMS or Sham vs DLS) on the probe tests.
Supplementary Figures

Figure Captions

Figure S1. Outcome devaluation procedure strongly affects food consumption both in the waiting cage and in the maze, independently on the training and on the striatal treatment. The number of animals using the response strategy submitted to the devaluation of the outcome procedure was: Sham trained for 26 days =22; Sham trained for 61 days =15; DMS trained for 26 days =15; DLS trained for 61 days =9. For each experiment the study was run once and then replicated with another squad of animals. Outcome devaluation decreased food consumption both in the waiting cage (A,C) and in the maze (B,D) independently on the number of training days (26 days: (A,B) or 61 days: (C,D) and independently on the treatment (Sham vs BoNT/A within the DMS, (A,B) or the DLS, (C,D). [A: Outcome (F1/33=173.841; p<0.0001); Day (F1/33=292.262; p<0.0001); B: Outcome (F1/33=30.209; p<0.0001); Day (F1/33=33.149; p<0.0001); Day x Treatment (F1/33=4.65=0.04); Day x Outcome (F1/33=32.787; p<0.0001); Treatment x Day x Outcome (F1/33=3.91; p=0.056); C: (Outcome (F1/20=355.496; p<0.0001); Day (F1/20=467.98; p<0.0001); Outcome x Day (F1/20=355.496; p<0.0001); D: Outcome (F1/20=13.121; p=0.0017); Day (F1/20=15.33; p=0.0009); Outcome x Day (F1/20=11.410; p=0.003)]. The only minor difference was a further reduction of food consumption in the maze of DMS lesioned animals as compared to sham animals; * p<0.05 Tukey Honestly Significant Difference (HSD) post hoc analysis to compare Pre-dev vs. Post-dev within group; # p<0.05 Tukey Honestly Significant Difference (HSD) post hoc analysis to compare BoNT/A vs. Sham, within pre-dev/post-dev treatment.

Figure S2. Dorsolateral and dorsomedial striatal lesion on performance across 26 or 61 days of training. Percentage correct trials in mice trained in the dual-solution plus-maze task. Pre-training dorsomedial (A) or dorsolateral striatal (C) injection of BoNT/A did not influence the percentage of correct trials during training. (B) Percentage of mice using a place or a response strategy on probe test 1 (day 27) in sham and DMS BoNT/A injected groups. (D) Percentage of mice using a place or a response strategy after overtraining on probe test 1 (day 61) in the sham or in the DLS BoNT/A injected group.