Features of compulsive checking behavior mediated by nucleus accumbens and orbital frontal cortex

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FEATURES OF COMPULSIVE CHECKING BEHAVIOR MEDIATED BY NUCLEUS ACCUMBENS AND ORBITAL FRONTAL CORTEX

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Abstract

The quinpirole sensitization model of obsessive-compulsive disorder (OCD) was used to investigate the functional role that brain regions implicated in a neuroanatomical circuit of OCD may play in compulsive checking behavior. Following repeated injections of saline or quinpirole (0.5 mg/kg, twice a week, x 8 injections) to induce compulsive checking, rats received N-methyl-D-aspartate lesions of the nucleus accumbens core (NAc), the orbital frontal cortex (OFC) and the basolateral amygdala (BLA), or sham lesions. When retested 17 days post-surgery, results showed effects of NAc and OFC but not BLA lesion. NAc lesions affected measures indicative of the amount of checking behavior, while OFC lesions affected indices of staying away from checking. The pattern of results suggested that functional roles of NAc and OFC in checking behavior are to control vigor of motor performance and focus on goal-directed activity, respectively. Furthermore, similarities in behavior between quinpirole sham rats and saline NAc lesion rats suggested that quinpirole may drive the vigor of checking by inhibition of NAc neurons, and that NAc may be a site for the negative feedback control of checking.

Introduction

Amongst psychiatric disorders, biological research of obsessive-compulsive disorder (OCD) is somewhat privileged since there is remarkably good theoretical agreement of the neuroanatomical network underlying this disorder. Most models posit that OCD emanates from some perturbation in cortical-basal ganglia circuits (Wise & Rapoport, 1989; Modell *et al.*, 1989; Baxter *et al.*, 1992; Insel, 1992; Graybiel & Rauch, 2000; Saxena *et al.*, 2001; Szechtman & Woody, 2004; Aouizerate *et al.*, 2004; Huey *et al.*, 2008). However, there is less agreement as to what is the function of relevance to OCD that this network performs—some suggest that it is the mediation of innate motor programs (typical of OCD rituals) (Wise & Rapoport, 1989); others that it is the mediation of implicit learning (Rauch *et al.*, 2001); and still others that it is the mediation of security motivation (Szechtman & Woody, 2004). Moreover, there is no agreement as to which working component of the circuit is dysfunctional — some models identify the fault to be a "starting" mechanism which activates the network too readily (Wise & Rapoport, 1989) while others consider the problem to lie with a "stop" mechanism which fails to terminate network activity appropriately (Szechtman & Woody, 2004).

Such questions of biological mechanisms can be pursued experimentally in animal models. Here, we ask whether three brain areas postulated as parts of the neural network underlying OCD play a role in expression of compulsive checking behavior in an animal model, and if so, what functional role these regions subserve to produce this profile of behavior. The experimental approach involved excitotoxic lesions of the basolateral amygdala (BLA), the nucleus accumbens

core (NAc) and the orbital frontal cortex (OFC), brain regions considered of relevance to OCD (Graybiel & Rauch, 2000; Szechtman & Woody, 2004; Aouizerate *et al.*, 2004; Huey *et al.*, 2008).

The preparation used was the quinpirole sensitization rat model of OCD (Szechtman *et al.*, 1998; Dvorkin *et al.*, 2006b) in which repeated treatment with the D2/D3 dopamine agonist quinpirole induces compulsive checking behavior. In the rat, compulsive checking behavior is characterized by exaggerated pre-occupation with one place in the environment (a large open field), to which the animal returns repeatedly. Such behavior in the rat is similar to OCD checking in the human, according to four lines of evidence (reviewed in Man *et al.*, 2004; Szechtman & Eilam, 2005; Korff & Harvey, 2006; Joel, 2006a; Westenberg *et al.*, 2007). First, the temporal structure of quinpirole-induced compulsive checking and its organization in the environment satisfies performance criteria which define the salient features of an OCD compulsion (Szechtman *et al.*, 1998), namely, an exaggerated preoccupation with the item(s) of concern, a ritual-like quality in motor performance, and environmental dependence for display of the behavior. Second, the motivational basis of quinpirole-induced and OCD checking appear similar (Szechtman *et al.*, 1998; Szechtman & Woody, 2004; Woody & Szechtman, 2005; Boyer & Lienard, 2006; Feygin *et al.*, 2006; Whishaw a0 *et al.*, 2006), in that both represent an exaggerated form of normal checking of stimuli related to safety and security (the "home base" in the case of the rat model). Third, quinpirole-induced compulsive checking is subject to similar modulation as OCD compulsions in that the performance of each is modulated by external stimuli and can be suppressed albeit temporarily (Szechtman *et al.*, 2001; Ben Pazi *et al.*, 2001; Zadicario *et al.*, 2007). Finally, treatments that are therapeutically useful for OCD, are also effective in attenuating quinpirole-induced compulsive checking; for example, clomipramine (Szechtman *et al.*, 1998; Foa

et al., 2005), nicotine (Tizabi *et al.*, 2002; Salin-Pascual & Basanez-Villa, 2003; Lundberg *et al.*, 2004), and deep brain stimulation (Greenberg *et al.*, 2006; Winter *et al.*, 2008; Mundt *et al.*, 2009).

Materials and methods

Subjects

A total of 168 experimentally naïve Long-Evans rats (Charles River, St. Constant, Quebec, Canada) weighing 250-300g at start of experiment entered the study. Rats were individually housed in a temperature-controlled colony room (22℃) under a 12 h light-dark cycle, with free access to food and water. Rats were allowed to acclimatize to colony room for 1 week following arrival and were handled 2-3 min daily for 5 days before start of the experiment. All treatment and testing was conducted during light hours. Animals were housed and tested in compliance with guidelines described in Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

Drugs

Quinpirole hydrochloride (Sigma Aldrich) was dissolved in physiological saline and injected subcutaneously at a dose of 0.5 mg/kg. Rats were injected and tested on a twice weekly schedule. The particular dose and injection regimen of quinpirole had been used in our prior studies and was selected because the 0.5 mg/kg dose of quinpirole is representative of behavioral effects induced by doses of the drug from 0.25 to 2.5 mg/kg, and because behavioral effects of chronic treatment reach a plateau after 8-10 drug injections administered 2-8 days apart (Szechtman *et al.*, 1994a; Szechtman *et al.*, 1994b; Dvorkin *et al.*, 2006a; Perreault *et al.*, 2007).

Apparatus

Animals were tested after each injection in a large open field consisting of a solid surface top table (160 x 160 and 60 cm high). The table top was constructed of material used in making kitchen counter-tops – it was smooth, non-porous, composed of unsaturated polyester and acrylic resin blends (Acryflek Industries), and had a custom blue color to facilitate video detection of dark and white objects. Four small Plexiglas/glass boxes (approximately 8 x 8 x 7.5 cm) were present at same fixed location of the open field throughout study: two at corners and two at places near center of the open field. The open field platform was subdivided virtually into 25 rectangular places (locales) used to define the location of the animal in the field. Open field and objects were wiped clean after each rat with a diluted solution of an antibacterial cleaner (Lysol). Behavior was videotaped continuously by a camera affixed to the ceiling, providing a stationary top view of the entire open field and the rat in it. Videotapes were converted to MPEG files (Canopus MPEGPro EMR realtime MPEG-1 MPEG-2 encoder) and these digitized videos were used to automatically track the trajectories of locomotion using EthoVision 3.1 (Noldus Information Technology bv, Netherlands) system (Noldus *et al.*, 2001; Spink *et al.*, 2001). Spatial sensitivity of the tracking system was 8 mm x 8 mm per pixel, with a temporal resolution of 30 frames per second.

Surgery

Neurotoxic lesions were performed using the excitotoxin N-methyl-D-aspartate (NMDA; Sigma Aldrich, Canada) that was dissolved in phosphate-buffered saline (PBS; Sigma Aldrich, Canada) at a concentration of 7 mg/ml for BLA lesion, 12 mg/ml for NAc and 20 mg/ml for OFC lesion. For sham lesions, an equivalent volume of PBS was injected. Intracranial injections of NMDA and PBS injections were administered using a 10 µl non-coring Hamilton syringe (Hamilton Company, U.S.A) mounted in a motorized Ultra Micro Pump (World Precision Instruments, U.S.A.) attached

to the arm of a Kopf Stereotaxic Apparatus (David Kopf Instruments, U.S.A.). Animals were anaesthetized using vaporized isofluorane (Pharmaceutical Partners of Canada, Canada) and lidocaine hydrochloride (0.002 mg; Astra Zeneca, Canada) was injected subcutaneously at the surgical site. The post-operative non-steroidal anti-inflammatory analgesic Anafen (0.05 mg/kg; Merial, Canada) was administered subcutaneously 10 minutes prior to end of surgery. Animals were allowed 14 days for recovery before resumption of behavioral testing.

Neurotoxin injections for BLA lesion were made bilaterally at two injection sites with following flat skull coordinates (Paxinos & Watson, 1998): (1) antero-posterior (AP), -2.5 mm from bregma; medio-lateral (ML), ± 4.8 mm; and dorso-ventral (DV), -8.1 mm from dura; (2) AP, -3.1 from bregma; ML, ± 4.8 mm; DV, -8.1 mm from dura. At both injection sites, 0.2 µl was injected at a rate of 0.2 µl/min, and cannula was left in place for 3 min to allow for the neurotoxin to sufficiently diffuse away from the cannula. To reduce post-operative seizures and to prevent distal neuronal damage due to excitotoxicity in limbic structures (Fuller & Olney, 1981), diazepam (5 mg/kg; Sabex Inc., Canada) was administered intraperitoneally to all animals 10 minutes prior to first intracranial injection. A second injection of diazepam (1 mg/kg) was administered 20 minutes after the initial diazepam injection.

Coordinates for NAc lesion were: AP, +1.2 mm from bregma; ML, ±1.9; DV, -7.0 mm from dura. At the injection site, 0.3 µl was injected at a rate of 0.1 µl/min, and needle was left in place for 5 min to allow for neurotoxin to sufficiently diffuse away from needle tip.

Coordinates for OFC lesion were: AP, +3.7 mm from bregma; ML, ± 2.6 mm; DV, -4.6 from dura. At the injection site, 0.35 µl of NMDA or PBS was injected at a rate of 0.1 µl/min, and the needle left in place for 5 minutes.

Histological analysis

Four days after final test, rats were euthanized using carbon dioxide. Brains were immediately removed and flash frozen in -60ºC methylbutane, placed on dry ice for 1 minute, wrapped in an aluminum foil and stored in a -80°C freezer until sectioning. Rat brain was mounted for sectioning using Tissue-Tek Optimum Cutting Temperature compound and placed in cryostat for 1 hour to thaw to -20˚C. The coronal plane was sectioned at 20 µm thickness, with every 3rd section collected on a gelatine coated slide and stored in a -35ºC freezer until immunohistochemistry.

Lesion location and size were visualized using neuronal nuclei (NeuN) protein immunohistochemistry: sections were stained using monoclonal mouse anti-neuronal nuclei (1 mg/ml; Chemicon International, U.S.A.) as the primary antibody, followed by a biotinylated monoclonal anti-mouse IgG (0.5 mg/ml; Vector Laboratories, Canada) as the secondary antibody according to a described procedure (Jongen-Relo & Feldon, 2002). Following NeuN staining, each section was examined for location and size of lesion using an Axiskope microscope and Axiovision 4.3 software system (Carl Zeiss Microimaging Inc., U.S.A). Lesion boundaries inside and outside region of interest (ROI) were demarcated, areas computed, and expressed as a percentage of ROI area. To compute ROI lesion area, brain sections at (or nearest) pre-determined atlas plates (Paxinos & Watson, 1998) were taken (for BLA: plates 27, 30, 32 and 34; for NAc: plates 11, 13 and 15; for OFC: plates 5, 6, and 7) and percent of the ROI lesioned at these plates was averaged to obtained mean percent of ROI lesioned in the animal. Bilateral lesions that encompassed at least 55% of ROI were considered to meet lesion criterion (see Table 1) and those rats were used for behavioral analyses.

Design and procedure

Study consisted of three separate experiments that had an identical design and procedure. Each experiment examined effects of a lesion in a distinct region of interest (ROI): the basolateral amygdala (BLA), the nucleus accumbens core (NAc), and the orbital frontal cortex (OFC). The design of each experiment had two fully crossed independent factors: Chronic Drug Treatment (saline *vs* quinpirole) and Lesion (sham lesion *vs* ROI lesion), forming four groups. A comparison of control groups across the three experiments allowed for the experiments to be combined into one study, yielding a design with two fully crossed independent factors: Chronic Drug Treatment (saline *vs* quinpirole) and ROI Lesion (sham lesion *vs* BLA *vs* NAc *vs* OFC), forming eight groups.

At start of experiment, rats were allocated into saline *vs* quinpirole treatment conditions at random, and received 8 injections. The purpose of this phase of experiment was to induce compulsive checking behavior in rats treated chronically with quinpirole. After this treatment, rats were assigned at random into a sham *vs* lesion group, underwent surgery, and were allowed to recover for two weeks before renewal of testing. Injection 9 (14 days post-surgery) and injection 10 (17 days post-surgery) were the same treatment as previous injections. For Injection 11, half of the animals in each group received an injection of saline while the rest of the animals received an injection of quinpirole. For injection 12, drug treatments were reversed. Results of behavioral analysis for injections 10 are presented in this report; injection 9 is omitted to reduce possible confounds from non-specific effects of surgery. Saline administration on injections 11 or 12 is referred to as "saline test" and results of this test are also discussed; results of quinpirole administration on injections 11 or 12 are not considered here as they are not directly pertinent to the current question.

At end of experiment, rats received an "object rotation test" which is used to assess whether checking behavior is influenced by changes in the environment; for this test, the four objects in the open field are rotated in space by 180 degrees and thus moved to different open field locales (Szechtman *et al.*, 1998). Results of the object rotation test are not presented as all groups showed the expected shift in checking behavior (Perreault *et al.*, 2007) and the findings of this test did not yield additional information of interest to a ROI lesion effect.

On the day of testing, animals were weighed, transported in their home cage to an adjoining non-colony testing room, and administered the appropriate injection. Immediately afterwards, rats were placed into the open field for 55 min and their behavior videotaped for further offline data analysis.

Data analysis

EthoVision 3.1 software was used to extract the time series of *x, y* coordinates of the rat from digitized video recordings as described previously (Dvorkin *et al.*, 2006b). Digitized tracking data were pre-processed to remove noise (by applying appropriate filters to smooth the *x,y* coordinates; (Hen *et al.*, 2004)), and obtained coordinates were divided into episodes of forward locomotion (called, *progression*) and episodes of small movements or immobility (called, *lingering*), as described (Golani *et al.*, 1993; Drai *et al.*, 2000; Drai & Golani, 2001). *Distance traveled* was computed as sum of the distances during progression and lingering. In addition, the area of *2 standard deviational ellipse* (2SDE) (Lefever, 1926) was computed. This metric is a measure of the extent of space covered by the trajectories of locomotion (Dvorkin *et al.*, 2006b), and is one of the basic types of descriptors of spatial distribution used in centrographic statistics; it represents

the area of an ellipse encompassing data points within 2 standard deviations of the mean along the long and the short axes of the ellipse (Ebdon, 1985).

Compulsive checking behavior

Using a virtual implementation of the coordinate system of 25 open field locales (places) (Szechtman *et al.*, 1998), frequency of visits and duration of stops in each locale were computed (the terms "visit" and "stop" are equivalent and are used interchangeably). The obtained values were used to identify the locale with highest cumulative frequency of visits as well as the place with maximal cumulative duration of stops. Checking behavior was defined with reference to the most visited locale (labeled, "key place" or "key locale"; these terms are equivalent), which was in almost all instances also the locale with longest duration of stops (Eilam & Golani, 1989; Szechtman *et al.*, 1998). If several locales had an equal number of visits then the locale with the higher cumulative duration of stops was used as the key locale. A visit to key place is also referred to as a "check" or "checking," and the following group of 4 criteria measures of checking behavior were computed: 1) *relative frequency of checking,* total number of visits to key locale normalized by the expected number of visit per visited locales (this measurement controls statistically for individual differences in overall amount of activity); 2) *relative recurrence time of checking*, mean duration of return times to key place normalized to mean of return times to every place that was visited more than once ("return time" is interval from departure to next arrival to the locale); 3) *stops before returning to key locale,* mean number of places visited between returns to key locale; and, 4) *length of check*, total duration of stay at key locale divided by frequency of visits there; this measure is also an indirect index of ritual-like behavior as very short duration of stay in the key locale is associated with appearance of motor rituals in quinpirole rats (Szechtman *et al.*, 1998;

Ben Pazi *et al.*, 2001). These dependent variables were defined during earlier studies of checking behavior (Szechtman *et al.*, 1998; Szechtman *et al.*, 2001; Tizabi *et al.*, 2002; Dvorkin *et al.*, 2006b) and capture the essential features defining OCD checking (Szechtman & Eilam, 2005; Eilam & Szechtman, 2005). In the animal model, compulsive checking behavior is identified by presence of a significant difference between quinpirole- and saline-treated rats—all 4 measures need to differ from controls for the claim of compulsive checking (Szechtman *et al.*, 1998), and hence the group of these 4 measures is termed "criteria measures" for compulsive checking.

Bouts of checking behavior

As another metric of compulsive checking behavior, we recently developed a method to identify whether sequences of checking are clustered into bouts, as described in detail elsewhere (Dvorkin *et al.*, 2006b). In brief, a bout of behavior is generally defined on the basis of the distribution of time intervals between behavioral events (inter-event intervals). This distribution is examined to locate and extract a timepoint which will produce a natural split between clusterings of inter-event intervals; specifically, that it will separate the time intervals into a class of intervals that are between the bouts of behavior (inter-bout intervals) and a class of intervals that belong within a bout of behavior (intra-bout intervals) (Tolkamp *et al.*, 1998; Tolkamp & Kyriazakis, 1999), and this principle was employed to identify bouts of checking behavior. The following bout-related measures were computed: *number of bouts* in a test session, *time to next checking bout* (calculated as the average duration of inter-bout intervals), and *rate of checks* within a checking bout. These measures index the amount or intensity of checking. Unless noted otherwise, each rat contributed a single score for computation of group means; that is, for measures occurring multiple times in a rat, the score for each rat was the mean of multiple occurrences.

We analyzed also distribution of the durations of lingering episodes during checking bouts. Lingering is the time spent in non-locomotor activity and it is then that a rat can perform various acts, such as sniffing, scanning, grooming, etc, as well as staying in place or resting (Eilam & Golani, 1989). Hence, this metric describes the rapidity of episodes of lingering, reflecting their intensity during bouts of checking. Because of the continuous nature of the measure, data were discretized into bins using SPSS Optimal Binning procedure. All test data were pooled (a total of 167,603 cases), and bin formation was set as optimal with respect to drug (saline *vs* quinpirole) and lesion (sham *vs* lesion) factors. This procedure yielded 8 bins (<0.7, 0.7-2.1, 2.1-4.4, 4.4-7.5, 7.5-12.1, 12.1-18.0, 18.0-45.8, >45.8 sec) that were used to obtain the relative distribution of the duration of lingering episodes for each animal and compute group means.

Statistics

For statistical analysis of the criteria measures, a separate two-way ANOVA with one factor being Chronic Drug Treatment (saline *vs* quinpirole) and the second factor being Region of Interest Lesioned (sham *vs* BLA *vs* NAc *vs* OFC) was performed for each dependent variable at each test. The same procedure was followed for analyses of checking bouts, except for number of bouts per session where total time spent in bouts was used as a covariate. When significant main or interaction effects were followed by post-hoc tests, simple effects were evaluated by comparing the relevant marginal means and non-overlapping 95% confidence intervals were considered to be statistically significant. Chosen level of significance was $P < 0.05$. Calculations were performed using SPSS 17 for Windows. Graphs show marginal means and standard error of the mean (SEM). For clarity of presentation, results of statistical analyses indicating the F values and the associated probabilities for main and interaction effects are shown in one location, Table 2.

Results

Histology

To be considered a proper lesion, at least 55% of the region of interest (ROI) had to be damaged, as measured by area of cell loss with NeuN staining (Figure 1). As shown in Table 1, mean percentage of damage was about 75% for BLA and NAc, and about 68% for OFC. NAc lesions produced little damage outside the core region while lesions of BLA were larger and produced some damage to areas ventral and lateral to BLA but spared central and medial amygdaloid nuclei. OFC lesions were also relatively large, and produced damage to various regions outside the lateral orbital area of the frontal cortex, impinging in different rats on the lower layers of primary and secondary motor cortex, claustrum, agranular insular cortex and dorsolateral orbital area. Table 1 gives the final number of rats in each group used in analyses of behavior.

Induction of compulsive checking behavior by quinpirole

When placed into a large open field, normal rats establish a "home base" from which they depart and to which they return often in their explorations of the environment (Eilam & Golani, 1989). A return to such a "key location" is referred to as "checking" or a "check" and the repertoire of behavior with reference to the key locale constitutes "checking behavior." Accordingly, normal rats show checking behavior. Chronic treatment with quinpirole exaggerates such checking behavior, and we have termed the behavior under quinpirole as "compulsive checking behavior" because of its similarity to compulsive checking of OCD patients. The similarity lies in the correspondence to the structure of OCD compulsive checking. The structure of OCD compulsive checking is characterized by the patient's preoccupation with and reluctance to leave the checked

object or place. The attributes defining this structure were operationalized into 5 performance criteria that could be measured, and rats were deemed to show compulsive checking behavior if they satisfied all 5 criteria (Szechtman et al., 1998). That is, for compulsive checking behavior, there had to be present: (1) an excessive number of returns to a place in the environment; (2) an excessively short time to return to this particular locale; (3) few visits elsewhere before returns to the key place; (4) a ritual-like quality to the performance of motor acts at the key location; and, (5) a change in behavioral activity if the environment was altered. In the animal model, compulsive checking behavior is evidenced by the presence of a significant difference between quinpirole- and saline-treated rats for each of the criterion measure. Because significant differences must exist for all 5 measures, this group of measures is termed here "criteria measures" for compulsive checking.

As expected, chronic treatment with quinpirole was effective in inducing compulsive checking in sham lesion rats, according to the above enumerated criteria. In particular:

(1) Quinpirole-treated sham lesion rats returned to the most visited place in the open field almost 5 times more often than did saline-treated sham lesion animals (79.7 ± 5.3 returns to key locale under quinpirole *vs.* 17.3 ± 1.1 returns under saline; t_{68} = 12.1, $P < 0.001$). Moreover, this measure of an elevated frequency of checking under quinpirole held true even when the *relative frequency of checking* was computed in which the number of returns to the key locale was normalized to the total number of visits shown by quinpirole and saline rats. Specifically, quinpirole-treated sham rats made 290.6 ± 15.5 returns to 20.1 ± 0.4 different locales in the open field, and thus on the basis of a uniform frequency distribution their expected number of returns to any locale in the open field was 14.5 ± 0.8

returns per locale (the corresponding values for saline-treated sham rats were: 100.2 ± 7.4 returns to 17.4 ± 0.5 different locales yielding 5.6 ± 0.3 expected returns per locale). Accordingly, the observed rate of returns to the key locale was 5.4-fold higher under quinpirole than would be expected and 3.1-fold higher under saline. This *relative frequency of checking* in quinpirole sham rats was significantly higher than the corresponding ratio of observed-to-expected returns to key locale in saline sham controls, as shown in Figure 2 (*left panel, top row, open bars*).

(2) The mean return time to the key locale was nearly 4-fold shorter in the quinpirole sham group compared to the saline sham controls (31.0 ± 3.2 s *vs.* 115.4 ± 7.0 s, t_{68} = 10.5, $P < 0.001$). Moreover, this measure of a faster recurrence time of checking under quinpirole held true even when the *relative recurrence time of checking* was computed in which the return time to the key locale was normalized to the mean of return times to places in quinpirole (131.4 ± 8.9 s) and saline (330.3 ± 18.6 s) sham rats. This *relative recurrence time of checking* was 0.232 ± 0.020 in quinpirole sham rats and was significantly quicker than the corresponding value of 0.367 ± 0.019 in saline sham controls, as shown in Figure 2 (*left panel, third row, open bars*).

(3) Quinpirole sham rats visited only 2.8 ± 0.2 places before returning to the key locale, in contrast to saline sham controls who stopped in 4.8 ± 0.2 locales before re-entering the key place. This difference in *stops between returns to key locale* was statistically significant, as shown in Figure 2 (*left panel, fourth row, open bars*).

(4) With regards to the criterion of a ritual-like quality to the performance of motor acts at the key location, this is indexed indirectly in the present study. Previous findings indicated that the display of motor-rituals by quinpirole rats is associated with a significantly reduced time that the quinpirole rats stay at the key locale during each visit, compared to the time spent by saline rats (Szechtman *et al.*, 1998; Ben Pazi *et al.*, 2001). Here, too, the mean duration of a visit to the key locale in the quinpirole sham group (14.4 ± 9.3 s) was significantly shorter than the corresponding duration in the saline sham controls (88.5 ± 8.8 s), as shown in Figure 2 (*left panel, second row, open bars*). Because this index reflects the time spent at the key locale, it is in essence the time spent in checking the key locale upon the return to it, and hence, this measure is referred to as *length of check*.

(5) Finally, the criterion that checking behavior exhibits dependence on environmental context was also met, as shown on the test when objects on the open field were moved to new locations (the "object rotation test"). As expected, quinpirole sham rats shifted their checking to a new location and visited the previous key locale less often (data not shown).

Thus, as expected, prior treatment with quinpirole was effective in establishing compulsive checking behavior, permitting the examination of the effects of lesion to the BLA, NAc and OFC on the criteria measures of compulsive checking. Because results of the object rotation test were not informative as to a lesion effect, this test is not considered further and only the first 4 criteria measures for compulsive checking are discussed below.

Effects of lesion

Figure 2 (*left panel*) shows the effects of the three lesions on the criteria measures for compulsive checking behavior. Inspection of the graphs shows that BLA lesions did not affect any measure of checking behavior. However, lesions of NAc and OFC did, producing statistically significant effects in saline-treated rats on different measures of checking behavior. NAc lesion increased *relative frequency of checking* and reduced *length of check* in saline treated animals. In contrast, OFC lesion elevated *relative recurrence time of checking* and increased the number of *stops before returning to check* in saline treated animals. This split of the criteria measures suggests that each set of measures may reflect a distinct functional process. Indeed, measures of checking behavior affected by NAc lesion can be seen as reflecting performance at the place being checked. In contrast, measures affected by OFC lesion can be seen as reflecting behavior whilst away from this locale of interest. Below, we use this framework to analyze further the effects of NAc and OFC lesions on the two sets of measures.

NAc lesion

A change in behavior marked by episodes of activity occurring more frequently and with shorter durations, suggests an increase in the intensity or vigor of performance. Consequently, the NAc-induced increase in frequency of checking with shortened duration of checks in saline rats (Figure 2), suggests an increase in the intensity of checking. Hence, NAc lesions appear to increase the intensity of checking behavior.

As shown in Figure 3, changes in parameters of checking bouts also suggest that NAc lesions increase the intensity of checking behavior: in saline rats, the lesion elevated *rate of checks* within a bout and *number of bouts*, as well as reducing *time to next checking bout*, all indications of more vigorous performance. Moreover, behavior of NAc rats did not have the usual distribution of

act durations but was dominated instead by brief acts (Figure 4)—a finding that points to behavior as characterized by a rapidity of brief acts, consistent with the impression gained by watching the lesioned rats. This too suggests more intense behavior because presumably such a profile of activity demands an increase in energy expenditure.

While the effects of NAc lesion were striking in saline rats, this was not the case in quinpirole-treated animals. As shown in Figure 2 for day 17 post-surgery, quinpirole-treated animals with NAc lesion did not differ statistically from quinpirole controls with sham lesion on any of the criteria measures of compulsive checking. However, this finding does not indicate that the NAc lesion was without any behavioral effects in quinpirole-treated rats, as evidenced by the following **two** results:

First, a subtle effect of NAc lesion in quinpirole rats **may be inferred from** the lesion-induced loss of statistical significance for *relative recurrence time of checking* in comparison to sham saline controls on day 17 post-surgery (Figure 2). **Such a finding may suggest** that the lesion prolonged the duration of time that the quinpirole rats stayed away from the key locale, albeit not so markedly as to differ from sham quinpirole controls. Second, as shown in Figure 3 for the test on day 17 post-surgery, there was a significant main effect of ROI lesion for number of bouts in a session, with the NAc lesion groups showing more bouts than any of the other groups. This finding suggests NAc lesion was effective in increasing the number of checking bouts even in quinpirole-treated rats, in whom this number was already elevated compared to saline controls.

OFC lesion

Increased *recurrence time of checking* combined with more *stops between returns to key locale* (Figure 2, *left panel*), suggests a decline in interest to return to the home locale. Hence, OFC lesion, by affecting behavior away from key locale, appears to have altered the focus on checking. Measures of checking bouts (Figure 3) and of act durations (Figure 4) were not altered by OFC lesion, consistent with notion that those measures reflect the intensity, and not the focus, of checking behavior.

As shown in Figure 2, on day 17 post-surgery, a lesion of OFC had effects in saline but not quinpirole-treated rats. The finding that when re-tested several days later (Figure 2, saline test) saline rats no longer showed lesion effects, raises the possibility of a quick functional recovery.

Spatial distribution of behavior

Induction of compulsive checking in the quinpirole model is associated with locomotor sensitization, with distance traveled rising 4 to 8 fold (Szechtman *et al.*, 1994b; Dvorkin *et al.*, 2006b). Present findings provide compelling evidence that locomotor hyperactivity, in and of itself, does not produce the behavioral profile of compulsive checking. Specifically, while NAc saline rats showed at least as much locomotion as quinpirole sham rats (Figure 5, *left graph*), they did not meet the **full set of** criteria for compulsive checking behavior (Figure 2). Moreover, compulsive checking in quinpirole rats is associated with a particular profile of paths distribution (compare in Figure 5, *top row*, the trajectories of the sham quinpirole rat to the trajectories of the sham saline rat). The characteristics of this quinpirole profile are reflected quantitatively in a smaller *2 standard deviational ellipse* (2SDE) compared to sham saline controls (Figure 5, *right graph*). However, the 2SDE of NAc saline rats remained at control levels and was significantly different from that of sham quinpirole rats (Figure 5, *right graph*). This difference is another piece

of evidence that mere locomotor hyperactivity does not lead to the characteristic path profile of compulsive checking behavior, as indeed may be apparent from an inspection of the trajectories of the NAc saline rat in Figure 5 (*top row*).

Discussion

The present study used an animal model of OCD to investigate whether NAc, OFC, and BLA play a role in compulsive checking behavior. Results show no effect of BLA lesion. In contrast, NAc and OFC lesions produced distinct and marked changes in checking behavior, but only in saline-treated rats. An examination of these effects with reference to quinpirole-induced compulsive checking raises the possibility that the roles of NAc and OFC in compulsive checking are to mediate vigor of motor performance and focus on goal-directed activity, respectively. As discussed, findings suggest further that the inhibition of NAc neurons is one of the mechanisms by which quinpirole induces compulsive checking, and that NAc may be a site for the negative feedback control of checking.

Lesion-induced changes in checking behavior

We utilized a rich set of measures to evaluate checking behavior. In addition to the group of 4 criteria measures establishing compulsive checking, several variables were employed to characterize the magnitude of checking. BLA lesions did not affect any measure but lesions of NAc and OFC did, each producing an effect on a distinct group of variables in saline-treated animals, In particular, NAc lesions increased the frequency of checking and reduced the length of checks—these effects suggest more vigorous performance of checking. In contrast, OFC lesions increased the time between checks (*relative recurrence time of checking*) as well as the number of

visits elsewhere before returning to check the key locale (*stops before returning to check*)—these effects suggest a lowered interest in checking or increased potency of other stimuli to distract from checking or both. Stated in another way, the above division suggests that NAc lesions impacted the intensity of checking behavior and OFC lesions affected the concentration on checking. This conclusion is strengthened by evidence obtained with bout-related measures of intensity which were affected by a lesion of NAc but not OFC.

The possibility that different lesions could impact distinct sets of the criteria measures is supported by prior findings which showed the same groupings of the criteria measures during the course of development of compulsive checking. Specifically, during the course of treatment with quinpirole, measures indicative of the amount of checking (frequency of returns to key locale and duration of staying there) showed sensitization while measures indicative of staying away from checking (return time to key locale and number of stops between returns to key locale) remained unchanged during the course of treatment (Dvorkin *et al.*, 2006b). This suggests that the two sets of variables are controlled by distinct processes and hence liable to fractionation into separate components by different lesions (Teitelbaum & Pellis, 1992; Teitelbaum & Stricker, 1994).

Thus, the present findings indicate not only that NAc and OFC both play a role in checking behavior but also that their roles are distinctly different from each other.

Sites of action of quinpirole in intact brain

The behavioral profile of lesion-induced changes suggests that the site of action of quinpirole to induce compulsive checking in normal rats includes the NAc. This site is suggested by findings that a lesion of NAc brought checking of saline rats nearly to the same level of intensity as shown by quinpirole-treated sham controls. The fact that a cell-body lesion yielded quinpirole-like effects,

argues furthermore for an inhibitory action of quinpirole on NAc neurons, releasing their inhibitory control over other neural sites to increase checking intensity. Such a quinpirole inhibitory mechanism is consistent with several lines of evidence: with electrophysiological findings that NAc applications of quinpirole inhibit neuronal firing of accumbal medium spiny neurons (Perez *et al.*, 2006); with findings from 2-deoxyglucose autoradiography that a specific effect of chronic treatment with quinpirole (compared to acute quinpirole) is a reduction of glucose metabolism in the nucleus accumbens (Carpenter *et al.*, 2003; Richards *et al.*, 2007); with the suggestion from mice over-expressing striatal D2 receptors that the basal metabolic rate for glucose is reduced there (Kellendonk *et al.*, 2006); and finally, with results of the present study showing that NAc lesions elevate checking under saline, without doing so under quinpirole.

Functional roles of NAc, OFC and BLA in compulsive checking

Lesion-induced division of the criteria measures suggests that the functional role played by NAc in compulsive checking behavior is to control the intensity or vigor of checking. Such a functional role is consistent with literature. Specifically, NAc is considered to transduce the impact of rewards and of the reinforcing effects of stimuli (Wise, 2004; Everitt & Robbins, 2005; Robbins & Everitt, 2007; Cools, 2008), producing an "activating, energizing, or invigorating effect" on motivated behavior (Salamone & Correa, 2002; Robbins & Everitt, 2007; Cools, 2008). This energizing effect is often manifested as an increase in vigor of motor performance (Niv *et al.*, 2007). Thus, both present behavioral data and literature support the suggestion that NAc plays a role in compulsive checking behavior by controlling the vigor of performance.

With regards to OFC, lesion-induced division of the criteria measures suggests that the functional role of OFC in compulsive checking is to control the concentration on checking. There

is support in the literature for such a role. Specifically, two prominent features of an OFC lesion are: deficits with reversal learning and problems with decision-making despite no apparent intellectual impairments (Clark *et al.*, 2004). Recent considerations have suggested that both of these features may in fact tap into closely related constructs (Clark *et al.*, 2004), a notion that was pursued in a recent review and led to the unifying concept that OFC processing is directed towards future potential goals where "OFC integrates information to derive the values of potential reward outcomes" (Wallis, 2007). In this schema, OFC provides an initial assessment of potential reward value and this appraisal is passed to other prefrontal areas for further evaluation and construction of implementation plans to reach the goal and obtain the reward (Wallis, 2007). In other words, OFC role is related to focusing on reaching the goal. For compulsive checking such focusing would be reflected through a concentration on reaching the key place to check it. Thus, both present behavioral data and literature support the suggestion that OFC plays a role in compulsive checking behavior by controlling goal-directed focus.

Interestingly, despite its involvement in mediating fear (LeDoux, 2000), BLA did not appear to have any identified role in the expression of checking. Conceivably, its role may not extend to affective processing once the environment is familiar or a behavior is well established. Indeed, the importance of neural sites may be different for the expression and for the acquisition of compulsive checking behavior, as is the case for other behaviors (Cardinal *et al.*, 2002). This may apply also for OFC since in another model of OCD (Joel, 2006b) where OFC was lesioned prior to acquisition of compulsive behavior, the formation of compulsive behavior was enhanced (Joel *et al.*, 2005). In contrast, in present study which examined expression of checking behavior, OFC lesions did not enhance checking.

We have inferred the functional roles of NAc and OFC in compulsive checking from a comparison of lesion effects in saline rats to behavior of sham controls treated with quinpirole. It may be argued that this approach is flawed because strong lesion effects were not observed in rats treated with quinpirole—the model preparation of compulsive checking—and hence no inferences are possible regarding the roles of NAc and OFC in compulsive checking. However, this expectation of a lesion effect is not a logical prediction of a model like the one considered here. Specifically, we reasoned that compulsive checking behavior reflects the co-joint activity of several component processes with separate neural mechanisms (rather than representing a unitary phenomenon). Consequently, lesion effects on compulsive checking as a unit are not predicted and indeed did not occur. The apparently contradictory observation that NAc is important in mediating vigor of checking and yet a lesion of it does not alter the vigor of compulsive checking is likely a consequence of quinpirole's inhibitory mode of action on the NAc, an effect that can be mimicked by a lesion, as suggested above. Some similar schemas may be hypothesized for the apparently contradictory finding that OFC lesions disrupt returns to the home base in saline rats and yet the OFC enhances focus on goal-directed activity under quinpirole.

Motivation, compulsive behavior and OCD

The present study suggests two elemental features of compulsive checking behavior, both greatly exaggerated: one for vigor of performance and one for degree of focus on goal-directed activity. Exaggerated vigor and focus are also the behavioral attributes of an organism who is highly motivated. This raises the question: Is "compulsive" behavior synonymous with "highly motivated" behavior?

According to some theories, checking is the behavioral output of a security motivation (Szechtman & Woody, 2004) or a precaution-hazard system (Boyer & Lienard, 2006) activated by potential danger. Since security motivation is vital to self-preservation, checking can be a highly motivated response. However, there exists something more when checking behavior is compulsive. Normally, a bout of motivated behavior is followed by a prolonged period of rest or "satiety" before a new bout starts again (Tolkamp *et al.*, 1998; Berridge, 2004). In our model, this satiety is indexed by *time to next checking bout* (Dvorkin *et al.*, 2006b), and is reduced markedly in quinpirole-treated compulsive rats. Accordingly, compulsive checking is indeed highly motivated behavior, but with a crucial difference—attenuated satiety. Such foreshortened satiety corresponds to notions of OCD as failures to attain the "sense of task completion" (Pitman, 1989) or a "feeling of knowing" (Szechtman & Woody, 2004).

Interestingly, saline rats with NAc lesions showed the same diminution in satiety as quinpirole-treated animals. This is of interest because satiety is considered to reflect the action of a negative feedback signal which shuts down checking behavior (Szechtman & Woody, 2004). Conceivably, NAc is also a site of the negative feedback satiety signal.

Finally, the present findings appear relevant for understanding therapeutic effects of deep brain stimulation in OCD (Sturm *et al.*, 2003; Greenberg *et al.*, 2006; Rauch *et al.*, 2006; Okun *et al.*, 2007). A recent study showed that deep brain stimulation of NAc is effective in attenuating quinpirole-induced compulsive checking in rats (Mundt *et al.*, 2009). Juxtaposed with current notion that quinpirole inhibits NAc to produce compulsive checking, a plausible mechanism behind therapeutic effects of deep brain stimulation may be in fact removal of NAc inhibition, possibly over ventral pallidum (Mundt *et al.*, 2009) and/or OFC (McCracken & Grace, 2007).

However, it is of importance to note that because compulsive checking behavior is comprised of several features controlled by distinct brain areas, more than one brain region may need targeting for successful treatment (McCracken & Grace, 2007; Harrison *et al.*, 2009).

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Abreviations

2SDE - standard deviational ellipse; BLA - basolateral amygdaloid nucleus; NAc - nucleus accumbens core; NeuN - neuronal nuclei; NMDA - N-methyl-D-aspartate; OCD - obsessive-compulsive disorder; OFC - orbital frontal cortex; PBS - phosphate-buffered saline; ROI - region of interest

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Figure Captions

Figure 1. A representative NeuN stained section of a BLA (*left*), NAc (*middle*) and OFC (*right*) lesion from a rat with percent ROI lesioned similar to mean lesion size indicated in Table 1. The dotted line demarcates the area of damage.

Figure 2. Performance on criteria measures of compulsive checking behavior shown by groups of rats on two tests. Blue bars represent groups with chronic saline treatment (left column of each panel, labeled "Chronic SAL") and red bars represent groups with chronic quinpirole treatment (right column of each panel, labeled "Chronic QNP"). Background color shows the drug injected on the test day: bluish background indicates injection of saline and reddish background injection of quinpirole (all groups received an injection of saline on the Saline Test, *right panel*). Same color convention is used in subsequent figures. Legend: Open bars = sham controls, right hatch = BLA lesion; cross hatch = NAc lesion, left hatch = OFC lesion. For comparisons following a significant interaction between ROI Lesion by Chronic Drug Treatment: * $P < 0.05$ *vs* sham controls, BLA lesion, and OFC lesion groups treated chronically with saline; ** $P < 0.05$ *vs* every group treated chronically with saline; *** $P < 0.05$ *vs* every other group; ## $P < 0.05$ *vs* every group treated chronically with quinpirole as well as sham controls and NAc groups treated chronically with saline; + $P < 0.05$ *vs* sham controls treated chronically with saline. For comparisons following a significant main effect of ROI Lesion: solid triangle down $P < 0.05$ *vs* sham controls, BLA lesion and OFC lesion groups. Significant main effects of Chronic Drug Treatment are not depicted on the graphs but were significant for every measure on the test 17 days post-surgery (*left column*),

and for none of the measures on the saline test (*right column*). *F* values and the associated probabilities for main and interaction effects are shown in Table 2.

Figure 3. Performance on measures of checking bouts shown by groups of rats on two tests. Same groups and color convention as in Figure 2. For comparisons following a significant interaction between ROI Lesion by Chronic Drug Treatment: * $P < 0.05$ *vs* sham controls, BLA lesion, and OFC lesion groups treated chronically with saline; + $P < 0.05$ *vs* sham controls treated chronically with saline; ++ $P < 0.05$ *vs* sham controls and BLA lesion group treated chronically with saline. For comparisons following a significant main effect of ROI Lesion: solid square $P < 0.05$ *vs* sham controls and BLA lesion groups; solid triangle down $P < 0.05$ *vs* sham controls, BLA lesion and OFC lesion groups; solid triangle up $P < 0.05$ *vs* sham controls and OFC lesion groups. Significant main effects of Chronic Drug Treatment are not depicted on the graphs but were significant for every measure on the test 17 days post-surgery. A value for *time to next bout* is not shown for saline OFC group on the test 17 days post-surgery (left column in left panel) as none of the rats initiated a second bout of checking. F values and the associated probabilities for main and interaction effects are shown in Table 2.

Figure 4. Distribution of durations of lingering episodes belonging to checking bouts on the test 17 days post-surgery. Groups with chronic saline treatment are drawn in shades of blue (*left side of graph*) and those with chronic quinpirole treatment in shades of red (*right side of graph*). **Inset**. Frequency of lingering episodes with the duration belonging to bin 2 (0.7-2.1 sec). Blue bars represent groups with chronic saline treatment and red bars with chronic quinpirole treatment. Open bars = sham controls, right hatch = BLA lesion; cross hatch = Nac lesion, left hatch = OFC lesion. * $P < 0.05$ *vs* sham controls, BLA lesion, and OFC lesion groups treated chronically with

saline; ** $P < 0.05$ *vs* every group treated chronically with saline; ^* $P < 0.05$ *vs* sham controls, NAc and OFC lesion groups treated chronically with quinpirole as well as sham controls, BLA and OFC lesion groups treated chronically with saline. *F* values and the associated probabilities for main and interaction effects are shown in Table 2. Sal = saline, QNP = quinpirole.

Figure 5. Locomotor trajectories of a representative rat from each group (*top row*) and amount of locomotion (*left*) and spatial distribution of locomotor trajectories (*right*) shown by groups of rats (*bottom row*). Rats from groups that received chronic saline treatment are demarcated by blue color and those that received chronic quinpirole treatment by red. Trajectories during the entire 55 min session are shown; each line represents a trajectory of locomotion. Green squares indicate locations of the 4 objects in the open field. * $P < 0.05$ *vs* sham controls, BLA lesion, and OFC lesion groups treated chronically with saline; ** $P < 0.05$ *vs* every group treated chronically with saline. The main effect of Chronic Drug Treatment was significant for locomotor distance (*left* graph) and 2 standard deviational ellipse (*right* graph). *F* values and the associated probabilities for main and interaction effects are shown in Table 2. Sham = sham lesion, BLA = lesion of basolateral amygdala, NAc = lesion of nucleus accumbens core, OFC = lesion of orbital frontal cortex.

Table 1: Number of rats in each group with proper lesion in the target region of interest (ROI Factor), and the percentage of the ROI lesioned (mean±sem and minimum).

ROI Factor	**Chronic Drug Treatment Factor**					
	Saline			Quinpirole		
	N	Mean ±sem	Minimum	N	Mean ±sem	Minimum
Sham-controls	39	0.8%±0.5%	0.0%	33	0.4%±0.4%	0.0%
BLA-Lesion	12	74.6%±3.8%	55.9%	11	76.8%±3.1%	60.3%
NAc-Lesion	8	72.5%±1.6%	67.7%	14	73.7%±2.3%	62.0%
OFC-Lesion	8	66.6%±4.4%	56.8%	13	67.9%±1.6%	55.1%

Table 2: Summary of statistical analyses for the dependent measures shown in Figures 2 to 5. Each dependent measure was analyzed in a Chronic Drug by Region of Interest (ROI) analysis of variance (ANOVA).

			Chronic Drug				ROI				Chronic Drug x ROI			
Fig. #	**Test**	**Dependent Measure**	df1	df2	F	*P* ≤	df1	df2	F	*P* ≤	df1	df2	F	*P* ≤
2	17 days post-surgery	Relative frequency of checking	1	127	117.229	**0.001**	3	127	2.039	0.112	3	127	6.292	**0.001**
		Length of check	1	127	37.941	**0.001**	3	127	4.555	**0.005**	3	127	4.557	**0.005**
		Relative recurrence of checking	1	127	28.571	**0.001**	3	127	3.058	**0.031**	3	127	4.839	**0.003**
		Stops before returning to check	1	127	78.117	**0.001**	3	127	2.819	**0.042**	3	127	3.961	**0.010**
	saline test	Relative frequency of checking	1	126	1.747	0.189	3	126	3.469	**0.018**	3	126	1.321	0.270
		Length of check	1	126	2.773	0.098	3	126	4.749	**0.004**	3	126	0.434	0.729
		Relative recurrence of checking	1	126	3.288	0.072	3	126	2.322	0.078	3	126	0.979	0.405
		Stops before returning to check	1	126	0.616	0.434	3	126	0.241	0.868	3	126	0.957	0.415
3	17 days post-surgery	Rate of checks	1	125	44.403	**0.001**	3	125	2.084	0.106	3	125	2.998	**0.033**
		Bouts in session	1	124	14.464	**0.001**	3	124	2.821	**0.042**	3	124	1.045	0.375
		Time to next checking bout	1	60	24.011	**0.001**	3	60	7.174	**0.001**	2	60	16.814	0.000
	saline test	Rate of checks	1	123	0.259	0.612	3	123	4.023	**0.009**	3	123	1.606	0.191
		Bouts in session	1	122	1.834	0.178	3	122	5.756	**0.001**	3	122	0.369	0.775
		Time to next checking bout	1	50	0.231	0.633	3	50	8.912	**0.001**	3	50	1.382	0.259
4	17 days post-surgery	Frequency of lingering	1	125	36.977	**0.001**	3	125	17.212	**0.001**	3	125	22.158	**0.001**
5	17 days post-surgery	Locomotor distance	1	130	44.758	**0.001**	3	130	11.514	**0.001**	3	130	14.833	**0.001**
		2 Standard deviational ellipse	1	130	104.139	**0.001**	3	130	1.909	0.131	3	130	0.300	0.825

Note: df1 and df2 refer to degrees of freedom for the numerator and denominator, respectively, for the indicated F statistic; the associated *P* value (or less) is indicated in column *P* ≤ . *P* values less than .05 are highlighted in bold font.

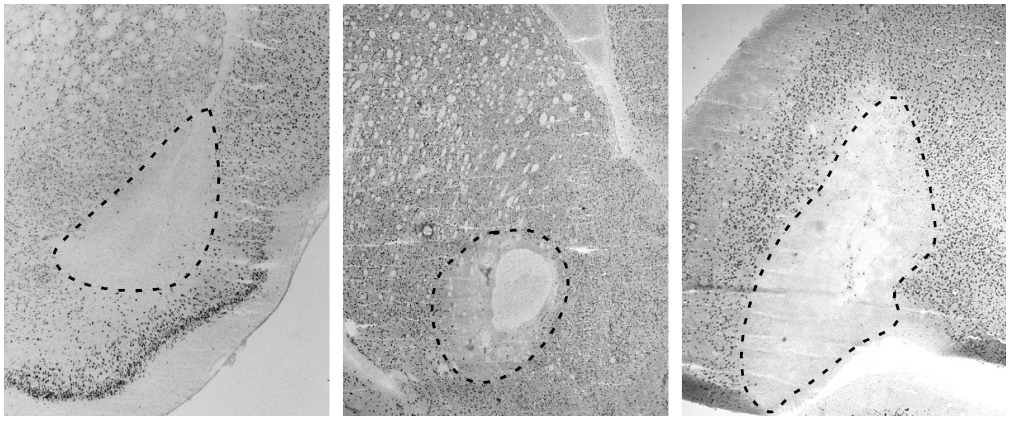

Figure 1. A representative NeuN stained section of a BLA (left), NAc (middle) and OFC (right) lesion from a rat with percent ROI lesioned similar to mean lesion size indicated in Table 1. The dotted line demarcates the area of damage.
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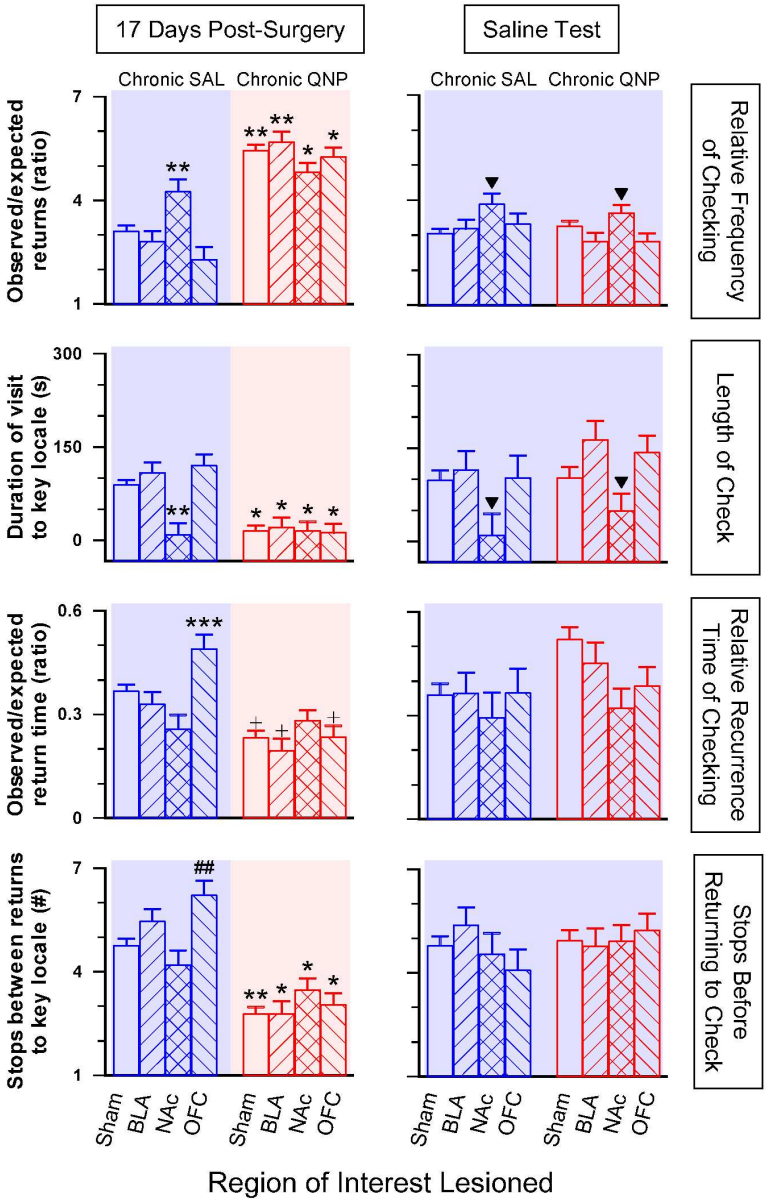

Figure 2. Performance on criteria measures of compulsive checking behavior shown by groups of rats on two tests.
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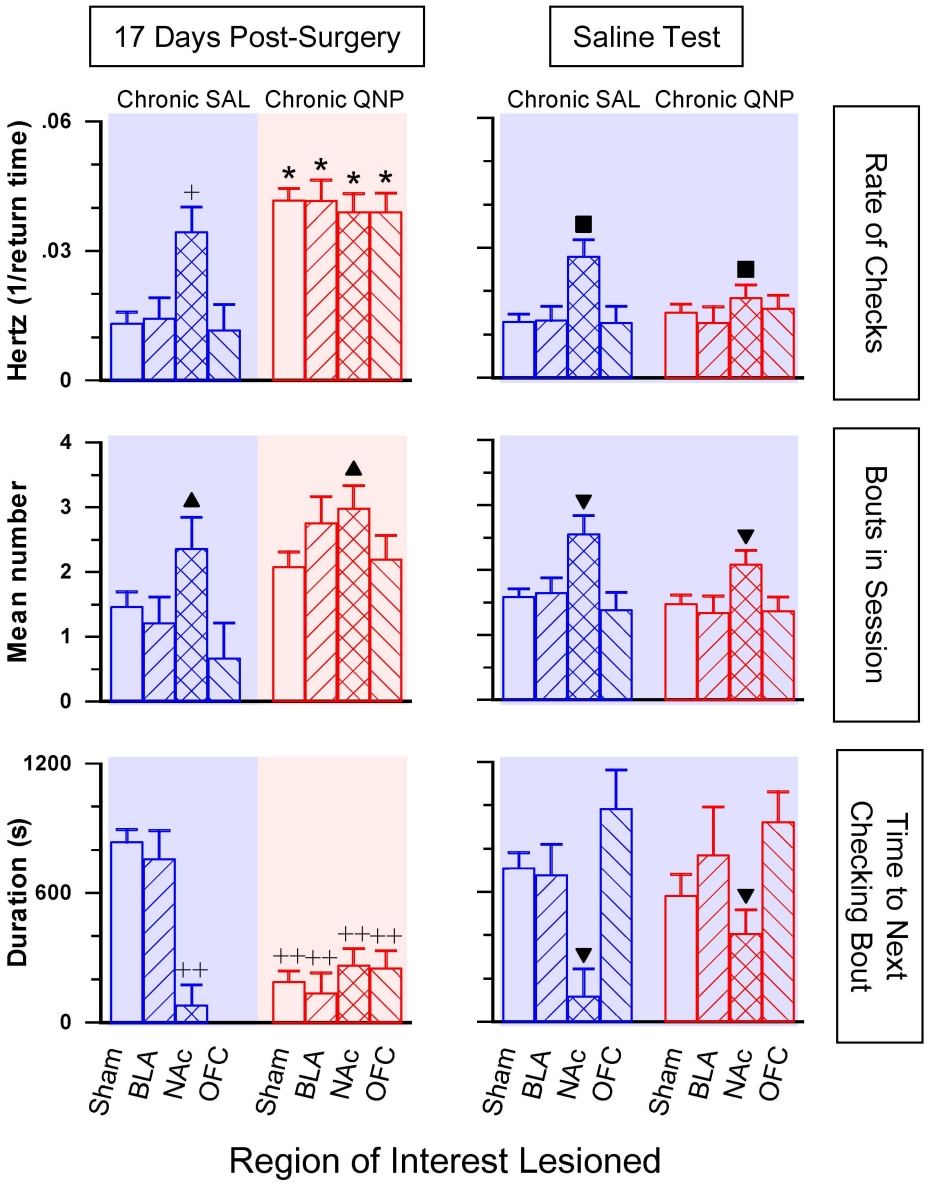

Figure 3. Performance on measures of checking bouts shown by groups of rats on two tests. Same groups and color convention as in Figure 2.
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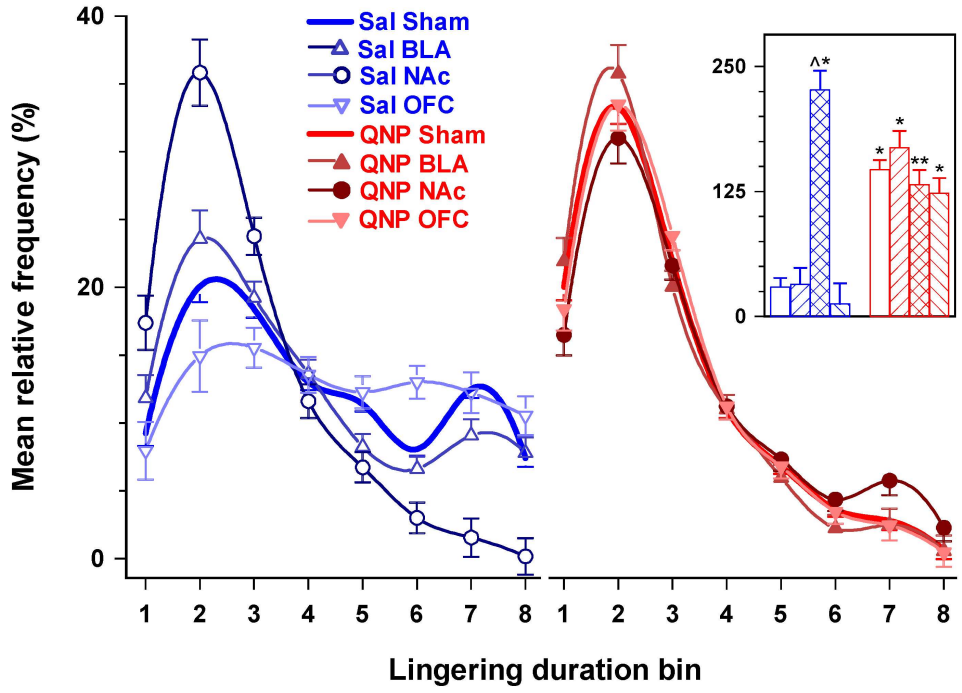

Figure 4. Distribution of durations of lingering episodes belonging to checking bouts on the test 17 days post-surgery.
123x88mm (600 x 600 DPI)

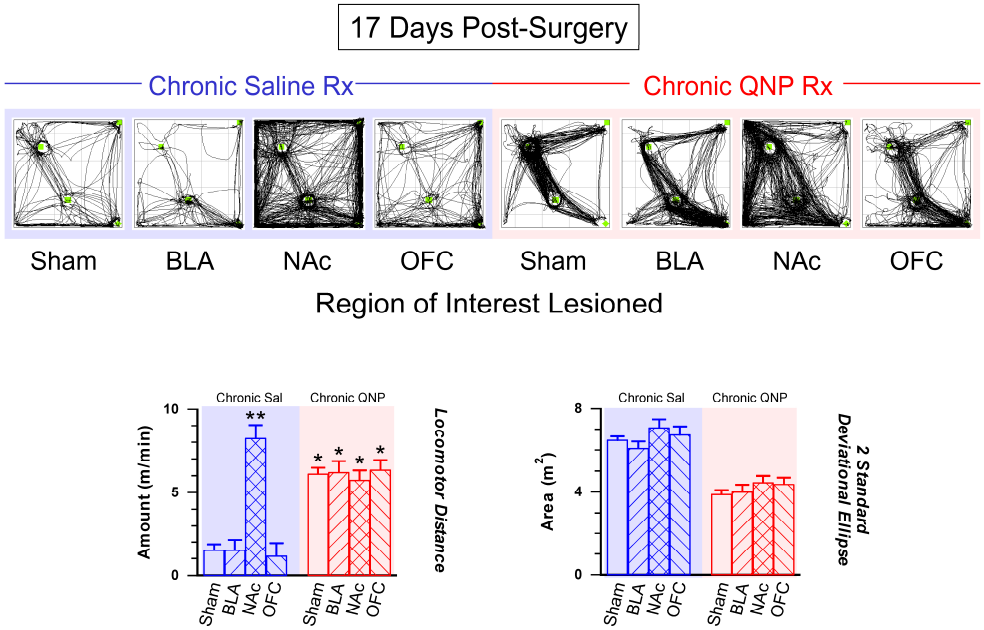

Figure 5. Locomotor trajectories of a representative rat from each group (top row) and amount of locomotion (left) and spatial distribution of locomotor trajectories (right) shown by groups of rats (bottom row).
247x161mm (600 x 600 DPI)