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Changes in gut microbiota during development of compulsive checking and locomotor sensitization induced by chronic treatment with the dopamine agonist quinpirole

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CHANGES IN GUT MICROBIOTA DURING DEVELOPMENT OF COMPULSIVE CHECKING AND LOCOMOTOR SENSITIZATION INDUCED BY CHRONIC TREATMENT WITH THE DOPAMINE AGONIST QUINPIROLE

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ABSTRACT

Chronic treatment of rats with the D2/D3 dopamine agonist quinpirole induces compulsive checking (proposed as animal model of obsessive-compulsive disorder; OCD) and locomotor sensitization. The mechanisms by which chronic quinpirole produces those behavioral transformations are not known. Here we examined whether changes in gut microbiota play a role in these behavioral phenomena by monitoring the development of compulsive checking and locomotor sensitization at the same time as measuring the response of gut microbiota to chronic quinpirole injections. Two groups of rats received 9 injections of saline (n=16) or quinpirole (n=15; 0.25 mg/kg), at weekly intervals for the first 5 weeks and then 2 injections per week until end of treatment. After each injection, rats were placed on a large open field for 55 min and their behavior was video recorded for subsequent analysis. Fecal matter was collected after each trial and frozen for bacterial community profiling of the 16S rRNA gene using paired end reads of the V3 region. Results indicated that the induction of locomotor sensitization and compulsive checking was accompanied by changes in several communities of bacteria belonging to the order Clostridiales (class Clostridia, phylum Firmicutes), and predominantly in *Lachnospiraceae* and *Ruminococcaceae* families of bacteria. It is suggested that changes in these microbes may serve to support the energy utilization requirements of compulsive checking and OCD.

KEYWORDS

Compulsive checking behavior; locomotor sensitization; quinpirole; gut microbiota; Lachnospiraceae; energy metabolism; hyperactivity; security motivation system; animal model; obsessive-compulsive disorder;

INTRODUCTION

An acute injection of the D2/D3 dopamine agonist quinpirole has a marked effect on the behavior of rats exploring a large open field: explored space becomes constricted such that the routes of travel are confined to only a limited portion of the environment, unlike the trajectories of locomotion of saline-treated animals that are dispersed widely throughout the arena and cover the entire perimeter boundary of the open field (Eilam *et al*, 1989b; Eilam *et al*, 1991). Also, an acute injection of quinpirole may change somewhat the amount of locomotion (Eilam *et al*, 1989b; Eilam and Szechtman, 1989d; Eilam *et al*, 1992) but with chronic injections of the drug, activity is strikingly elevated: there is a 3 to 8 fold increase in distance traveled after 8 to 10 injections of quinpirole, compared to the acute quinpirole response (Szechtman *et al*, 1994a; Szechtman *et al*, 1994c; Einat *et al*, 1996; Szumlinski *et al*, 2000; Lomanowska *et al*, 2004; Culver *et al*, 2008; Ballester Gonzalez *et al*, 2015). This augmented response is an instance of behavioral or locomotor "sensitization" – a general phenomenon of enhanced responding to psychostimulant drugs with chronic treatment (Robinson and Becker, 1986; Stewart and Badiani, 1993; Wise and Leeb, 1993). The phenomenon of behavioral sensitization has attracted much research interest because of its potential relevance to mechanisms of various psychiatric disorders such as psychosis, mania, and addiction (Ellinwood, 1968; Ellison, 1979; Post and Contel, 1981; Segal and Schuckit, 1983; Robinson and Berridge, 1993; Antelman *et al*, 2000; Shaldubina *et al*, 2002). With respect to behavioral sensitization to quinpirole, it too may be of relevance to a psychiatric disorder, namely, obsessive-compulsive disorder (OCD) (Szechtman *et al*, 1999; Eilam and Szechtman, 2005; Stuchlik *et al*, 2016).

The notion that behavioral sensitization to quinpirole may be of relevance to OCD emerged from the findings that the spatial-temporal structure of activity by quinpirole-sensitized rats meets the criteria of compulsive behavior performed by OCD patients (Szechtman *et al*, 1998; Eilam *et al*, 2012). Accordingly, it was proposed that the transformation in behavior induced by chronic quinpirole constitutes an animal model of OCD of use for probing the mechanisms of this disorder and of the symptom of compulsive checking in particular (Man *et al*, 2004; Korff and Harvey, 2006; Westenberg *et al*, 2007; Hoffman, 2011; Albelda and Joel, 2012; Alonso *et al*, 2015; Grados *et al*, 2016).

Much of the animal research on mechanisms of OCD and locomotor sensitization to quinpirole examine central processes. For instance, using the 2-deoxyglucose technique to measure local cerebral glucose utilization in quinpirole-sensitized animals (Carpenter *et al*, 2003; Richards *et al*, 2005), alterations had been found in cortical (the cingulate

cortex-area 1, frontal cortex-area 3, lateral orbital cortex, medial/ventral orbital cortex, and parietal cortex) and subcortical areas (ventral pallidum and nucleus accumbens); more recently, changes in the cortico-striato-thalamico-cortical circuit had been observed by PET neuroimaging in quinpirole-sensitized rats (Servaes *et al*, 2016). Others report in quinpirole-sensitized animals: increased high-affinity states of dopamine D2 receptors ($D2^{High}$) (Seeman *et al*, 2006; Perreault *et al*, 2007; Culver *et al*, 2008); decreased dopamine levels in the left pre-frontal cortex (Sullivan *et al*, 1998); reduced dopamine and glutamate neurotransmission in the nucleus accumbens (Escobar *et al*, 2015); as well alterations in dopamine-serotonin interaction (Alkhatib *et al*, 2013; Tucci *et al*, 2013; Johnson and Szechtman, 2016). However, it remains to be established which of those findings, if any, are necessary for the pathophysiology of compulsive checking (Szechtman *et al*, 2014).

While a research focus on central mechanisms underlying OCD and behavioral sensitization is a very reasonable strategy, a plausible contribution of peripheral mechanisms should not be discounted, given findings of peripheral immune factors altering behavior (Sakic *et al*, 1997; Kapadia and Sakic, 2011) and the possibility of gut-brain signaling modulating behavioral performance (Sekirov *et al*, 2010; Bercik *et al*, 2011; Collins *et al*, 2013; Bharwani *et al*, 2016; Foster, 2016; Stilling *et al*, 2016; Dinan and Cryan, 2017a). Moreover, there are suggestions for a role of peripheral mechanisms in quinpirole sensitization (Coscina *et al*, 1998; Baladi and France, 2009, 2010) and OCD (Swedo *et al*, 1998; da Rocha *et al*, 2008; Rees, 2014; Turna *et al*, 2016).

In the present study, we investigate the possibility of a role for peripheral mechanisms —and of gut microbiota in particular —in the phenomena of quinpirole-induced locomotor sensitization and compulsive checking. It was expected that if gut microbiota plays a role in these phenomena then during development of locomotor sensitization and compulsive checking a change in bacterial composition should be induced as a function of chronic quinpirole treatment. Results indicate that the induction of locomotor sensitization and compulsive checking was indeed accompanied by changes in gut bacterial composition, predominantly within the taxon Clostridiales (order).

MATERIALS AND METHODS

ANIMALS

A total of 32 experimentally naive Long-Evans male rats (Charles River, St Constant, Quebec, Canada), weighing 260–330 g at the start of testing, entered the study. Animals were housed individually in a climate controlled colony room on a 12 hour light/dark cycle (6 AM lights on, 6 PM lights off). Food and water were freely available.

Upon arrival, rats were allowed to habituate to the animal facility for 7 days and were then handled for approximately 2-5 minutes each day for 5 days in the week before start of behavioral testing. Testing occurred during the light phase. Animals were housed and tested as approved by the Animal Research Ethics Board, McMaster University in compliance with the Canadian Council on Animal Care guidelines.

DRUGS

(-)-Quinpirole hydrochloride (Q102; Sigma-Aldrich) was dissolved in 0.9% physiological saline, and administered at a dose of 0.25 mg/kg in a volume of 1 ml/kg through a subcutaneous injection under the nape of the neck. Control animals were similarly injected with 1 ml/kg of 0.9% physiological saline. Maximum effect of quinpirole for locomotor sensitization is observed at a dose of about 0.2–0.5 mg/kg (Szechtman *et al*, 1994b; Szechtman *et al*, 1994c; Szumlinski *et al*, 1997; Perreault *et al*, 2005; Dvorkin *et al*, 2006a); in studies of compulsive checking the typical dose employed is 0.5 mg/kg (Szechtman *et al*, 1998; Zadicario *et al*, 2007; Winter *et al*, 2008; Mundt *et al*, 2009; Dvorkin *et al*, 2010; Ballester Gonzalez *et al*, 2015) but lower doses (0.2-0.25 mg/kg) had been found effective as well (Alkhatib *et al*, 2013; Tucci *et al*, 2014).

APPARATUS

Animals were tested on a large open field (160 x 160 cm and 60 cm high table without walls) that was located in a non-colony room illuminated by usual overhead fluorescent lights, as described previously (Dvorkin *et al*, 2006b; Dvorkin *et al*, 2010). The table was divided virtually into a grid of 25 rectangular places (locales), but no actual lines were marked on the table surface. Four small Plexiglas/glass boxes (approximately 8×8×7.5 cm) were located at the same fixed location on the open field throughout the study: two at corners and two at places near the center of the open field (Szechtman *et al*, 1998). After each rat was tested, the table and objects were wiped clean with a diluted solution of an antibacterial cleaner (Lysol). Behavior on the open field was recorded by a stationary overhead camera interfaced with a computer that stored the video signal as MPEG files for offline analysis. These digitized videos were used to automatically track the trajectories of locomotion in the open field using EthoVision 3.1 (Noldus Information Technology, Netherlands) software (Noldus *et al*, 2001; Spink *et al*, 2001).

GUT MICROBIOTA PROFILING

Gut microbial composition was assessed by sequencing the bacterial 16S rRNA gene collected from fecal pellets deposited by the rat during the open field test. The collected fecal pellets were stored at −80 °C until molecular analysis of microbiota.

Bacterial DNA extraction was carried as previously described (Whelan et al., 2014). Bacterial community profiling of the 16S rRNA gene was performed using paired end reads of the V3 region. Triplicate amplifications were pooled for 250 nt paired-end MiSeq Illumina sequencing. This approach provides overlapping sequence reads of the V3 region, which can be used for correcting poor quality base calls and increasing sequencing accuracy. Sequence data were processed by an in-house bioinformatics pipeline (Whelan *et al*, 2014; De Palma *et al*, 2015) that incorporates quality filtering, sequence trimming and read assembly [Cutadapt (Martin, 2011) and PANDAseq (Masella *et al*, 2012)]. Bacterial sequences were clustered into *de novo* operational taxonomic units (OTUs) with the AbundantOTU+ algorithm (Ye, 2011). Taxonomy was assigned with the Ribosomal Database Project (RDP) Classifier in QIIME (Caporaso *et al*, 2010) and the Greengenes training set (February 2011 release). OTUs rarefied to 43,345 were used in statistical analysis; singletons and non-bacterial sequences were excluded. DNA extraction and 16S rRNA gene sequencing were carried out in the McMaster Genome Center (McMaster University). Raw sequence reads have been deposited in the NCBI Short Read Archive under BioProject xxxxxxxxxxx.

BEHAVIOURAL DATA ANALYSIS

EthoVision 3.1 software was used to extract the time series of *x, y* coordinates of the rat from digitized video recordings (Dvorkin *et al*, 2006b; Dvorkin *et al*, 2010). To remove noise, digitized tracking data were pre-processed (by applying appropriate filters to smooth the *x, y* coordinates) (Hen *et al*, 2004), and the obtained coordinates were divided into episodes of forward locomotion (called progression) and episodes of small movements or immobility (called lingering), as described previously (Golani *et al*, 1993; Drai *et al*, 2000; Drai and Golani, 2001). The coordinate system was mapped onto the 25 open field locales (places) (Szechtman *et al*, 1998), and the frequency of visits and duration of stops in each locale were computed (the terms 'visit' and 'stop' are equivalent and are used interchangeably). Checking behavior was defined with reference to the most visited locale (labeled 'key place' or 'key locale'; these terms are equivalent), which in most instances is also the locale with the longest total duration of stops (Eilam and Golani, 1989a; Szechtman *et al*, 1998). A visit to the key place is also referred to as a 'check' or 'checking', and the following set of four measures of checking behavior were computed. (1) Frequency of checking: total number of visits to the key locale. (2) Length of check: total duration of stay at the key locale divided by the frequency of visits there; this measure is also an indirect index of ritual-like behavior as the appearance of motor rituals in quinpirole-treated rats is associated with a very short duration of stay in the key locale (Szechtman *et al*, 1998; Ben Pazi *et al*, 2001). (3) Recurrence time of checking: mean duration of return times to the key locale ('return

time' is the interval from departure to next arrival at the locale). (4) Stops before returning to check: mean number of places visited between returns to the key locale. Compulsive checking behavior is identified by the presence of a significant difference between quinpirole- and saline-treated rats – all four measures need to differ from controls for the claim of 'compulsive' checking (Szechtman *et al*, 1998), and hence the set of these four measures is termed 'criteria measures' for compulsive checking.

In addition to the above criteria measures, we also evaluate "time to next checking bout" (Dvorkin *et al*, 2006b). This measure is greatly reduced in quinpirole-sensitized rats and has been proposed to index the third constitutive component of compulsive checking behavior—"satiety" or rest after checking (Dvorkin *et al*, 2010). The computation of checking bouts is detailed in Dvorkin *et al.* (2006b) and a modification in Tucci *et al.* (2013).

Total distance traveled was calculated as the sum of distances moved in progression segments and lingering episodes. For the spatial distribution of routes of travel, two indices were used: path stereotypy ratio and area of 2 standard deviational ellipse (2SDE), computed according to the method detailed elsewhere (Dvorkin *et al*, 2006a). The first index, path stereotypy ratio, reflects the relative frequency of repetitions of travel along the same paths while the second index is a measure of the extent of the area covered by the trajectories of locomotion.

DESIGN AND PROCEDURE

Two groups of rats were tested: the experimental group (N=15) received injections of quinpirole (QNP), while the control group (N=16) received a similar regimen of saline injections. Animals were assigned to treatment groups at random. Originally the two groups had an equal number of subjects but one rat from the quinpirole group was removed from the experiment early on due to concerns with its health.

The typical regimen of quinpirole injections in studies of locomotor sensitization or compulsive checking are twice weekly injections (Szechtman *et al*, 1998; Dvorkin *et al*, 2006b). The behavioral effects of chronic treatment with quinpirole reach a plateau at approximately 8 injections administered 2–8 days apart (Szechtman *et al*, 1994b; Szechtman *et al*, 1994c; Perreault *et al*, 2005; Dvorkin *et al*, 2006b). The regimen of quinpirole injections chosen for the present study fell within these parameters but the typical regular schedule of drug administrations was modified to accommodate the requirements of a second objective for which these rats were being tested. Specifically, the experiment served also to evaluate gene expression changes as a function of chronic treatment with quinpirole, an objective that required the sampling of blood. Because a

two week interval between samplings was required, rats were tested on the open field once a week for the first 5 quinpirole treatments and twice per week for injections 6 to 9. Blood was sampled from the tail vein on the day following injections 1, 3, 5 and 9 (the gene expression findings will not be discussed here). Fecal pellets were collected after each test but were analyzed only for injections 1, 5 and 9 as these three time points were expected to provide a good snapshot of the start, middle and end of the sensitization process.

For all tests the same procedure was followed: Animals were weighed, transported in their home cage to an adjoining non-colony experimental testing room, and administered the appropriate injection. Immediately afterwards, the rat was placed into the open field for 55 min and its behavior videotaped for offline analysis. Each rat was run on the same assigned days of the week, at approximately the same time of day, and by the same experimenter for 9 trials. Each experimenter was assigned a balanced number of rats from every experimental group.

STATISTICAL ANALYSIS

To assess the development of checking behavior and locomotor sensitization across injections 1 to 9, regression estimates for each dependent variable were computed for each rat. The obtained individual slopes and intercepts were then analyzed statistically using a Group (saline *vs.* quinpirole) 1-way ANOVA. To correct for skew in the data, the variables *length of check* and *time to next checking bout*, were log transformed for statistical analysis. QIIME software was used for group comparison of OTUs using the Kruskal Wallis (KW) test. The OTU data identified as significant by KW were imported into SPSS (IBM SPSS Statistics 23) and analyzed with a 2x3 repeated measures ANOVA, where the Drug factor had two levels (saline vs quinpirole) and the repeated measures Injection factor had 3 levels (injection 1, injection 5 and injection 9).

RESULTS

INDUCTION OF COMPULSIVE CHECKING AND LOCOMOTOR SENSITIZATION

To examine whether changes in gut microbiota accompany the behavioral response to quinpirole, we first establish that in the present experiment the current protocol of quinpirole injections was effective in inducing the expected development of compulsive checking as well as locomotor sensitization and alteration in the spatial distribution of locomotion.

DEVELOPMENT OF COMPULSIVE CHECKING

Figure 1 (*left* panel) displays the criteria measures defining compulsive checking—*frequency of checking, length of check, recurrence time of checking,* and *stops before returning to check*. The graph shows the profile of these measures during injections 1 to 9 for the saline (*open circles*) and quinpirole-injected rats (*open squares*). Compulsive checking is expected after 8 to 10 injections of quinpirole; evidence for compulsive checking requires the presence of a significant difference between the quinpirole- and saline-treated rats on all 4 criteria measures (Szechtman *et al*, 1998; Dvorkin *et al*, 2006b; Dvorkin *et al*, 2010; Szechtman *et al*, 2017). As evaluated on injection 9 and indicated by the light gray rectangles in the figure, the quinpirole and saline groups were as expected significantly different from each other on all 4 criteria measures (t-tests, $p \leq .003$). Hence, this study regimen of quinpirole injections was effective in inducing compulsive checking.

Figure 1 (*right* panel) shows another variable—*duration of rest* after a bout of checking that has been suggested to constitute a component of compulsive checking and which characteristically is short in quinpirole-treated rats (Dvorkin *et al*, 2006b; Dvorkin *et al*, 2010). In the present study, as expected, and as shown by the light gray rectangle in the figure, the duration of rest on injection 9 was significantly shorter in the quinpirole-treated rats compared to the saline controls (*t*-test, $t(9) = 2.028$, $p = 0.037$, 1-tail).

The time course of development of compulsive checking can be assessed by examining the regression line for each criterion measure across injections. Prior analyses show that chronic treatment with quinpirole produces two distinct effects on these regression lines (Tucci *et al*, 2014; Ballester Gonzalez *et al*, 2015): For *frequency of checking* and *length of check*, repeated injections of quinpirole alter the slope but not the intercept of the regression line, compared to the saline group. For *recurrence time of checking* and *stops before returning to check*, quinpirole produces a shift in the intercept of the regression line without effect on the slope, compared to the saline group. The *frequency of checking* and *length of check* are variables that index the vigor in the motor performance of checking while *recurrence time of checking* and *stops before returning to check* are variables related to the focus on the task of checking (Dvorkin *et al*, 2010). Accordingly, the findings of altered slope but not intercept for measures of vigor indicate that only the vigor of checking sensitizes with repeated injections of quinpirole. In contrast, the findings of altered intercept but not slope for measures of focus indicate that quinpirole increases focus acutely, and this acute effect persists unabated throughout the course of chronic quinpirole treatment (Dvorkin *et al*, 2006b; Tucci *et al*, 2014; Ballester Gonzalez *et al*, 2015).

Figure 1 shows the calculated regression lines for each measure of compulsive checking for the saline- and quinpirole-injected groups in the present study, indicated by the *solid thin* line and the *solid thick* line, respectively. Parameters of these regression lines as well as the statistical analysis of these parameters are shown in Table 1. As can be seen, the regimen of quinpirole injections used in the present study yielded the same pattern of results as noted above, except that the intercepts of the regression lines for *frequency of checking* were also significantly different between the saline and quinpirole groups (Table 1). Nevertheless, it is evident that the quinpirole treatment used in the present study produced the expected profile of changes across injections during development of compulsive checking.

LOCOMOTOR SENSITIZATION AND ROUTES OF TRAVEL

Figure 2a shows the trajectories of locomotion shown by a rat injected with saline (*top* row) and a rat injected with quinpirole (*bottom* row) during the course of 9 treatments. The chosen rats are nearest their group mean of distance travelled in the experiment (saline mean ± SEM = 43.0 ± 6.6 m; quinpirole mean ± SEM = 236.2 ± 38.9 m) and illustrate the key features of changes across injections in their respective groups. Quantitative analyses for these routes of travel are presented in Figure 2bcd and involve variables shown previously to exhibit a specific profile which accompanies quinpirole-induced compulsive checking (Eilam *et al*, 1989c; Szechtman *et al*, 1994c; Dvorkin *et al*, 2006a; Dvorkin *et al*, 2006b; Dvorkin *et al*, 2010). In particular, it was shown previously that routes of travel by quinpirole rats with compulsive checking are characterized by: (i) elevated amount of locomotion, as measured by distance travelled; (ii) shrinkage of explored space, as measured by 2 standard deviational ellipse (2SDE) and, (iii) restriction of locomotion to repeated travel along a few routes only, as measured by path stereotypy. Such changes in each variable are also evident in Figure 2a by a visual comparison of routes of travel on injection 9 under saline *versus* quinpirole. More locomotion (and hence a higher value for distance travelled) is evident by greater density of trajectories in the quinpirole rat compared to the saline control. Shrinkage of explored space in the quinpirole rat (and hence a lower value for 2SDE) is evident by paths being distributed over a relatively narrow area of the open field *versus* paths of the saline rat being dispersed over the entire environment and edges of the perimeter boundary. Finally, more repetitions of travel along a few routes (and hence a higher value for path stereotypy) is evident by few yet thick trajectories in the quinpirole rat compared to many and thin trajectories of locomotion in the saline rat. Statistical comparisons of these measures on injection 9 for saline and quinpirole rats showed the expected significant group differences (*t*-tests, $p<.001$, light gray rectangles in Figure 2bcd), indicating the efficacy of the quinpirole treatment used in the present study.

The regression lines in Figure 2bcd show the time course of change in these measures during the course of treatment; the parameters of the regression lines are presented in Table 1. Comparison of the parameters from the present study to prior findings (Ballester Gonzalez *et al*, 2015) suggests that while the present regimen of quinpirole injections was effective in producing the salient features on routes of travel (locomotor sensitization, shrinkage of explored space and increase in path stereotypy), the intensity of these effects was smaller compared to findings in Ballester Gonzalez *et al* (2015), especially in terms of a smaller increase in distance travelled and in path stereotypy across injections.

EFFECTS OF QUINPIROLE ON GUT MICROBIOTA

Table 2 presents the list of 25 gut bacteria clusters identified through statistical analyses as likely altered because of chronic treatment with quinpirole. These clusters passed the following statistical analyses to emerge as potentially related to repeated injections of quinpirole:

First, profiling of bacterial composition produced 4313 operational taxonomic units (OTUs) appropriate for statistical analyses by Kruskal-Wallis (KW). The KW non-parametric 1-way analysis of variance treated the data as emanating from 6 independent groups, formed from the combination of two treatments (saline vs quinpirole) and 3 injections (injection 1, injection 5 and injection 9); OTUs with non-zero values in at least 20% of samples entered the statistical analysis. The KW analysis yielded 232 OTUs which showed a significant main effect ($p<.05$).

Second, the statistically significant OTUs from KW analysis were re-analyzed using a 2x3 repeated measures ANOVA, where Saline vs Quinpirole was the between-subjects Drug factor and Injections 1, 5 and 9 was the within-subjects repeated measures Injection factor. This statistical analysis yielded a total of 219 OTUs with a significant main or interaction effects: the main effect of Drug was found in 50 OTUs, the main effect of Injection in 178 OTUs, and a significant Drug by Injection interaction for 25 OTUs. These 25 OTUs are shown in Table 2 as potentially related to chronic injections of quinpirole because no drug effect is expected 55 min after injection 1 (when the first fecal pellets were collected) but to emerge over days as a function of quinpirole injections, yielding an interaction effect.

As is evident from Table 2, 22 of the 25 significant OTUs are from the phylum Firmicutes. The three remaining OTUs are one each from the phyla Deferribacteres, Proteobacteria, and Tenericutes.

All of the 22 OTUs from the phylum Firmicutes belong to the class Clostridia, order Clostridiales. At a still finer taxonomic level, the highest frequency of OTUs (9 in total) is in the family *Lachnospiraceae*, followed by 7 OTUs in the family *Ruminococcaceae* (Table 2). Two examples of the change in OTUs from *Lachnospiraceae* are displayed graphically in Figure 3.

Although all the above OTUs are statistically significant from our analysis, it is evident that many of the OTUs have low rarefied counts (Table 2). These OTUs may represent low abundant taxa, and as such they are subject to sampling noise in rarefication process.

DISCUSSION

In the present study the changes in behavior to repeated injections of quinpirole, namely, the development of compulsive checking and of locomotor sensitization, were monitored at the same time as was the response of gut microbiota to these drug injections. Behavior and microbiota were monitored concurrently as it was expected that if they are linked then evidence of an association should be present. Indeed, the present findings provide evidence consistent with the hypothesis of a relationship between changes in gut microbiota and development of locomotor sensitization or compulsive checking or both. Results showed that changes in behavior that occur after several injections of quinpirole were temporally accompanied by changes in several clusters of gut bacteria. Specifically, after some injections of quinpirole significant responses were found in 25 OTUs, with the vast majority of OTUs (22) belonging to the order Clostridiales and the class Clostridia in the phylum Firmicutes. At a finer level of taxonomy, 9 OTUs were in the *Lachnospiraceae* family and 7 OTUs were in the *Ruminococcaceae* family of bacteria.

Of possible relevance to the present findings, an effect on these gut microbiota has been reported in the following two studies. In one study, Ning *et al* (2017) report that in rats treated repeatedly with the indirect dopamine agonist methamphetamine and tested for conditioned place preference, there was an associated alteration in the intestinal microbiota, and amongst those changes there was an increase in OTUs from the family *Ruminococcaceae.* In another recent study of gut microbiota, Hill-Burns *et al* (2017) confirmed the findings of a prior study (Keshavarzian *et al*, 2015) of reduced levels of *Lachnospiraceae* in patients with Parkinson Disease (PD). Together with our

results, these findings point towards a likely relationship between dopamine activity and effect on those particular colonies of gut microbiota.

One vital role of gut microbiota is to metabolize resistant starches and dietary fibers through fermentation and decomposition, yielding as end products short chain fatty acids (SCFAs) which include acetate, propionate and butyrate (den Besten *et al*, 2013; Ohira *et al*, 2017). Of the two most abundant phyla in the intestine, the Bacteroides phylum has as its metabolic end product mainly acetate and propionate and the Firmicutes phylum produces largely butyrate (den Besten *et al*, 2013). *Lachnospiraceae* and *Ruminococcaceae* are the most abundant Firmicutes families in gut environments (Biddle *et al*, 2013; Meehan and Beiko, 2014). Recently, much interest has been focused on the butyrate producing bacteria, though *Lachnospiraceae* and *Ruminococcaceae* are not the only families to do this and not all members have butyrate-producing pathways (Biddle *et al*, 2013; Vital *et al*, 2014). Butyrate (also known as butanoic acid, butanoate, and butyric acid) is a short-chain fatty acid that provides energy for other microbes and host cells, promotes energy expenditure, as well as facilitating fatty acid oxidation and lipolysis (Gao *et al*, 2009; Sekirov *et al*, 2010; Williams *et al*, 2011; den Besten *et al*, 2013; Meehan *et al*, 2014; Hong *et al*, 2016; Stilling *et al*, 2016).

While the present study provides evidence consistent with an association between the behavioral and gut microbiota phenomena, it does not address the question of the cause-effect direction underlying the observed association nor the putative mechanisms. The change in bacterial composition could be a direct effect of the drug on gut microbiota, driving the change in behavior. Alternatively, the change in bacterial composition could be contributing to the change in behavior but itself be a consequence of the injection effect on host physiology and/or nervous system. It is also plausible that the change in bacterial composition and the change in behavior are two totally independent and unrelated effects of the drug treatment. As a correlational study the present results do not provide evidence addressing these or any variation of these possibilities. Nevertheless, we can offer some suggestions that can provide a useful framework to generate hypotheses for further studies. Below, we first consider two contrasting perspectives to frame the connecting relationship between gut microbiota and behavior and then use one of them to speculate on a plausible mechanism underlying the observed association between gut microbiota and development of compulsive checking and locomotor sensitization.

TWO PERSPECTIVES

There is a rapidly expanding literature reporting on studies that show specific changes in gut microbiota and perturbations in particular behaviors, and vice-versa, raising the concept of an active and bidirectional microbiota-gut-brain axis (Collins *et al*, 2012; De Palma *et al*, 2014; Foster, 2016; Dinan and Cryan, 2017b; Zhu *et al*, 2017). Below, we outline two possible but polar models linking microbiota and behavior: one model in which gut microbiota are sufficient to produce a change in behavior and another one in which microbiota are neither necessary nor sufficient for the change in behavior.

MODEL OF MICROBIOTA STIMULATING BEHAVIOR

One way to conceptualize an observed association between gut microbiota and behavior is the perspective that gut microbes drive behavior. This perspective holds that there is a direct pathway from some particular gut microbiota to stimulation of specific neural circuits mediating unique behavioral actions. An extreme example of such a schema is illustrated by recent studies on manipulation of host biology and behavior through infection by the brain parasite *Toxoplasma gondii (T. gondii)* (Torrey *et al*, 2007; Kaushik *et al*, 2012; Flegr, 2013; Evans *et al*, 2014). *T. gondii*-infected rats and mice lose their natural avoidance of predator odor and open spaces, resulting in increased feline predation of the infected rodents and the ensuing passage of the parasite into the feline host for the next stage of its life cycle (Kaushik *et al*, 2012). The mechanism by which the parasite induces the particular change in rodent behavior involves cyst localization to specific neuroanatomical regions and interaction thein with the behaviorally functional neural circuit (Prandovszky *et al*, 2011; Kaushik *et al*, 2012; Evans *et al*, 2014). Because *T. gondii* enters the host through the intestinal tract, it affects behavior through its initial effects on the intestinal tract (Severance *et al*, 2016). A conceptually similar infectious model had been proposed for producing OCD (Rotge *et al*, 2010).

A growing body of literature is showing that without acquisition of infection, non-pathogenic alteration in the composition of the commensal bacteria can also change behavior. A particularly dramatic illustration of this relationship is a study showing that colonization of germ-free BALB/c mice with microbiota from two different mice strains produced strain-specific behavioral phenotypes in the recipient animals (Bercik *et al*, 2011). Although the mechanisms linking commensal bacteria and behavior are yet unidentified, there is evidence for multiple ways by which gut microbiota could activate brain circuits mediating behavior: Considering that microbiota elaborate a wide range of appropriate signaling molecules (monoamines, SCFAs and hormones) and have epitopes recognizable by both intestinal epithelial and mucosal immune cells, it follows that gut microbiota have access to neuronal (vagus, enteric nervous system), endocrine,

as well as immune channels for communication with the brain (Roshchina, 2010; Sekirov *et al*, 2010; Collins *et al*, 2012; Erny *et al*, 2015; Neuman *et al*, 2015; Stilling *et al*, 2016; Woo and Alenghat, 2017).

In all, according to the "microbiota stimulating behavior" model, an observed association between gut microbiota and behavioral phenotype indicates a relatively direct effect of the specific microbes on brain-specific neural circuits driving the behavioral phenotype.

MODEL OF INFRASTRUCTURAL SUPPORT

In contrast to the above model of behavioral activation by a relatively direct pathway from the gut to the brain, a model of "infrastructural support" does not draw a causal pathway from the change in microbiota to an associated change in behavior. Rather, it considers that the association reflects only some supportive role played by the microbiota in the performance of the behavior and the alteration in microbiota is neither necessary nor sufficient to drive the observed behavioral change. Although this model holds that the microbiota change is not causative of the change in behavior, it leaves open that the two phenomena can be relatively strongly coupled and that the microbiota change is important for the change in behavioral phenotype, as elaborated below.

The infrastructure model is built on the observation that normally gut microbiota have a symbiotic association with their host and play a prominent role in nutrition, immune regulation and maintenance of barrier function (Jandhyala *et al*, 2015). As such, rather than drive behavior directly, the role of alterations in gut microbiota may be to provide the infrastructural support needed for the organism to work and function in an adaptive fashion as demanded by circumstances. For instance, when the type of diet consumed changes then the composition of gut commensals changes accordingly to process the new nutrients (Turnbaugh *et al*, 2009; Wu *et al*, 2011; David *et al*, 2014; Wang *et al*, 2014).

Accordingly, the perspective of the infrastructural support model holds that an observed association between gut microbiota and behavioral phenotype indicates the presence of an accommodation of gut microbiota for supporting the physiological demands of the behavioral phenotype. The two phenomena are fully independent, but can become coupled in time.

PRIMING OF ENERGY SYSTEMS FOR RAPID DEPLOYMENT

In the present study the observed association between microbiota and behavior was produced by chronic quinpirole experience. Our hypothesis for this finding is that it reflects the infrastructure role of the altered microbiota in energy metabolism facilitating the behavioral phenotype. The rationale for this hypothesis is as follows.

The enhanced motor performance of quinpirole-sensitized rats raised the suggestion (Szechtman *et al*, 1994c) that the dopamine agonist acts on central energy control mechanisms that enable motor vigor (Vissing *et al*, 1989a; Vissing *et al*, 1989b; Scheurink and Steffens, 1990; Schwartz *et al*, 1992; Szechtman *et al*, 1994c), consistent with exacerbated "feelings of energy" often experienced with psychostimulant drugs (Smith and Beecher, 1960; Laties and Weiss, 1981; Post and Contel, 1983). Measurements of energy metabolism in quinpirole sensitized rats yielded findings consistent with an enhanced sense of energy in that in quinpirole- sensitized rats, energy metabolism (as measured by VCO_2/VO_2 respiratory quotient) is shifted towards utilization of a more energy-rich fuel, free fatty acid (FFA).

The present findings that most of the quinpirole-related changes in gut microbiota occurred in *Lachnospiraceae* and *Ruminococcaceae* bacteria may constitute in part the mechanism for the shift noted above towards utilization of FFA by quinpirole-sensitized rats. As noted, microbes from these families produce butyrate that provides energy for other microbes and host cells, promotes energy expenditure, as well as facilitating fatty acid oxidation and lipolysis (Gao *et al*, 2009; Williams *et al*, 2011; Meehan *et al*, 2014; Hong *et al*, 2016). Hence, one of the functional effects of quinpirole-induced altered microbiota may be to promote the shift towards FFA utilization observed in quinpirole-sensitized rats (Coscina *et al*, 1998).

If the change in microbiota does indeed contribute to the above shift in energy utilization with quinpirole then in addition to enabling sensitized locomotion, the microbiota plays a role also in the performance of compulsive checking and hence by implication from the animal model, in OCD. This suggestion follows from the security motivation theory of OCD (Szechtman and Woody, 2004; Woody and Szechtman, 2005) and in particular from a consideration of the physiological processes fueling the security motivation system (SMS). As elaborated elsewhere (Woody and Szechtman, 2011), the physiology of an activated SMS primes energy resources for rapid mobilization should maximal exertion becomes necessary if a real threat is encountered, and the "fight or flight" response is engaged (Cannon, 1927). An activated SMS primes, but does not mobilize the energy resources, because motor activity engendered by "security motivation – for example, checking for predators – is not physically strenuous and hence not highly demanding of energy resources" (Woody *et al*, 2011). Nevertheless the

motor actions of security motivation (precautionary and probing behaviors) do require an elevation in energy, an energy expenditure demand that can be met by a greater supply and utilization of FFA. Accordingly, the observed changes in *Lachnospiraceae* and *Ruminococcaceae* bacteria may serve to support the energy needs of compulsive checking and OCD.

COMPETING INTERESTS

The authors declare no competing interests.

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FIGURE CAPTIONS

FIGURE 1

Effects of chronic treatment with quinpirole on the criteria measures for compulsive checking. See Table 1 for parameters of the indicated regression lines. Each symbol is the mean value for the indicated dependent variable at the indicated injection obtained from all rats showing a value. Regression lines were computed from the regression parameters of each individual rat as described in Methods. *Open circles*, Saline control group injected repeatedly with saline; *open squares*, Quinpirole group injected repeatedly with quinpirole (0.25 mg/kg). The corresponding regression lines are: *thin solid line*, saline group; *thick solid line*, quinpirole group. *Light gray vertical rectangle* indicates that the groups evaluated at injection 9 were statistically different ($p<0.05$, *t*-tests).

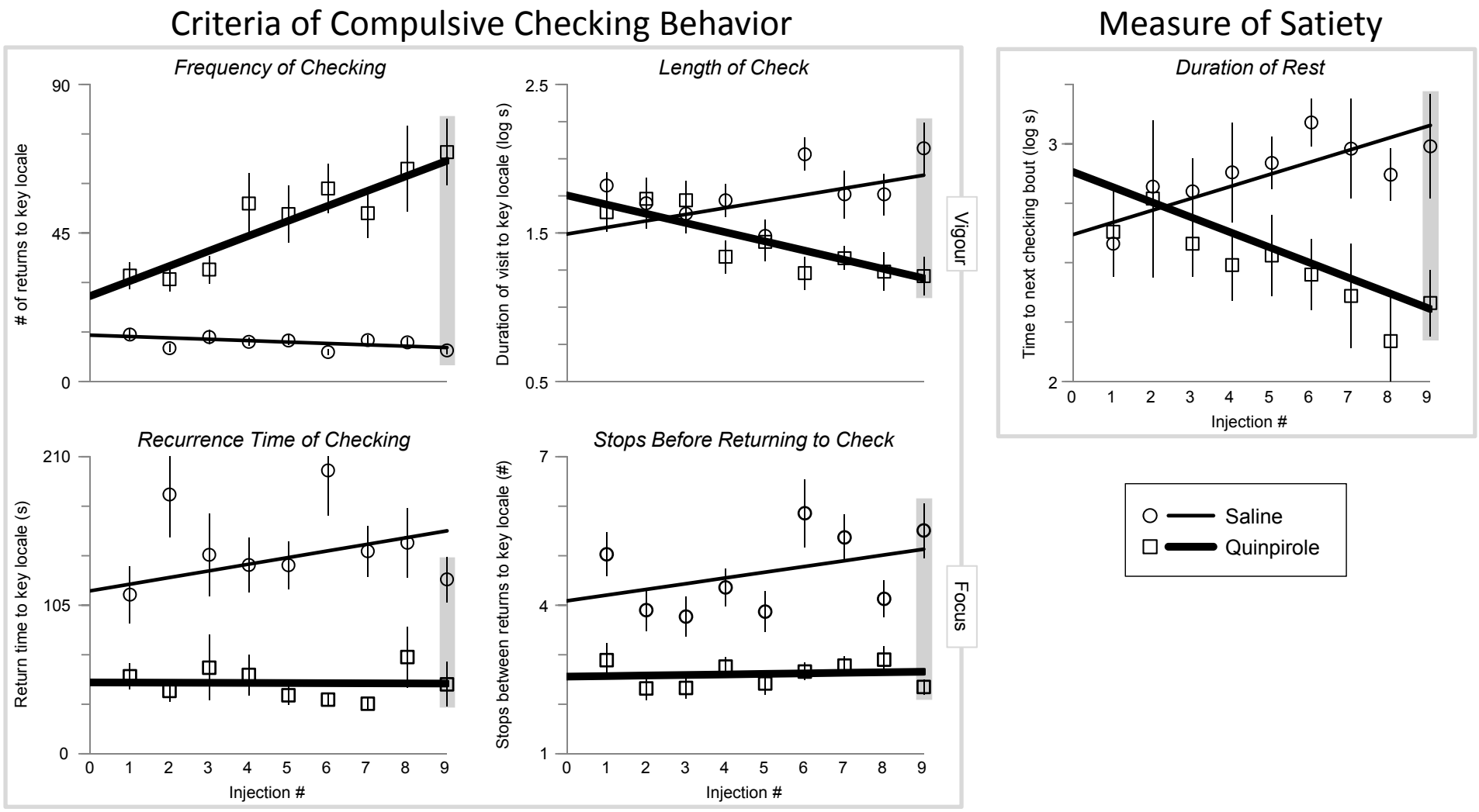
FIGURE 2

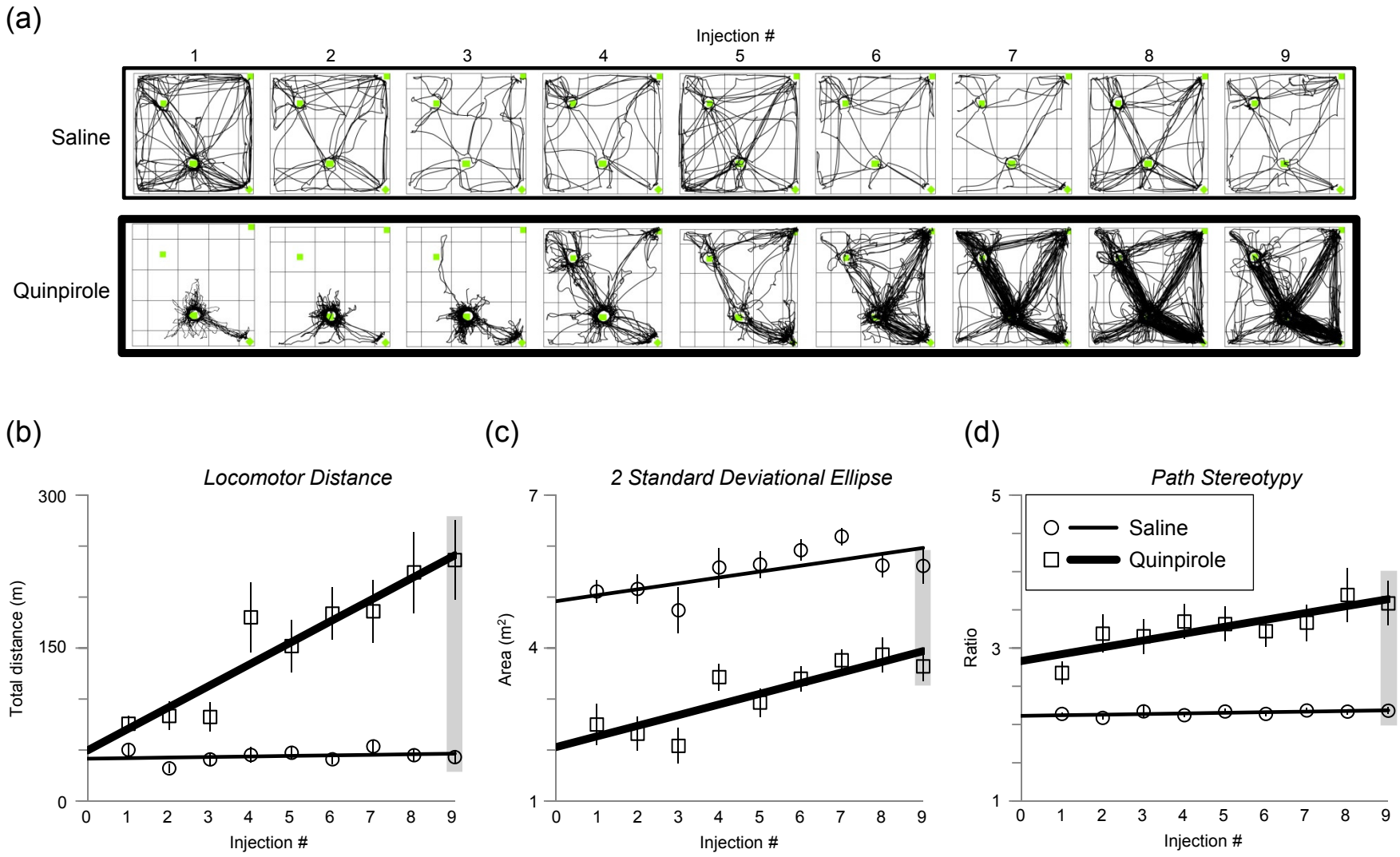
Effects of chronic treatment with quinpirole on the routes of travel (***a***) and on measures of distance travelled (***b***), 2 standard deviational ellipse (***c***) and path stereotypy (***d***). Routes of travel are shown as path plots for a representative rat treated with saline (top row) or quinpirole (bottom row). Locomotor trajectories during the entire 55 min session for injections 1 to 9 are shown. Each line represents a trajectory of locomotion and the density of trajectory lines corresponds to amount of locomotion. Gray squares indicate locations of the four objects in the open field. *Open circles*, Saline control group injected repeatedly with saline; *open squares*, Quinpirole group injected repeatedly with quinpirole (0.25 mg/kg). The corresponding regression lines are: *thin solid line*, saline group; *thick solid line*, quinpirole group; see Table 1 for parameters of the regression lines. *Light gray vertical rectangle* indicates that the groups evaluated at injection 9 were statistically different ($p<0.05$, *t*-tests).

FIGURE 3

Two examples of OTUs from the family *Lachnospiraceae* that changed with repeated injections of quinpirole. Data are from Table 2.

Figure 1, 2, and 3




(a)
Injection #
1
2
3
4
5
6
7
8
9
Saline
Quinpirole
(b)
Locomotor Distance
Total distance (m)
300
150
0
Injection #
(c)
2 Standard Deviational Ellipse
Area (m²)
7
4
1
Injection #
(d)
Path Stereotypy
Ratio
5
3
1
Saline
Quinpirole
Injection #

Lachnospiraceae

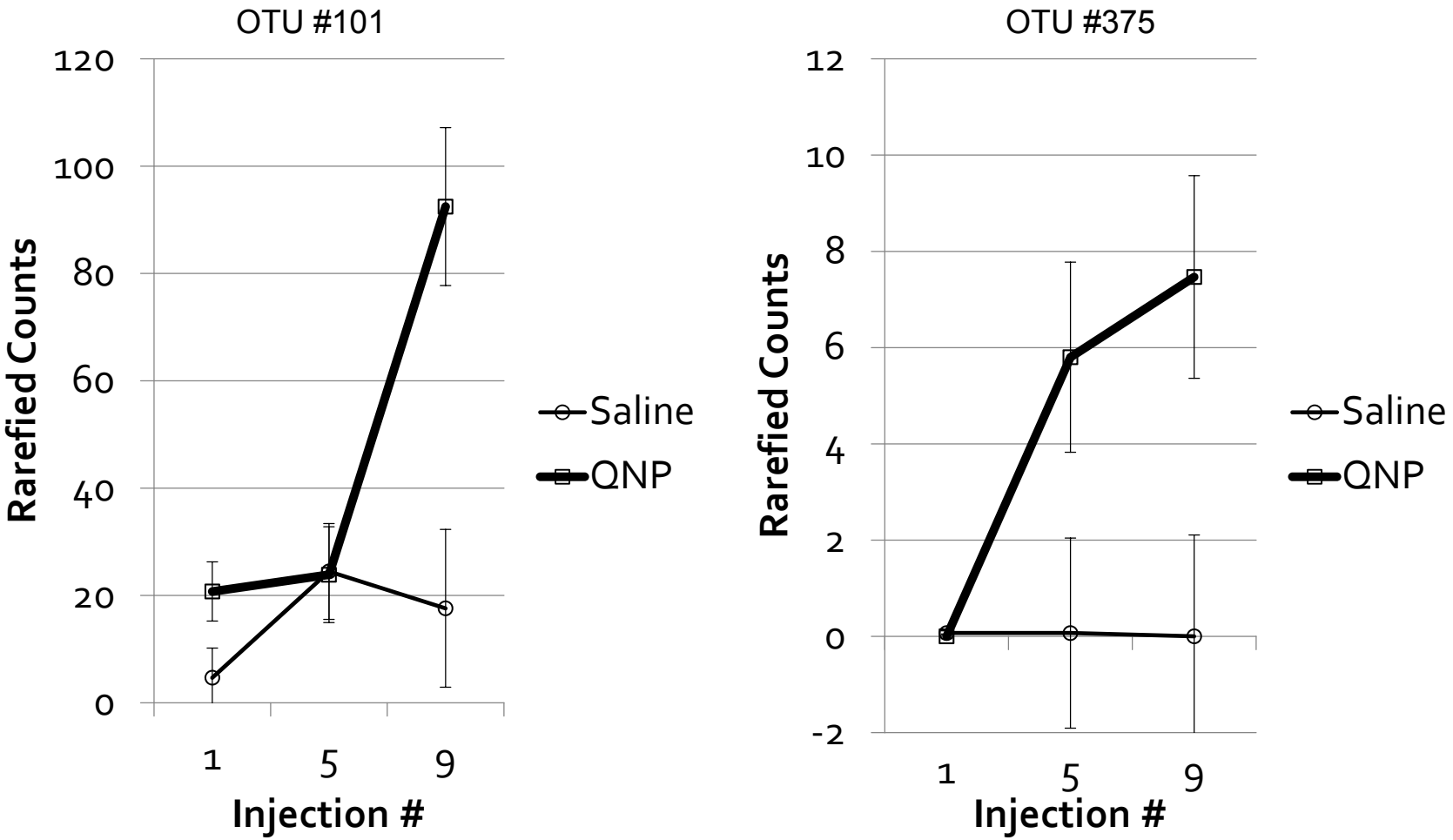


Table 1 **Parameters of regression lines in figures 1 and 2 for compulsive checking and for measures of locomotion.**

Compulsive Checking and Locomotion Measures	Regression Parameters[1]	Group[2]		Group Effect[3]		
		Saline	Quinpirole	F(1,29)	*P*	η_p^2
Frequency of checking	intercept	**14.03** ± 2.95	**25.91** ± 3.05	7.847	*0.009*	0.213
	slope	-0.42 ± 0.94	**4.56** ± 0.97	13.620	*0.001*	0.320
Length of check (log s)	intercept	**1.49** ± 0.15	**1.75** ± 0.15	1.505	0.230	0.049
	slope	0.04 ± 0.03	**-0.06** ± 0.03	7.278	*0.012*	0.201
Recurrence time of checking (s)	intercept	**115.18** ± 18.50	**50.39** ± 19.11	5.932	*0.021*	0.170
	slope	4.71 ± 2.77	-0.09 ± 2.86	1.452	0.238	0.048
# of stops before returning to check	intercept	**4.09** ± 0.29	**2.56** ± 0.30	13.896	*0.001*	0.324
	slope	**0.12** ± 0.05	0.01 ± 0.06	1.840	0.185	0.060
Time to next checking bout (log s)	intercept	**2.62** ± 0.23	**2.88** ± 0.20	0.741	0.400	0.036
	slope	0.05 ± 0.03	**-0.06** ± 0.03	7.694	*0.012*	0.278
Distance (m)	intercept	**41.48** ± 11.96	**49.66** ± 12.35	0.227	0.638	0.008
	slope	0.55 ± 3.31	**21.26** ± 3.42	18.902	*0.000*	0.395
2SDE	intercept	**4.92** ± 0.35	**2.06** ± 0.36	32.788	*0.000*	0.531
	slope	**0.12** ± 0.05	**0.21** ± 0.05	1.673	0.206	0.055
Path stereotypy (ratio)	intercept	**2.11** ± 0.17	**2.83** ± 0.18	8.614	*0.006*	0.229
	slope	0.01 ± 0.04	**0.09** ± 0.04	2.524	0.123	0.080

[1] Estimates of group slope and intercept are means (and SEM) of individual rat regression parameters fitted to the dependent variable data across injections 1 to 9. Values in bold font are significantly different from 0.

[2] Saline group (n=16) refers to control group that was injected chronically with saline; Quinpirole group (n=15) refers to rats that were injected chronically with quinpirole (0.25 mg/kg).

[3] Groups were evaluated using a 1-way analysis of variance and the obtained F values, statistical significance and partial eta squared values (η_p^2) are indicated. Degrees of freedom for *Time to next checking bout (log s)* were 1 and 20. Significant *P* values ($p<0.05$) are indicated by italic font.

Table 2 OTUs where a 2x3 ANOVA yielded a statistically significant Drug by Injection interaction

Taxonomy[1]	OTU #[2]	Injection[3]	Group[4]		Drug Effect[5]			Injection Effect[5]			Drug x Injection Interaction[5]		
			Saline	Quinpirole	F(1,28)	*P*	η_p^2	F(2,56)	*P*	η_p^2	F(2,56)	*P*	η_p^2
Firmicutes/Clostridia													
o__Clostridiales; f__Clostridiaceae; g__Sarcina	7	1	847.3 ± 233.0	1201.4 ± 233.0	6.285	0.*018*	0.183	3.822	0.*028*	0.120	3.315	*0.044*	0.106
		5	839.5 ± 878.6	3896.0 ± 878.6									
		9	1170.6 ± 734.0	3252.6 ± 734.0									
o__Clostridiales	26	1	154.0 ± 61.0	116.5 ± 61.0	9.519	0.*005*	0.254	3.957	0.*025*	0.124	4.937	0.*011*	0.150
		5	930.5 ± 164.0	91.7 ± 164.0									
		9	463.1 ± 161.3	258.3 ± 161.3									
o__Clostridiales; f__Ruminococcaceae; g__	44	1	56.2 ± 13.7	33.4 ± 13.7	2.662	0.114	0.087	16.104	0.*000*	0.365	3.978	0.*024*	0.124
		5	87.3 ± 26.9	143.9 ± 26.9									
		9	113.9 ± 28.1	200.2 ± 28.1									
o__Clostridiales; f__Lachnospiraceae	101	1	4.7 ± 5.5	20.7 ± 5.5	9.627	0.*004*	0.256	10.336	0.*000*	0.270	8.472	0.*001*	0.232
		5	24.5 ± 8.9	23.9 ± 8.9									
		9	17.6 ± 14.7	92.5 ± 14.7									
o__Clostridiales; f__Ruminococcaceae; g__Oscillospira	118	1	4.4 ± 1.4	5.3 ± 1.4	5.235	0.*030*	0.158	4.680	0.*013*	0.143	3.179	0.*049*	0.102
		5	6.5 ± 19.0	39.3 ± 19.0									
		9	11.6 ± 18.4	80.7 ± 18.4									
o__Clostridiales; f__Lachnospiraceae; g__Moryella	140	1	5.5 ± 4.3	12.9 ± 4.3	11.131	0.*002*	0.284	10.492	0.*000*	0.273	3.212	0.*048*	0.103
		5	8.2 ± 4.7	21.3 ± 4.7									
		9	17.5 ± 9.2	54.3 ± 9.2									
o__Clostridiales; f__Lachnospiraceae; g__	156	1	1.1 ± 0.6	1.5 ± 0.6	6.899	0.*014*	0.198	7.325	0.*001*	0.207	5.691	0.*006*	0.169
		5	2.7 ± 2.7	6.3 ± 2.7									
		9	6.5 ± 20.3	77.9 ± 20.3									
	176	1	7.1 ± 3.4	6.4 ± 3.4	7.684	0.*010*	0.215	1.223	0.302	0.042	3.896	0.*026*	0.122
		5	11.8 ± 4.2	4.5 ± 4.2									
		9	28.7 ± 7.4	0.1 ± 7.4									
	177	1	0.7 ± 1.4	4.3 ± 1.4	8.063	0.*008*	0.224	8.792	0.*000*	0.239	6.424	0.*003*	0.187
		5	1.7 ± 4.9	16.9 ± 4.9									
		9	4.5 ± 12.4	53.4 ± 12.4									
o__Clostridiales; f__; g__	197	1	9.5 ± 2.1	14.5 ± 2.1	0.020	0.890	0.001	4.192	0.*020*	0.130	7.040	0.*002*	0.201
		5	17.2 ± 2.0	10.3 ± 2.0									
		9	7.6 ± 1.9	10.3 ± 1.9									

o__Clostridiales; f__Ruminococcaceae	252	1	4.3 ± 1.5	4.5 ± 1.5	7.951	0.*009*	0.221	6.322	0.*003*	0.184	3.328	0.*043*	0.106
		5	2.3 ± 1.3	7.7 ± 1.3									
		9	5.7 ± 3.4	17.0 ± 3.4									
o__Clostridiales; f__Ruminococcaceae; g__	262	1	0.1 ± 0.9	2.9 ± 0.9	8.998	0.*006*	0.243	4.390	0.*017*	0.136	4.547	0.*015*	0.140
		5	0.0 ± 3.0	12.4 ± 3.0									
		9	0.0 ± 5.1	18.9 ± 5.1									
o__Clostridiales; f__Lachnospiraceae; g__	375	1	0.1 ± 0.0	0.0 ± 0.0	6.208	0.*019*	0.181	4.089	0.*022*	0.127	4.199	0.*020*	0.130
		5	0.1 ± 2.0	5.8 ± 2.0									
		9	0.0 ± 2.1	7.5 ± 2.1									
o__Clostridiales; f__Ruminococcaceae	433	1	1.1 ± 0.5	1.6 ± 0.5	18.831	0.*000*	0.402	1.387	0.258	0.047	6.327	0.*003*	0.184
		5	0.7 ± 0.6	3.1 ± 0.6									
		9	0.1 ± 0.7	4.5 ± 0.7									
o__Clostridiales	454	1	0.2 ± 0.2	0.5 ± 0.2	6.647	0.*015*	0.192	5.232	0.*008*	0.157	3.216	0.*048*	0.103
		5	0.4 ± 0.7	2.7 ± 0.7									
		9	0.6 ± 0.9	3.5 ± 0.9									
o__Clostridiales; f__Ruminococcaceae	462	1	0.0 ± 0.1	0.3 ± 0.1	9.140	0.*005*	0.246	8.857	0.*000*	0.240	5.426	0.*007*	0.162
		5	0.3 ± 0.5	2.1 ± 0.5									
		9	0.6 ± 1.1	5.1 ± 1.1									
o__Clostridiales; f__Clostridiaceae	484	1	0.2 ± 0.2	0.4 ± 0.2	6.805	0.*014*	0.196	7.008	0.*002*	0.200	4.447	0.*016*	0.137
		5	0.4 ± 0.6	1.8 ± 0.6									
		9	0.5 ± 0.7	3.3 ± 0.7									
o__Clostridiales; f__Lachnospiraceae; g__	550	1	0.0 ± 0.0	0.1 ± 0.0	4.996	0.*034*	0.151	7.183	0.*002*	0.204	3.692	0.*031*	0.117
		5	0.1 ± 0.1	0.3 ± 0.1									
		9	0.6 ± 1.0	3.5 ± 1.0									
o__Clostridiales; f__Lachnospiraceae	565	1	0.1 ± 0.1	0.2 ± 0.1	8.082	0.*008*	0.224	10.698	0.*000*	0.276	5.012	0.*010*	0.152
		5	0.1 ± 0.3	1.0 ± 0.3									
		9	0.5 ± 0.5	2.5 ± 0.5									
	636	1	0.1 ± 0.2	0.3 ± 0.2	2.773	0.107	0.090	9.414	0.*000*	0.252	4.383	0.*017*	0.135
		5	0.3 ± 0.2	0.3 ± 0.2									
		9	1.9 ± 0.4	0.6 ± 0.4									
o__Clostridiales; f__Clostridiaceae; g__Clostridium	796	1	0.1 ± 0.1	0.2 ± 0.1	8.313	0.*007*	0.229	5.424	0.*007*	0.162	3.958	0.*025*	0.124
		5	0.1 ± 0.2	0.6 ± 0.2									
		9	0.1 ± 0.2	1.1 ± 0.2									
o__Clostridiales; f__Ruminococcaceae; g__Clostridium	813	1	0.1 ± 0.1	0.2 ± 0.1	1.800	0.190	0.060	5.224	0.*008*	0.157	4.388	0.*017*	0.135
		5	0.1 ± 0.1	0.1 ± 0.1									

		9	0.7 ± 0.2	0.2 ± 0.2									
Tenericutes/Mollicutes													
o__RF39; f__; g__	60	1	50.3 ± 21.2	46.6 ± 21.2	3.139	0.087	0.101	0.679	0.511	0.024	3.765	0.*029*	0.119
		5	65.9 ± 40.2	75.0 ± 40.2									
		9	192.5 ± 56.3	0.1 ± 56.3									
Proteobacteria/Betaproteobacteria													
o__Burkholderiales; f__Alcaligenaceae; g__	68	1	75.1 ± 23.5	101.6 ± 23.5	1.157	0.291	0.040	6.631	0.*003*	0.191	3.545	0.*036*	0.112
		5	53.6 ± 8.4	20.5 ± 8.4									
		9	80.1 ± 11.4	36.9 ± 11.4									
Deferribacteres/Deferribacteres													
o__Deferribacterales; f__Deferribacteraceae; g__Mucispirillum	365	1	1.9 ± 1.3	1.3 ± 1.3	3.534	0.071	0.112	2.514	0.090	0.082	3.729	0.*030*	0.118
		5	1.5 ± 0.7	1.9 ± 0.7									
		9	1.1 ± 2.3	8.3 ± 2.3									

[1] Bold font indicates the phylum/class. Taxa in the designated phylum/class are indented in successive rows and are identified with the prefix "o_" (order), "f_" (family), and "g_" (genus).

[2] Operational Taxonomic Unit (OTU) assigned the taxonomic identification indicated in the left column. OTU numerals correspond to the rank order of counts in bacterial clusters (from highest to lowest counts). Table is sorted from lowest to highest OTU within phylum/cluster.

[3] Injection is the number of administrations of saline or quinpirole received at the indicated open field test. Injection is a repeated measures factor with 3 levels (injection 1 vs injection 5 vs injection 9).

[4] Saline refers to the control group that was injected chronically with saline, and Quinpirole is the experimental group treated chronically with quinpirole (0.25 mg/kg); they constitute the chronic drug treatment factor, with two levels (Saline vs Quinpirole). Values are the adjusted marginal means and 1 SE from the Drug by Injection ANOVA. Numbers are the counts in the rarefied OTU table with 43335 reads/sample and index a measure of the abundance of bacteria in the assigned OTU.

[5] Groups were evaluated using a drug-by-injection analysis of variance and the F values obtained, statistical significance, and partial eta squared values (ηp^2) are indicated. Significant differences (P <0.05) are marked in italic font.

Dear Dr Willner

Thank you very much for the encouraging and helpful comments of the reviewers. We have incorporated most of the suggestions made by the reviewers:

Reviewer 1:

1. Reviewer suggests that it may be useful to present additional analyses of the microbiota data and in particular by profiling at higher taxonomic classifications. Although we did do such analyses they were not very revealing and helpful, probably because the changes we report are confined to relatively few OTUs and thus likely overshadowed at grosser levels of analysis. Moreover, we are really interested in changes that appear as a result of several injections of quinpirole and those are difficult to discern from the effects of time in the type of analyses that the reviewer suggests. Hence, we did not include such analyses in the paper. However, we are depositing the raw sequence data in the NCBI data base and thus they are available to additional analyses by any interested researcher.
2. Reviewer suggests that we relate our quinpirole findings to other studies where the effects of dopaminergic drugs on gut microbiota were investigated, and the reviewer points to 3 such studies. We thank the reviewer for the suggestion and pointing us to those studies. We did not find a way to relate our findings to the results of those studies that used dopamine blockers; the third study where cocaine was used did not measure gut microbiota. However, following up on the reviewer's suggestion we found a recent study examining the effects amphetamine on gut microbiota and we included it in the revised ms. Moreover, we also included in the revised ms a comparison to studies of Parkinson patients where the microbiota findings were in the opposite direction to the quinpirole results (page 12, 2nd paragraph of the Discussion).
3. Reviewer did not consider that the link with T. gondii was very convincing in our exposition of the "infection model". We have re-written the sections describing the plausible models to make them clearer. In the revised description the model is called "MODEL OF MICROBIOTA STIMULATING BEHAVIOR" and we believe presents a more convincing picture of the plausible linkage between microbiota and behavior considered in the literature (pages 13-15, section "Two perspectives" was reworded substantially).

Reviewer 2.

1. Reviewer suggests that we should be more consistent and use "locomotor sensitization" rather than just "sensitization" throughout the ms. We agree and made those changes in the revised ms.
2. Reviewer points to an unexplained XXX in the Results section. We apologize for this mistake. The XXX was a placeholder to input the appropriate value but clearly I missed doing this before submitting the ms. The appropriate value (4313) is now indicated (page 11, 3rd paragraph).
3. Reviewer suggests that we elaborate more on our findings and in particular on the functions of the altered microbiota. We thank the reviewer for this suggestion and in the revised ms present more information (page 13, 2nd paragraph). We also expanded the following paragraph (page 13,

3rd paragraph) presenting the possible interpretations for the observed association between gut microbiota and behavior.

4. Reviewer found that the gray vertical rectangles were not clear in Figures 1 and 2. We made those rectangles darker and hopefully easier to see.

In addition to those changes we also revised Table 2 to make it clearer by providing more annotations and by presenting the data sorted by OTU rank as that gives a better picture of the size of the observed changes. We also made some stylistic changes on re-reading the ms.

As noted above, we are depositing the raw sequence data to NCBI database and that is indicated in the ms on page 6: "Raw sequence reads have been deposited in the NCBI Short Read Archive under BioProject xxxxxxxxxxx." That sentence is highlighted in yellow as the deposit is not yet complete and we do not have the actual project ID to include in the ms. However, the submission to NCBI should be completed in a few days and I can input the project ID at the page proofs stage or if a second revision of the ms is needed.

We thank again the reviewers and you for the comments on the ms and we trust that we have addressed all the issues.

With many thanks and best wishes

Henry Szechtman