Schedule-induced polydipsia as a model of compulsive behaviour: behavioural inflexibility, excessive habit formation and neurobiological substrates

La polidipsia inducida por programa como modelo de conducta compulsiva: inflexibilidad conductual, excesiva formación de hábitos y sus correlatos neurobiológicos

Doctoral Thesis

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«You cannot get through a single day
without having an impact
on the world around you.

What you do makes a difference,
and you have to decide what kind
of difference you want to make»

Jane Goodall

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Abstract / Resumen

ABSTRACT

Compulsive behaviour has been defined as actions inappropriate to the situation, which persist, have no obvious relationship to the overall goal and which often result in undesirable consequences. The presence of this symptom is characteristic of various psychiatric disorders, such as obsessive-compulsive-disorder, body dysmorphic disorder, hoarding disorder, hair-pulling disorder and skin-picking disorder, which comprise the "Obsessive-compulsive and related disorders" cluster in the Diagnostic and Statistical Manual of Mental Disorder (5^{th} edition). However, in OCD patients, comorbidity with other psychiatric disorders, such as anxiety, eating disorders, depression, impulse control disorders or substance abuse, is often present in the majority of the cases. Therefore, there is a growing interest in identifying transdiagnostic, neurobehavioural domains, such as behavioural inflexibility and excessive habit formation, for developing accurate translational models to investigate the complex neuropathology of compulsivity. This behaviour is associated with altered levels of monoamines, particularly serotonin (5-hydroxytryptamine- 5-HT) and its receptor system. Since the gut microbiota regulates 5-HT precursor metabolism (tryptophan-TRP) and may affect global 5-HT synthesis, an altered functioning of the microbiota-gut-brain axis may be involved in compulsivity.

In the present Doctoral Thesis, we studied compulsive behaviour with an animal model, Schedule-induced Polydipsia (SIP), characterized by the development of persistent and excessive drinking under intermittent food-reinforcement schedules. The amount of water consumed in SIP varies between- and within- strains of rats, which allows the classification of two populations according to these individual differences: High Drinkers (HD) and Low Drinkers (LD) rats. The main objectives of the Doctoral Thesis were: First, to explore neurobehavioural domains, such as behavioural flexibility and habit formation, and neurobiological substrates that could underlie the vulnerability to develop compulsive drinking in SIP. Second, given the close relationship between compulsivity and the serotonergic system, to investigate the effects of serotonin reductions by chronic dietary TRP depletion on compulsive drinking in SIP and on the gut microbiota.

The results from the first experimental series revealed that HD rats exhibit behavioural inflexibility in a reversal-learning task and excessive habit formation in a reinforcer devaluation task. Also, this group showed increased plasma levels of the stress hormone corticosterone after the SIP exposure, indicating that SIP might represent a stressful context for vulnerable individuals. In addition, HD rats had normal serum sodium concentrations, which provide evidence that the behavioural, neurochemical and neuroanatomical markers for compulsivity in SIP are not as a consequence of brain damaged associated to hyponatremia. Moreover, we identified specific microstructural patterns of licking among different rats strains, showing that the Wistar strain had increased frequency and intensity of licking compared with the remaining strains, which we relate to excessive habit formation of licking in SIP. Hyperactivity of the lateral orbitofrontal cortex and the basolateral amygdala, brain areas that are involved in behavioural flexibility and goal-directed control over habitual actions, was observed in those rats showing higher frequent and intense licking in SIP.

In the second experimental series, we found that the chronic TRP depletion, which reduced effectively brain and plasma 5-HT levels, affected the vulnerable Wistar HD rats compared with non-vulnerable Wistar LD and Lister Hooded rats. The TRP-depleted Wistar HD rats increased compulsive licking in SIP, had a reduction of the striatal 5-HT_{2A} receptor *binding* and showed a less balanced faecal bacterial community structure compared with the remaining groups. Moreover, HD compared with LD rats showed a reduced bacterial diversity, irrespectively of the diets.

According to the results obtained, we can conclude that behavioural inflexibility and excessive habit formation are neurobehavioural markers for predicting compulsive behaviour. Moreover, several biomarkers for compulsivity were identified, such as hyperactivity of the lateral orbitofrontal cortex and the basolateral amygdala, reduced serotonin 2A receptor subtype in the striatum, and reduced bacterial diversity of the gut microbiota. Furthermore, we provided evidence of the relationship between the serotonergic system and compulsive behaviour, showing that serotonin reductions increased compulsive behaviour and unbalanced the gut microbiota of vulnerable populations.

RESUMEN

La conducta compulsiva se ha definido como acciones inapropiadas para la situación, que son persistentes, no tienen una relación obvia con el objetivo de dicha conducta, y que a menudo tienen consecuencias indeseables. Este síntoma está presente en varios trastornos psiquiátricos, como el trastorno obsesivo compulsivo (TOC), el trastorno dismórfico corporal, el trastorno de acumulación, tricotilomanía y el trastorno por excoriación, que están agrupados en la categoría "Trastorno Obsesivo-Compulsivo y trastornos asociados" del Manual Diagnóstico y Estadístico de los Trastornos Mentales (quinta edición). Sin embargo, en la mayoría de casos con diagnóstico de TOC suele haber comorbilidad con otros trastornos psiquiátricos, como ansiedad, trastornos alimentarios, depresión, trastornos del control de impulsos o abuso de sustancias. Por ello, hay un creciente interés en identificar dominios neuroconductuales transdiagnósticos, como la inflexibilidad conductual o la formación excesiva de hábitos, para desarrollar modelos traslacionales eficaces que permitan estudiar la compleja neuropatología de la compulsividad. Esta conducta está relacionada con niveles alterados de monoaminas, concretamente de la serotonina (5-hidroxitriptamina-5-HT) y sus receptores. Dado que la microbiota intestinal regula el metabolismo del precursor de 5-HT (triptófano-TRP) y puede afectar a la síntesis global de 5-HT, un mal funcionamiento del eje microbiota-intestino-cerebro puede estar involucrado en la compulsividad.

En la presente Tesis Doctoral estudiamos la conducta compulsiva con un modelo animal denominado Polidipsia Inducida por Programa (PIP), que se caracteriza por el desarrollo de bebida persistente y excesiva bajo un programa de reforzamiento intermitente de comida. La cantidad de agua ingerida en SIP varía entre razas de ratas y entre los individuos de una misma raza, permitiendo la clasificación de dos poblaciones según sus diferencias individuales: ratas Altas Bebedoras (AB) y Bajas Bebedoras (BB). Los principales objetivos de la Tesis Doctoral fueron: Primero, explorar los dominios neuroconductuales, como la flexibilidad conductual y la formación de hábitos, y los sustratos neurobiológicos que están a la base de la vulnerabilidad a desarrollar bebida compulsiva en PIP. Segundo, dada la estrecha relación entre la compulsividad y el sistema serotoninérgico, investigar los efectos de la reducción de serotonina mediante la exposición crónica a una dieta carente de TRP en la bebida compulsiva en PIP y en la microbiota intestinal.

Los resultados de la primera serie experimental revelaron que las ratas AB exhibieron inflexibilidad conductual en una tarea de aprendizaje inverso, y excesiva formación de hábitos en una tarea de devaluación del reforzador. Este grupo también mostró elevados niveles en plasma de la hormona de estrés corticosterona tras la exposición a la PIP, lo cual indica que la PIP puede representar un contexto estresante para individuos vulnerables. Además, las ratas AB tuvieron una concentración normal de sodio en suero, lo que aporta pruebas de que los marcadores conductuales, neuroquímicos y neuroanatómicos de compulsividad en la PIP no son consecuencia de daño cerebral asociado a hiponatremia. Asimismo, identificamos patrones microestructurales específicos de lametones en diferentes razas de ratas, mostrando que la raza de ratas Wistar tenía alta frecuencia e intensidad de lametones comparada con el resto de razas, lo cual interpretamos como una formación de hábito descontrolado en PIP. En las ratas que mostraron alta frecuencia e intensidad de lametones en PIP se observó una hiperactividad anormal de la corteza orbitofrontal lateral y de la amígdala basolateral, áreas cerebrales que están involucradas en la flexibilidad conductual y el control de acciones dirigidas a meta.

En la segunda serie experimental encontramos que la depleción crónica de TRP, la cual redujo de forma efectiva los niveles de serotonina en el cerebro y en plasma, afectó las ratas AB Wistar en comparación con las ratas BB Wistar y Lister Hooded. Las ratas depletadas de TRP AB Wistar incrementaron los lametones en PIP, tuvieron una reducción del *binding* del receptor 5-HT_{2A} y mostraron una estructura de la comunidad bacteriana fecal menos equilibrada comparadas con el resto de grupos. Además, las AB en comparación con las ratas BB mostraron una menor diversidad bacteriana, independientemente de la dieta.

Según los resultados obtenidos, podemos concluir que la inflexibilidad conductual y la excesiva formación de hábitos son marcadores neuroconductuales que predicen la conducta compulsiva. Además, identificamos varios marcadores biológicos de la compulsividad, como hiperactividad de la corteza orbitofrontal y de la amígdala basolateral, niveles reducidos del receptor serotoninérgico 2A en el estriado y diversidad bacteriana reducida de la microbiota intestinal. También aportamos evidencias sobre la relación entre el sistema serotoninérgico y la conducta compulsiva, mostrando que la reducción de serotonina incrementa la conducta compulsiva en PIP y desequilibra la microbiota intestinal en poblaciones vulnerables.

2

General Introduction

1. Compulsive behaviour

1.1. A definition of compulsive behaviour

Compulsive behaviour has been defined as “actions inappropriate to the situation which persist, have no obvious relationship to the overall goal and which often result in undesirable consequences” (Dalley et al., 2011). The presence of this symptom is characteristic of various psychiatric disorders, such as obsessive-compulsive disorder (OCD), body dysmorphic disorder (BDD), hoarding disorder, hair-pulling disorder and excoriation (skin-picking) disorder, categorized in the newly created "Obsessive Compulsive and Related Disorders" (OCRDs) chapter in the *Diagnostic and Statistical Manual of Mental Disorders*, 5th Edition (DSM-5) (American Psychiatric Association, 2013). In the DSM-5, OCD is defined by the presence of obsessions and/or compulsions that are time consuming, distressing and disabling (American Psychiatric Association, 2013). OCD is the paradigmatic example of compulsivity (Robbins and Crockett, 2010) and affects approximately 1-3% of the population (Karno et al., 1988).

Compulsivity often occurs together with impulsivity, which has been defined as “actions which are poorly conceived, prematurely expressed, unduly risky or inappropriate to the situation and that often result in undesirable consequences” (Durana & Barnes, 1993). Prototypical clinical disorders expressing impulsive behaviour include attention deficit/hyperactivity disorder, substance abuse and schizophrenia (Bari & Robbins, 2013). A high level of comorbidity exits between impulsive and compulsive disorders, indicating that both of them may be underpinned by a common behavioural disinhibition resulted from a failure in “top-down” control of fronto-striatal brain circuits (Fineberg et al., 2014). For instance, the majority of OCD cases often have comorbid psychiatric disorders, such as anxiety, impulse control, substance abuse or personality disorders (Ruscio et al., 2010; Torres et al., 2016). Moreover, approximately 30% of schizophrenia cases report OCD symptomatology and

14% have been diagnosed with OCD as well (Swets et al., 2014). This issue has led to a shift away from traditional psychiatric classification systems, in favour of a new biological approach based on “transdiagnostic” neurobehavioural domains (Robbins et al., 2012). The term "transdiagnostic" has been used to refer psychological processes that are present in a range of disorders and reflect causal, functional mechanisms for co-occurrence (Harvey et al., 2011; Sauer-Zavala et al., 2017). The use of such domains would benefit not only the clinical practice facilitating the early detection and therapeutic interventions, but also the preclinical research developing robust animal models of compulsivity and evidence-based pharmacological treatments (Gillan et al., 2017; Robbins et al., 2012).

1.2. Pharmacological treatments and the serotonergic system

As a neurotransmitter, serotonin (or 5-hydroxytryptamine, 5-HT) has been implicated not only in physiological processes such as body temperature, sleep, appetite, pain and motor activity (Marazziti, 2017), but also in higher brain functions, including cognition and emotional behaviour (Švob Štrac et al., 2016). Over the last decades, it has been suggested that compulsivity might be related to the functioning of the 5-HT system, based on the observation that OCD patients typically respond to pharmacotherapy with non-selective 5-HT reuptake inhibitors (SRIs), including the tricyclic antidepressant clomipramine, as well as the selective 5-HT reuptake inhibitors (SSRIs) such as paroxetine, fluvoxamine, fluoxetine, citalopram, escitalopram and sertraline (Grant et al., 2014; Robbins et al., 2012; van Dijk et al., 2010). In short, SSRIs work by blocking the reuptake of 5-HT in the synapses back into the pre-synaptic cell, thereby increasing the level of extracellular serotonin in the synapse (Sangkuhl et al., 2009).

At least 14 different pre- and post-synaptic 5-HT receptors have been discovered (Nichols & Nichols, 2008). Evidence from animal and human studies have suggested a possible involvement of 5-HT2 receptors in compulsive behaviours (Fineberg et al., 2010), and more specifically of the serotonin 2A

receptor subtype (Aznar & Klein, 2013). The 5-HT_{2A} receptors, located mostly in different parts of the cortex, basal ganglia and slightly less in the hippocampus (Hoyer et al., 2002), seem to have an excitatory role in the prefrontal cortex (PFC) by modulating synchronous neuronal activity through regulation of glutamate release, and this PFC activation regulates amygdala reactivity (Aznar & Klein, 2013). In fact, it has been proposed that the anti-compulsive effects of SSRIs are mediated by the activation of 5-HT_{2A} receptors in the PFC (El Mansari et al., 1995; El Mansari & Blier, 2006). However, not all OCRDs respond similarly to the same pharmacological treatments. Although OCD and BDD typically respond to SRIs and SSRIs treatments (Grant et al., 2014), not all patients improve with these first-line treatments. In fact, some data suggest that only about 40% of OCD patients experience partial remission (Fineberg et al., 2015). In contrast, hair pulling and skin picking disorders, which are also characterised by lack of impulse control and addictive symptomatology, respond better to combination treatment with drugs acting on dopamine, glutamate, opioid and noradrenergic systems, like impulse-control disorders or even behavioural addictions (Fineberg et al., 2018). The pharmacological treatment response may be of particular interest for studying the underpinning biological mechanisms, and provide evidence of the need for evaluating transdiagnostic domains to develop effective pharmacological treatments.

The biosynthesis of 5-HT is limited by the availability of the essential amino acid tryptophan (TRP), which must be supplied in the diet (Ruddick et al., 2006). TRP is converted to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase, which is the rate-limiting step in the pathway, and the aromatic amino acid decarboxylase subsequently converts 5-HTP into 5-HT (O'Mahony et al., 2015). A common method for studying the psychobiology of the serotonergic system in human and animal studies is though dietary depletion of the precursor TRP. This non-invasive procedure has been shown to produce a reduction of central 5-HT synthesis, content and release (Biggio et al., 1974; Carpenter et al., 1998; Gessa et al., 1974; Stancampiano et al., 1977a,b), without affecting

dopamine (DA) and noradrenaline neurotransmission (Ardis et al., 2009; Cox et al., 2011). Although acute TRP depletion (ATD) have shown brain 5-HT reductions in rats and humans (Brown et al., 1988; Carpenter et al., 1998; Lieben et al., 2004), when the dietary TRP-deficient diet was applied chronically in rats produced stronger effects, reducing brain 5-HT levels to 35-40% at 14 days (Fadda et al., 2000), and to 75% at 5-weeks exposures (Vergnes & Kempf, 1981). Studies in rodents have demonstrated that chronic dietary TRP depletion increased aggressiveness, locomotor activity (Vergnes and Kempf, 1981) and sexual behaviour (Fratta et al., 1977), suggesting a possible lack of inhibitory control. However, no previous research has tested the effect of chronic TRP depletion on compulsivity.

2. Fractionating compulsivity: Behavioural inflexibility and excessive habit formation

Neurobehavioural measures of compulsivity have typically assessed the perseverative component of the construct in relation to the ability to flexibly adapt behavior after negative feedback (Fineberg et al., 2014). Behavioural inflexibility can be measured by perseveration in tasks in which altered reinforcement contingencies demand to switch the responding strategy. The simplest version of this is extinction, or reinforcer omission (Robbins & Crockett, 2010). Another measure of behavioural inflexibility is provided by reversal learning in a two-choice discrimination paradigm, in which a previous association between one stimulus and a rewarding outcome (e.g. pressing the right lever to obtain a pellet), and the other and reward omission or punishment (e.g. pressing the left lever results in a time-out), are reversal, so the individual needs to adapt its behaviour to changes in reward contingency (Fineberg et al., 2011).

Repetitive performance of behaviours without apparent adaptive function may be underlying not only inflexibility to switch actions, but also a lack of sensitivity to goals. For instance, many OCD patients continue to perform repetitive responses despite being aware that those actions have no relation to the desirable outcome. According to associative learning theories of instrumental behaviour, actions can be supported by 2 systems: a goal-directed system and a habitual system (de Wit & Dickinson, 2009; Dickinson & Balleine, 1993). Goal-directed behaviour is mediated by knowledge of the causal relationship between the action and its consequences, and that is only performed when those consequences are in line with current needs and desires, whereas habitual behaviour refers to the responses (R) triggered by environmental stimuli (S) regardless of the current desirability of the consequences (Robbins et al., 2012). The repetitive past experience with reward (positive reinforcement), or by the omission of an aversive event (negative reinforcement), produce strong S-R associations mediating habits. According to this theory, compulsive behaviour would be driven by an imbalance between the goal-directed learning system and the habitual system (Everitt & Robbins, 2005; Robbins et al., 2012). The dominance of habits over goal-directed behaviour has traditionally been studied through the reinforcer devaluation paradigm, which measures sensitivity of the response to motivational change. In a typical outcome devaluation methodology, the value of the reinforcer of action is reduced (by i.e. selective satiation), and the experimenter assesses if the animal's behaviour change under extinction (i.e. reducing presses of the lever associated to the devalued condition) (Gillan et al., 2016). It is well established that overtraining rats to press a lever for a reward renders performance of that action habitual and insensitive to post-training changes in the value of the reward (Adams and Dickinson, 1981; Balleine, 2001).

Several neurobehavioural domains have been proposed as transdiagnostic biological markers that contribute toward vulnerability to compulsivity in a broad range of compulsive disorders, including behavioural inflexibility, excessive habit formation, attentional inflexibility, inflexible fear conditioning and motor

desinhibition (Fineberg et al., 2018) (see table 1 for a summarize of findings). From those neuropsychological domains, behavioural inflexibility and excessive habit formation are of particular interest for research on compulsivity (Robbins et al., 2012). In the following sections, we will review the main findings regarding the brain areas and the neurochemical mechanisms involved in behavioural inflexibility and excessive habit formation as neuropsychological traits of compulsive behaviour.

Table 1. Fractionating compulsivity according to altered neurobehavioural domains: Definitions, tasks, brain circuits and neurochemistry.

Neurobehavioural Domains	Definitions	Tasks	Brain Circuits	Neurochemistry
Behavioural inflexibility	Inability to adapt behaviour after negative feedback	Discrimination Reversal-Learning Tasks	OFC, Caudate, BLA	Serotonin (OFC) Dopamine (Caudate) ?
Excessive Habit Learning	Lack of sensitivity to goals, contingencies or outcomes of actions	Reinforcer Devaluation Tasks	Habits circuit: SMA, Posterior Putamen, CA Goal-directed actions circuit: OFC, Caudate, BLA	Dopamine ? Serotonin ?
Attentional Inflexibility	Inability to switch attention between stimuli	Extra-dimensional attentional set-shifting tasks	vlPFC, Dorsal Caudate	Dopamine
Inflexible fear conditioning	Inflexible fear learning and inadequate safety signalling	Pavlovian fear reversal	OFC, Caudate, Insula, ACC, BLA	Dopamine ? Acetylcholine ?
Motor desinhibition	Pre-potent motor disinhibition	Go/No-go task Stop signal reaction time task	rIFC, SMA, Caudate	Noradrenaline

ACC = Anterior Cingulate Cortex; BLA = Basolateral amygdala; CA = Central nucleus of the amygdala; OFC = Orbitofrontal cortex; SMA = Supplementary motor area; vlPFC = Ventrolateral prefrontal cortex; rIFC = Right inferior frontal Cortex

2.1. Neurobiological substrates of behavioural flexibility

2.1.1. Brain circuit of behavioural flexibility

Preclinical and clinical studies have investigated the neural correlates of behavioural flexibility, showing an implication of a fronto-striatal-limbic circuit that includes the orbitofrontal cortex (OFC), the dorsomedial striatum and the basolateral amygdala (BLA) for flexible actions. In animal studies, lesions of the OFC impaired reversal learning in monkeys (Dias et al., 1996; Izquierdo et al., 2004; Rudebeck & Murray, 2008) and rodents (Bisonnette et al., 2008; Boulougouris et al., 2007; Chudasama & Robbins, 2003; McAlonan & Brown, 2003), whereas lesions of the medial PFC (which includes the prelimbic and inflalimbic cortex) did not increase perseverative responding (Bisonnette et al., 2008; Boulougouris et al., 2007; Chudasana & Robbins, 2013). Similarly, human subjects with lesions of the OFC cortex had impaired reversal learning compared with a group with dorsolateral frontal lobe damage and normal controls (Fellows & Farah, 2003). In functional magnetic resonance imaging (fMRI) studies, the OFC was activated during reversal learning in healthy subjects (Cools et al., 2002; Hampshire & Owen, 2006), whereas OCD patients and their unaffected relatives have both shown reduced activation of the OFC during visual reversal learning (Chamberlain et al., 2008; Remijnse et al., 2006).

Furthermore, some authors have also implicated the striatum in behavioural flexibility (Divac et al., 1967; Winocur & Mills, 1969; Winocur & Eskes, 1998). The striatum is divided into the dorsal striatum, which includes most of the caudate (dorsomedial striatum-DMS) and putamen (dorsolateral striatum-DLS), and the ventral striatum, which comprises the nucleus accumbens (NAc), the ventromedial parts of the caudate and putamen, and the striatal part of the olfactory tubercle (Joel & Weiner, 2000). In monkeys, lesions of the medial caudate and the nucleus accumbens, which receive projections from the OFC in these animals (Roberts et al., 2007), increased perseveration in the reversal-

learning task, similar to lesioned-OFC animals, compared with control animals (Clarke et al., 2008; Man et al., 2009). Further studies found that lesions of the DMS induced perseverative responding in reversal learning in rats, whereas NAc or DLS lesions did not (Castañe et al., 2010). Taking together, these results provide evidence of a fronto-striatal circuit mediating behavioural flexibility.

On the other hand, the role of the amygdala in reversal learning is of particular interest for the projections that receives from the OFC (Krettek & Price, 1977; Shi & Cassell, 1998), and its implication in emotional processes (Kluver & Bucy, 1939). Lesions of the amygdala do not disrupt reversal learning (Izquierdo & Murray, 2007; Rudebeck & Murray, 2008) or instrumental extinction in monkeys (Izquierdo & Murray, 2005). Interestingly, humans and monkeys with lesions of the amygdala had increased tendency to switch choice behaviour compared with controls (Hampton et al., 2007; Rudebeck & Murray, 2008). Specifically, the facilitation of reversal learning may depend on the damage in the BLA (Stalnaker et al., 2007), which is critical for associative learning (Everitt et al., 2000; Gallagher, 2000). The impairment of reversal learning in rats with OFC lesions was abolished when accompanied by lesions in the BLA (Stalnaker et al., 2007), while BLA lesions alone had no effect on reversal learning (Schoenbaum et al., 2003; Stalnaker et al., 2007). Therefore, it have suggested that the OFC may promote reversal learning by facilitating recognition of errors though outputs to the BLA, that would encode the reversed cue-outcome associations (Schoembaum et al., 2007).

2.1.2. Neurochemical mechanisms in behavioural flexibility

Strong evidence from preclinical and clinical research suggests that the 5-HT system is implicated in the modulation of reversal learning in the OFC. Selective depletion of 5-HT in the OFC has shown impairment of reversal learning in primates and rats (Clarke et al., 2007, 2004; Lapiz-Bluhm et al., 2009). Moreover, the systemic treatment with the SSRI citalopram reversed the impairment in reversal learning induced by chronic stress in rats, and this

improvement was associated with an increase in 5-HT release in the OFC (Lapiz-Bluhm & Morilak, 2010). On the other hand, systemic administration and microinjections in the OFC of the selective 5-HT_{2A} receptor antagonist M100907 in rats impaired reversal learning (Boulougouris et al., 2008; Furr et al., 2012). Also, highly perseverative rats in reversal-learning task showed reductions in both serotonin 5-HT_{2A} receptor binding in the OFC and tryptophan hydroxylase levels in the dorsal raphe nucleus (Barlow et al., 2015). Similarly, positron emission tomography studies in drug-naive OCD patients revealed a reduced 5-HT_{2A} receptor availability in the frontal cortex (Perani et al., 2008), with specific correlations between OFC 5-HT_{2A} receptor availability and clinical severity and early age of onset (Perani et al., 2008; Simpson et al., 2011). Therefore, a malfunction of the OFC mediated by alterations of the 5-HT_{2A} receptor subtype might be good biological marker for behavioural inflexibility in compulsive-spectrum disorders.

Although DA depletion of the OFC in monkeys did not alter reversal learning (Walker et al., 2009), dopamine depletion of the medial caudate produced increased perseverative responding compared with 5-HT depleted and control monkeys (Clarke et al., 2011). These results suggest a different involvement of both neurotransmitters depending on the brain region within the cortico-striatal-limbic circuit of behavioural inflexibility.

2.2. Neurobiological substrates of goal-directed and habitual learning

2.1.1. Brain circuits of goal-directed and habitual learning

Goal-directed learning and habitual learning have two distinctive neural pathways. Preclinical and clinical studies have converged on the importance of the caudate nucleus (DMS) and medial OFC for goal-directed control over action, and the putamen (DLS) and supplementary motor area (SMA) for the gradual build-up of stimulus-response habit links over time (Gillan et al., 2016;

Fineberg et al., 2018). Rats with lesions of the DLS significantly reduced responding after outcome devaluation compared with DMS-lesioned and sham rats (Yin et al., 2004), suggesting that the DLS is necessary for habit formation. In mice, chemogenetic inhibition of the OFC disrupted goal-directed actions, whereas optogenetic activation of the OFC specifically increased goal-directed actions (Gremel & Costa, 2013), pointing towards an implication of this prefrontal cortex area in goal-directed learning. Similar results have been found in fMRI studies regarding goal-directed and habitual learning. Healthy participants had increased activity in the OFC during goal-directed learning (de Wit et al., 2009; Valentin et al., 2007), and in the posterior putamen and SMA during habit formation (Floyer-Lea & Matthews, 2004; Tricomi et al., 2009; Wunderlich et al., 2012a; Lee et al., 2014). In structural MRI, vulnerability to habitual behaviour was predicted by estimated white matter tract strength in the premotor cortex seeded from the posterior putamen, whereas flexible goal-directed behaviour was predicted by estimated tract strength in the OFC cortex seeded from the caudate (de Wit et al., 2012a).

Regarding the amygdala, rats with lesions of the BLA showed insensitivity to outcome devaluation compared with central nucleus of the amygdala (CA) lesioned and sham rats (Corbit & Balleine, 2005). Moreover, rats with lesions of the CA did not exhibit acquisition of habitual behaviour after overtraining, remaining goal-directed and sensitive to outcome devaluation (Lingawi and Balleine, 2012). Thus, the BLA seems to be important for the acquisition of goal-directed actions, whereas the CA seems to be critical for the formation of habits by its interaction with the DLS (Lingawi & Balleine, 2012). Additionally, rats exposed to chronic stress have shown insensitivity to outcome devaluation and altered neuronal density in prefrontal and striatal areas (Dias-Ferreira et al., 2009), suggesting a link between anxiety and goal-directed vs. habitual learning. Although fMRI studies have found hyperactivity of the amygdala during experimental symptom provocation in OCD patients (Adler et al., 2000; Banca et al., 2015; de Wit et al., 2015; Simon et al., 2010, 2014; van den Heuvel, 2004),

the literature regarding the amygdalar substrates of goal-directed and habitual learning in vulnerable populations to compulsivity is limited.

Nowadays, one of the main questions is whether compulsivity is driven by alterations of the neural substrates mediating goal-directed control over habitual processes or, conversely, by an excessive build-up of stimulus-response habits (or both). Some studies have provided evidence about the first hypothesis. For instance, in OCD patients, excessive habits were associated with hyperactivation of the medial OFC and the caudate nucleus (Gillan et al., 2015). Moreover, a transdiagnostic study found that subjects diagnosed with binge eating disorder, drug abuse and OCD had a common bias on goal-directed learning, and lower grey matter volumes in the medial OFC and caudate was associated with a habit formation bias (Voon et al., 2015). Those altered brain areas, the medial OFC and caudate, has been shown in animals and healthy subjects to be involved in goal-directed control, indicating a possible malfunction of goal-directed learning that leads to excessive habits in compulsive-spectrum disorders. Another study in OCD patients observed alterations in both goal-directed and habitual learning circuits. In this study, the experimental provocation of autobiographical compulsions in OCD patients was shown to reduce neural activation in brain regions implicated in goal-directed behavioural control (OFC, caudate nucleus) with concordant increased activation in regions implicated in habit learning (pre-SMA, putamen) (Banca et al., 2015). Still, more research is needed to elucidate the brain areas mediating the excessive habit formation observed in compulsive-spectrum disorders.

2.1.2. Neurochemical substrates of goal-directed and habitual learning

The dopaminergic system has been shown to play a role in both habitual and goal-directed behaviours. In rodents, chronic amphetamine administration resulted in accelerated habit formation (Nelson & Killcross, 2006), which was reversed by a dopamine D1 receptor antagonist (Nelson & Killcross, 2013). Similarly, rats subjected to DA depletion of the nigrostriatal pathway (from the

pars compacta to the dorsal striatum through the medial forebrain bundle) failed to acquire habitual behaviour, compared with sham rats that were insensitive to reward devaluation as expected after overtraining (Faure et al., 2005). Moreover, intra-amygdalar infusions of DA or noradrenaline agonists in rats biased behaviour toward habitual strategies in a water-maze task (Packard & Wingard, 2004; Packard et al., 1994). However, preclinical and clinical studies present contradictory results. In humans, drugs enhancing dopaminergic function (L-DOPA) increased model-based goal-directed learning (Wunderlich et al., 2012b), and this increment was associated with higher levels of presynaptic ventral striatal dopamine (Deserno et al., 2015). Similarly, acute tyrosine (DA precursor) depletion impaired goal-directed control on the fabulous fruit task, a human task that detects goal-directed and habitual tendencies to respond (de Wit et al., 2012b).

Although the 5-HT system is considered to have a functional relationship with the brain DA system (Zhou et al., 2005), little research has been done regarding the role of the serotonergic system in modulating goal-directed and habitual learning. In recent human studies, acute TRP depletion induced a shift from goal-directed to habitual control over action in healthy humans on the fabulous fruit task (Worbe et al., 2015). In addition, this shift after acute TRP depletion affected appetitive goal-directed behaviour but did not altered aversive goal-directed behaviour (Worbe et al., 2016). However, the serotonergic and dopaminergic mechanisms modulating goal-directed and habitual learning are unclear.

3. Microbiota-gut-brain axis

There is a growing interest among the scientific community in investigating the role of gut microbiota in neuropsychiatric disorders. Mounting preclinical evidence suggests that the gut microbiota modulate brain development, function and behaviour by immune, endocrine and neural pathways (Kelly et al., 2015).

This bidirectional system of communication has been called "microbiota-gut-brain axis", and represents a new biological axis for developing diet-based therapies to influence brain function and behaviour (Foster et al., 2016). Much of our understanding related to gut microbiota comes from germ-free (GF) mice studies. These animals, which are in sterile environments and have no commensal bacteria, have shown differences in exploration, anxiety-like behaviour and social behaviour compared with their control specific pathogen-free mice (Bercik et al., 2011; Clarke et al., 2013; Crumeyrolle-Arias et al., 2014 Desbonnet et al., 2013; Heijtz et al., 2011; Neufeld et al., 2011). Moreover, different stress paradigms, such as maternal separation (O'Mahony et al., 2009), prolonged restrain stressors (Bangsgaard Bendtsen et al., 2012) and social stressors (Bailey et al., 2011), have been shown to reduce the microbiota composition and the abundance of bacterial species in rodents.

The vast majority of 5-HT is located in the gut, where it is synthesised from TRP (Mawe & Hoffman, 2013). The gut microbiota regulates TRP metabolism, and may affect global 5-HT synthesis in the enteric and central nervous systems. In fact, the correct functioning of the microbiota-gut-brain axis depends on appropriate 5-HT signalling (O'Mahony et al., 2015). Since an altered serotonergic function has been related to compulsivity (Robbins et al., 2012; van Dijk et al., 2010), an altered gut microbiota may be involved in this symptom. However, little research has been done regarding the link between gut microbiota and compulsive behaviour (Rees, 2014; Turna et al., 2016). So far, the role of gut microbiota in compulsivity has been studied in mice with the marble-burying test. In this test, non-aversive glass marbles are placed in a chamber, and the number of buried marbles is counted as a measure of perseveration (Thomas et al., 2009). GF mice had a higher number of buried marbles (Nishino et al., 2013) and increased amount of time on self-grooming compared with controls (Desbonnet et al., 2013). In addition, innately anxious male BALB/c mice reduced the number of marbles buried after fed with probiotics, in a similar manner that did the administration of the SSRI escitalopram (Savignac et al.,

2014). Moreover, a mice study showed that 2 weeks of pre-treatment with probiotics reduced compulsive behaviours (such as perseverative open-field locomotion, stereotypic turning and marble burying) induced by the acute 5-$HT_{1A/1B}$ agonist RU24969, in a similar way to mice pre-treated for 4-weeks with fluoxetine (Kantak et al. 2014). So far, no human studies have explored the gut microbiota in OCD patients. However, some evidence regarding the possible beneficial effect in compulsivity has been shown in healthy humans, which had a reduction of the "obsessive-compulsive" sub-scores on the Hopkins symptoms checklist after 30-days probiotics administration (Messaoudi et al., 2011). Moreover, a study found that 35.1% of OCD patients, compared with 2.5% of controls, met the criteria for irritable bowel syndrome (Masand et al., 2006), which has been linked to altered intestinal bacterial overgrowth (Stern and Brenner, 2018). Although SSRIs are capable of decreasing pain and other symptoms associated with chronic gastro-intestinal disorders (Vanuytsel et al., 2014), little is known about the effects of 5-HT depletion on the gut microbiota of vulnerable populations to compulsive behaviour.

4. Schedule-induced Polydipsia as a model of compulsive behaviour

4.1. History and hypotheses

In 1961, John L. Falk discovered a fascinating phenomenon when studying fluid regulation in rats exposed to intermittent food reinforcement schedules with a water bottle available in the experimental setting (Falk, 1961). Food-deprived rats (80-85% from free feeding) drank an enormous amount of water under these conditions, which can reach one-half of their body weight in water (Falk, 1966). During daily 3-h sessions, rats consumed about 92 ml of water, over 3 times their usual daily consumption. The drinking is not regulatory, given that animals are not water deprived (Falk, 1961). This phenomenon, known as Schedule-induced Polydipsia (SIP), has been proposed as a prototype of adjunctive behaviour, a category of behaviour that would include all activities occurring in reinforcement

schedules with no direct relationship to reinforcement delivery (Falk, 1971). The phenomenon of adjunctive behaviour has been documented in different animal species, mice, rats, and monkeys (Falk, 1961; Grant et al., 2008; Mittleman et al., 2003), with different reinforcement schedules and types of reinforcers (Falk & Kupfer, 1998). Although the state of food deprivation or the inter-pellet interval length reproduced by different fixed time (FT) or fixed interval (FI) schedules influence the acquisition and expression of adjunctive drinking in SIP (Falk, 1966, 1971; Flores & Pellón, 1995; Flory 1971; Killeen, 1970; López-Crespo et al., 2004), the optimal FT interval for inducing a high rate of drinking behaviour has been found to be FT 30s and 60s schedules (Moreno & Flores, 2012).

Over the last decades, several hypotheses have been proposed to explain the phenomenon of SIP. One of these hypotheses interprets SIP as a coping response related to stress and the hypothalamic–pituitary–adrenal (HPA) axis (Brett & Levine 1979; Dantzer et al., 1988; Mittleman et al., 1988). This anxiolytic approach is based on studies reporting lower plasma levels of the stress hormone corticosterone in rats exhibiting excessive drinking in SIP than in animals with lower drinking rates or without a bottle in the operant chamber (Brett & Levine, 1981, 1979; Dantzer et al., 1988). However, other studies reported similar or higher levels of this stress hormone in rats acquiring SIP than in rats under conditions without a water bottle or without a schedule of intermittent reinforcement (Mittleman et al., 1988; Tazi et al., 1986; Wallace et al., 1986), indicating contradictory results regarding this possible coping response. An alternative hypothesis relates adjunctive behaviour to the incentive motivational properties provided by the intermittent release of the food pellets (Killeen et al., 1978). According to this view, adjunctive behaviour is due to the motivational excitation that accompanies the delivery of each food pellet, an excitation that produces a displacement behaviour occurring predominantly in the earlier part of inter-reinforcement intervals (Killeen & Sitomer, 2003). As the amount of adjunctive drinking in greater with short inter-pellet intervals length (e.g. FT-30), it is proposed as an operant behaviour that is reinforced by the delivery of the

pellet (Killen et al., 1978; Killeen & Pellón, 2013), instead of being induced by the conditions of stimulation.

Interestingly, the SIP context seems to be anxiogenic, based on the observation that rats exposed to FT-30s without water access exhibited increased corticosterone levels compared with rats non exposed to the intermittent food-reinforcement schedule (López-Grancha et al., 2006). Moreover, the amount of water consumed in SIP varies across strains of rats showing specific phenotypes, indicating that is sensitive to genetic vulnerabilities. The following section will review the main findings regarding strain-dependent differences in SIP.

4.2. Genetic vulnerabilities in rat strains

The acquisition of SIP and the amount of fluid intake vary across different inbred and outbred rat strains. For instance, selective breeding of Roman high (RHA) and low-avoidance (RLA) rats for rapid vs extremely poor acquisition of active avoidance behaviour in a shuttle-box displayed differences in SIP acquisition (Moreno et al., 2010). The phenotype of RLA rats is characterized by increased anxiety in different paradigms, a passive coping style, and increased HPA-axis activation, compared with RHA rats (Carrasco et al. 2008; Steimer and Driscoll 2003). In contrast, RHA rats show a phenotype characterized by higher novelty seeking, susceptibility to addictive drugs (Escorihuela et al., 1999; Fattore et al., 2009), and impulsive behaviour in two impulsivity tasks: the delay discounting task and the 5-choice serial reaction time task (5-CSRTT). Delay discounting refers to the degree to which immediate outcomes exhibit more influence over behaviour than outcomes which are delayed. Impulsive choice, in the context of delay discounting, is generally considered as an increased preference for immediate over delayed outcomes, even when the delayed outcomes are more advantageous (Mar & Robbins, 2007). On the other hand, the visuospatial attention 5-CSRTT requires that the animal identify which of the five holes has been briefly illuminated, and nose-poke into the correct hole to obtain a reward. Impulsive or premature responses are considered when the rat prematurely

responds before the presentation of the visual stimulus, while a perseverative response is considered when the rat keep responding in an aperture after a correct response and before the food collection (Robbins, 2002). RHA, which showed increased choices of the immediate reward in the delay discounting task and increase premature responses in the 5-CSRTT, displayed higher SIP drinking compared with RLA rats.

Moreover, the inbred Fisher 344 strain, which exhibits hyperresponsiveness of the HPA-axis (Dhabhar et al., 1993), had a faster SIP acquisition and higher water consumption than their control Lewis rat strain (Decarolis et al., 2003; Stöhr et al., 2000). The spontaneously hypertensive rat (SHR), which is typically compared to the normotensive Wistar-Kyoto rat (WKY), has been proposed as a rodent model of ADHD for showing hyperactivity, deficient sustained attention and impulsivity (Sagvolden, 2000). The SRH rats, which displayed higher impulsive choice in the delay-discounting task (Fox et al., 2008), had increased SIP acquisition than WKY control rats (Íbias and Pellón, 2011, 2014). Thus, the SIP model may be sensitive in detecting genetic susceptibilities to inhibitory control deficits among different rat strains.

However, pigmented rat strains such as Lister Hooded and Long Evans have been less well studied in SIP (Montes de Oca, 2013). In general, pigmented rats show increased locomotor activity in an open field test and less anxiety by showing more time spent in the open arms of the elevated plus maze task than albino rats (Broersen & Uyling, 1999; Clemens et al., 2014; Onaivi et al., 1992; Turner and Burne, 2014). Previous studies have shown that Wistar rats had poorer behavioural flexibility in a visual discrimination reversal task (Kumar et al., 2015), and increased perseverative swimming in a probe trial of the Morris Water Maze in which the platform is removed (Entlerova et al., 2013), compared with Long Evans rats. Although these results suggest a possible compulsive phenotype of Wistar rats compared with Long Evans rats, these two rat strains have not been compared in SIP acquisition. Furthermore, there are enormous

individual differences within the same strain in the amount of water consumption. The following section will focus on the characterization of two different populations of albino Wistar rats selected according to their drinking rates.

4.3. Individual differences in the Wistar strain in SIP

Important individual differences in SIP have been observed among individual exposed to identical intermittent food reinforcement schedules. Not all animals develop adjunctive drinking, and even those animals that show SIP acquisition exhibit considerable variability in the amount of water consumed (Moreno & Flores, 2012; Flores et al., 2014). As consistent and stable individual differences are observed in the rate of drinking after 15–20 SIP sessions, Moreno and Flores (2012) proposed a classification of the animals in High Drinkers (HD) and Low Drinkers (LD) based on whether their rates of drinking (average for each animal on the last 3 sessions) were above or below the group median, respectively. The differences in SIP between HD and LD rats are not related to regulatory drinking intake, as shown by the lack of differences in water intake between these groups in home cages during 24 hours and during 1 hour after 23 hours of water restriction (Flores et al., 2014).

Previous research has explored the performance of Wistar HD and LD rats in different behavioural paradigms in order to characterize these populations. In the open field test, HD and LD rats showed similar locomotor activity and novelty reactivity in a 60-min period (Moreno et al., 2012). Moreover, both groups were tested in the elevated plus maze, which is an apparatus of four arms (two open and two enclosed) that measures anxiety by exposing the animals to the conflict of exploring vs. avoiding open spaces (Handley & Mithani, 1984). The percentage of time spent on the open arms, the indicator of lower anxiety levels, was similar in HD and LD rats (López-Grancha et al., 2008). These groups were also tested in the conditioned place preference paradigm, which evaluate reward seeking by conditioning one compartment to an addictive drug

such as d-amphetamine, and the other compartment to saline. Rats were allowed to freely explore the entire apparatus for 15 min and the time the animal spent in each of the compartments was recorded. HD and LD spent similar time in the reward-associated compartment, indicating similar reward seeking processes (Flores et al., 2014).

Although the behavioural traits related with novelty reactivity, anxiety or reward seeking were similar in HD and LD rats, further studies evaluated differences in inhibitory control in impulsivity paradigms. In the 5-CSRTT, HD rats showed a greater increase in perseverative responses under extinction condition (disconnecting the pellet dispenser) (Moreno et al., 2012). Although one study found that HD rats exhibited increased impulsive choices in the delay discounting task compared with LD rats (Cardona et al., 2011), these results were not replicated later (Ibías & Pellón, 2014). The following section will provide evidence for the HD rats as a vulnerable population to compulsivity.

4.4. HD rats as a vulnerable population to compulsivity

As the SIP drinking is an excessive, persistent and maladaptive behaviour (Moreno & Flores, 2012), it has been proposed as an useful model to study those neuropsychiatric disorders characterized by the presence of compulsivity such as OCD (Schechter et al., 2008; Rosenzweig-Lipton et al., 2007; Woods-Kettelberger et al., 1997; Moreno & Flores, 2012; Platt et al., 2008), schizophrenia (Hawken et al., 2011, 2013) or alcohol and drug abuse (Ford, 2014; Gilpin et al., 2008; Mittleman et al., 2003, 2011; Myracle et al., 2005; Wayner, 2002).

As previously seen, Moreno et al. (2012) found that HD rats had resistance to extinction in the 5-CSRTT. Moreover, HD rats have shown increased lever pressing under the reversal condition in the reversal-learning task, indicating deficits in behavioural flexibility, compared with LD rats (Navarro et al., 2017). Although the reinforcer devaluation paradigm has not been studied in SIP

previously, HD rats have shown vulnerability to develop habitual responding in other tasks. For instance, HD rats had increased lever pressing under a variable-interval 60s schedule, which is a schedule of reinforcement that produces constant and stable rate of responding (Navarro et al., 2017). Moreover, rats exhibiting SIP acquisition had a preference for response-learning strategies, rather than place-learning strategies, in a spatial Y-maze task (Gregory et al., 2015). In this task, rats learn to locate a food pellet placed at the end of one arm of the Y-maze. Place learning is an hippocampal-dependent process that relies on extra-maze cues to learn a spatial location, while response learning depends on the dorsolateral striatum and is exhibited by automatic responses, i.e. a motor action of turning right or left, to reach the rewarded arm (Packard & McGaugh, 1996). Thus, the response-learning strategy observed in rats developing SIP suggests possible tendencies in engaging in excessive habitual behaviour.

On the other hand, HD rats have shown specific neurochemical and neuroanatomical differences compared with LD rats. In autoradiography analysis, HD rats showed reduced D1 receptor binding in the nucleus accumbens, substantia nigra, ventral tegmental area and medial prefrontal cortex, and higher dopamine D2 receptor binding in the amygdala, compared with LD rats (Pellón et al., 2011). In fact, HD showed hyperactivity in serotonergic and noradrenergic pathways in the amygdala, and hypoactivity in dopamine pathways in the nucleus accumbens and the prefrontal cortex in post-mortem analyses of these animals compared to LD rats (Moreno et al., 2012). Additionally, the DLS has been involved in SIP, since rats exposed to SIP had increased spine density in this striatal region compared with a control group (Ibías et al., 2015). Furthermore, different neuroanatomical activity has been observed in HD compared with LD rats, showing increased c-Fos expression in prefrontal cortex areas (Gregory et al., 2015; Pellón et al, 2011). Moreover, less myelination of the basolateral amygdala has been found in HD rats compared with LD rats (Navarro et al., 2017), which is an area related to acquisition of goal-directed actions (Balleine, 2005). Taking together, the differences observed in the functioning of fronto-

striatal-limbic circuits in HD animals, which are related to those brain areas that may be altered in compulsive spectrum disorders, provides evidence of SIP as a useful model for studying compulsive behaviour (Moreno et al., 2012).

From a pharmacological perspective, antipsychotics drugs or chronic treatment with SSRIs, have effectively reduced drinking behaviour in SIP without affecting water or food regulatory intake (Didriksen et al., 1993; Mittleman et al., 1994; Platt et al., 2008; Woods et al., 1993). When comparing HD and LD rats, serotonergic drugs such as the SSRI citalopram or 5-$HT_{2A/C}$ receptor agonist DOI hydrochloride ((±)-2,5-dimethoxy-4-iodoamphetamine) reduced compulsive drinking in HD with no effect in LD rats. The reduction produced by DOI was blocked selectively in HD rats by serotonin 5-HT_{2A} receptor antagonist ketanserin and M100907, but not by serotonin 5-HT_{2C} receptor antagonist SB242084 administration (Navarro et al., 2015). These results suggest an implication of the 5-HT_{2A} receptor in compulsive drinking in SIP.

All previous findings suggest that SIP is a suitable animal model for investigating behavioural and biological markers of compulsivity, including the involvement of the serotonergic system in the neuropathology of compulsive-spectrum disorders. Therefore, the present Doctoral Thesis aims at researching compulsivity through the animal model SIP, as we will see next in the "Approach and Objectives" chapter.

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3

Approach and Objectives

As we have seen in the General Introduction, there is a growing interest in identifying pathophysiological mechanisms underpinning compulsivity as transdiagnostic domains, i.e. behavioural flexibility and habit formation, for developing accurate translational models to investigate the complex neuropathology of this behaviour. From preclinical and clinical studies, convergent evidence points towards a failure in fronto-striatal-limbic circuits mediating behavioural flexibility and goal-directed vs habitual learning, leading to a lack of flexible, contingency-dependent instrumental behaviour in favour of excess habit generation in compulsive-spectrum disorders. However, the neurobehavioural, neuroanatomical and neurochemical substrates underlying behavioural inflexibility and excessive habit formation in psychiatric disorders characterized by compulsivity are under investigation.

In addition, the serotonergic system has been implicated in compulsivity, based on the observation that compulsive spectrum disorders typically respond to selective serotonin reuptake inhibitors (SSRIs). The literature has identified a modulatory role of the serotonergic system in behavioural flexibility and, possibly, in goal-directed and habitual behaviour. However, the underlying serotonergic mechanisms implicated in those neurobehavioural domains are unclear. Still, it has been hypothesised that the anti-compulsive effects of the SSRIs are mediated by enhancing behavioural flexibility and goal-directed learning through the activation of serotonin 2A receptors in prefrontal areas implicated in those neurobehavioural domains. Still, further investigation is needed to identify the role of this receptor subtype in compulsive behaviour.

Further, there is a growing interest in studying the role of microbiota-gut-brain axis in neuropsychiatric disorders. Preclinical evidence indicates that the modulation of the gut microbiota could alter different behaviours such as locomotor activity, anxiety-like behaviour or social behaviour. However, the literature regarding the microbiota-gut-brain axis in compulsive spectrum disorders is limited.

In the present Doctoral Thesis, we studied compulsive behaviour with an animal model, schedule-induced polydipsia (SIP), which is able to identify individual differences between- and within-strains of rats in water consumption under a fixed time schedule of food reinforcement. Some rats develop a non-regulatory, excessive, persistent and maladaptive drinking during the inter-pellet intervals (High Drinkers- HD), whereas other rats develop low rates or no acquisition of drinking in the same circumstances (Low Drinkers- LD). The SIP literature has characterized HD as resistant to extinction and inflexible to change behaviour when it is no longer reinforced, showing fronto-striatal-limbic alterations compared with LD rats. However, no previous research has tested these populations in goal-directed and habitual learning through a reinforcer devaluation paradigm. Moreover, SIP may be useful to evaluate the modulatory effect of the serotonergic system in compulsive drinking, and to identify gut microbiota alterations underlying the differences between HD and LD rats.

Therefore, in the present Doctoral Thesis, we used the animal model of compulsive behaviour SIP to assess the following general and specific objectives:

General Objective 1

Experimental Series I: Habit formation in compulsive drinking in SIP and neuroanatomical activation

The first experimental series explored neurobehavioural domains, such as behavioural flexibility and habit formation, and neurobiological substrates that could underlie and predict the vulnerability to develop compulsive drinking in SIP.

Specific objectives (Chapter 4):

1. To evaluate behavioural flexibility in a reversal-learning task in Wistar HD and LD rats before the SIP exposure.
2. To evaluate habit formation in a reinforcer devaluation task between Wistar HD and LD rats before the SIP exposure.
3. To compare plasma corticosterone levels, as a measure of the hypothalamic-pituitary-adrenal axis activity, in Wistar HD and LD rats, before and after the SIP exposure
4. To assess blood glucose levels, as a measure of the metabolic function, in Wistar HD and LD rats in SIP, before and after the SIP exposure.
5. To identify low serum sodium levels, as a measure of hyponatremia, in Wistar HD and LD rats after the SIP exposure.

Specific objectives (Chapter 5):

1. To compare the SIP microstructural pattern of licking in different rat strains.
2. To investigate excessive habit formation in different rat strains and populations clustered by their frequency and intensity of licking.
3. To compare the SIP acquisition of excessive and habitual drinking in populations of Wistar rats clustered by their SIP microstructural pattern of licking.
4. To compare the c-Fos expression, as a measure of neuronal activity, during SIP in different brain areas related to behavioural flexibility and goal-directed learning in populations of Wistar rats clustered by their SIP microstructural pattern of licking.

General Objective 2

Experimental Series II: Serotonergic involvement in compulsive drinking in SIP and in gut microbiota

The second experimental series investigated the effects of serotonin reductions by chronic dietary tryptophan (TRP) depletion on compulsive behaviour in SIP and on the gut microbiota.

Specific objectives (Chapter 6):

1. To compare strain-dependent differences in SIP acquisition between Wistar and Long Evans rats.
2. To evaluate strain-dependent effects of chronic dietary TRP depletion on compulsive drinking in SIP and locomotor activity in the open-field test, in HD and LD rats.
3. To measure strain-dependent effects of chronic dietary TRP depletion on serotonin 2A and 1A receptor binding, in HD and LD rats.
4. To assess the effects of chronic dietary TRP depletion on serotonin, dopamine and noradrenaline levels in different brain areas.

Specific objectives (Chapter 7):

1. To identify differences in the faecal bacterial community structure of Wistar HD and LD rats.
2. To evaluate the effects of chronic dietary TRP depletion on the faecal bacterial community structure of Wistar HD and LD rats.
3. To assess the effects of chronic dietary TRP depletion on plasma serotonin and brain-derived neurotrophic factor levels in Wistar HD and LD rats.

Experimental Series I:

Habit formation in compulsive drinking in SIP and neuroanatomical activation

4

Behavioural and biological markers for predicting compulsive drinking in schedule-induced polydipsia

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Submitted

Abstract

Schedule-induced polydipsia (SIP), characterized by the development of persistent and excessive drinking under intermittent food-reinforcement schedules, is an animal model of compulsive behavior that can differentiate two populations: high drinkers (HD) and low drinkers (LD). The aim of the present study was to identify behavioral and biological markers to predict the vulnerability to developing compulsive drinking in SIP. Adult male Wistar rats were first trained in a spatial-discrimination serial reversal-learning task and in a reinforcer devaluation task to measure behavioral flexibility and habit formation, respectively. Subsequently, the rats were tested using the SIP protocol and identified as HD or LD based on their drinking rates. The performance of HD and LD rats in the two previous tasks was then analyzed. Before and after SIP exposure, blood glucose and plasma corticosterone (CORT) levels were measured. Additionally, serum electrolyte levels, including sodium, potassium, and chloride, were analyzed after SIP. HD rats showed higher behavioral inflexibility by exhibiting increased perseverative responses in the reversal-learning task and insensitivity to reinforcer devaluation during extinction under selective satiation. Glucose and CORT levels were similar in HD and LD rats before SIP exposure. After SIP exposure, however, HD rats exhibited increased plasma CORT levels, indicating that the SIP protocol provides a stressful situation for vulnerable individuals. The HD group did not exhibit hyponatremia (i.e., reduced serum sodium levels) when compared to LD rats after 20 daily SIP sessions. The results of the present study demonstrated that HD rats exhibit deficits in behavioral flexibility and habit formation before SIP. Moreover, these results highlight the importance of measuring different behavioral and biological markers for predicting the vulnerability to developing compulsivity, and for enhancing the understanding of the pathophysiology of compulsive spectrum disorders.

Introduction

Compulsions are repetitive mental or overt acts that are experienced as being urge-driven, either in response to an obsession or according to a rule that must be rigidly applied, and are aimed at reducing anxiety or distress, or preventing a feared event from occurring (Gillan et al., 2017). The presence of this symptom is characteristic of various psychiatric disorders, such as obsessive-compulsive disorder (OCD), body dysmorphic disorder, hoarding disorder, hair-pulling disorder, and skin-picking disorder, which comprise the newly created Obsessive Compulsive and Related Disorders cluster in the *Diagnostic and Statistical Manual of Mental Disorders, 5th Edition* (American Psychiatric Association, 2013). However, compulsivity is also present in other psychiatric disorders including schizophrenia, pathological gambling, eating disorders, depression, and substance addiction (Skodol & Oldham, 1996). Furthermore, 30% of schizophrenia patients report obsessive-compulsive symptomatology, and 14% have been found to have OCD (Swets et al., 2014). In OCD, comorbidity with other psychiatric disorders, such as anxiety, impulse control, or substance use or personality disorders is often present in the majority of cases (Ruscio et al., 2010; Torres et al., 2016). Therefore, there is growing interest in identifying pathophysiological mechanisms underpinning compulsivity as a transdiagnostic, neuropsychological domain, which may be beneficial for developing accurate translational models for investigating the complex neuropathology of compulsive behavior (Fineberg et al., 2018; Gillan et al., 2017). In fact, mounting evidence suggests that the neurobehavioural mechanisms mediating cognitive flexibility and habit formation (the shift from goal-directed to habitual responding) contribute to vulnerability to compulsive activity in a broad range of compulsive disorders (Fineberg et al., 2014).

Previous research has reported that the onset of OCD is often preceded by stressful events (Toro et al., 1992). Moreover, OCD patients experience an increase in symptomatology under stressful situations (Coles et al., 2005), and suffer more from the stresses of daily life than healthy controls (Coles et al.,

2005; Findley et al., 2003). Studying the involvement of the hypothalamic-pituitary-adrenal (HPA) axis, the primary mammalian system of stress response (de Kloet et al., 2005), could contribute towards understanding the pathophysiology of compulsivity given that hyperactivity of this axis has been reported in OCD patients (Faravelli et al., 2012). On the other hand, a few studies have associated OCD with a substantial risk for metabolic and cardiovascular complications (Albert et al., 2013), even after considering several covariates, shared familial confounders, and excluding relevant comorbid disorders (Isomura et al., 2018). Additionally, individuals with type 2 diabetes mellitus, who have poor glycemic control, experience more OCD symptomatology than do control patients (Kontoangelos et al., 2013). However, data on this relationship are very limited. Therefore, studying the involvement of the HPA axis and metabolic abnormalities may contribute to identifying biomarkers of compulsivity.

Because of its characteristics of "excessiveness" and "persistence," schedule-induced polydipsia (SIP) is a useful model to study neuropsychiatric disorders characterized by the presence of compulsive behavior (Flores et al., 2014; Ford, 2014; Gilpin et al., 2008; Hawken et al., 2011; Hawken & Beninger, 2014; Merchán et al. 2018; Moreno & Flores, 2012). The SIP model is characterized by the development of excessive drinking in food-deprived animals exposed to intermittent food reinforcement schedules (Falk 1971, 1961). Important differences among individual subjects in the amount of fluid intake and licks support the differentiation of two phenotypes of rats, one with high or excessive drinking (HD), and a second group with low or no SIP acquisition (LD) (López-Grancha et al., 2008). Previous research in our laboratory found that HD rats exhibit increased behavioral inflexibility in a spatial reversal-learning task (Navarro et al., 2017), altered habit formation measured by resistance to extinction in the 5-choice serial reaction time task (5-CSRT) (Moreno et al., 2012), and increased lever pressing under a variable-interval 60-s (VI-60s) schedule of reinforcement (Navarro et al., 2017), when compared with LD rats. However, no previous study has tested the performance of HD and LD rats in reinforcer devaluation, a traditional protocol that assesses sensitivity of the

response to motivational change (Gillan et al., 2016) to evaluate excessive habit formation in vulnerable populations in SIP.

A similar phenomenon, known as psychogenic polydipsia, is characterized by compulsive non-regulatory fluid consumption with no obvious medical etiology, and is present in > 20% of chronic psychiatric patients, and has been associated with schizophrenia diagnosis and other compulsive spectrum disorders (de Leon et al., 2002, 1994; Iftene et al., 2013). Among these individuals, a substantial minority develops clinically significant hyponatremia (i.e., low blood sodium levels), which is associated with confusion, delirium, and ataxia that may progress to seizures, coma, and death (de Leon et al., 1994). Furthermore, severe hyponatremia or rapid correction of hyponatremia is associated with osmotic damage to myelinated structures in the brain (Penders et al., 2015). There is a large body of research demonstrating neurochemical and neuroanatomical alterations in HD rats (Flores et al., 2014; Moreno & Flores, 2012). For example, HD rats in SIP exhibit less myelination in the striatum and basolateral amygdala (Navarro et al., 2017), increased spine density in dorsolateral striatum neurons (Íbias *et al.* 2015), and elevated neuronal activity in the prefrontal cortex (Pellón et al., 2011), the lateral orbitofrontal cortex (Gregory et al., 2015; Merchán et al., 2018), and basolateral amygdala (Merchán et al. 2018). The differences observed in HD rats have not been associated with hyponatremia because this group does not exhibit any symptoms, such as disorientation, ataxia or seizures, after SIP (Navarro et al., 2017), and its regulatory levels of drinking are not different from those of LD rats in the home-cage (Flores et al., 2014). Nevertheless, no previous studies have measured the levels of serum electrolytes, such as sodium, potassium and chloride, after SIP in HD and LD rats.

In the present study, we investigated whether HD rats exhibit differences in behavioral and biological markers for compulsive behavior before SIP exposure compared with LD rats. Furthermore, we evaluated changes in plasma corticosterone (CORT), blood glucose, and serum electrolyte levels of sodium, potassium, and chloride in HD and LD rats after SIP exposure. We hypothesized

that, before SIP exposure, HD rats will present behavioral inflexibility and insensitivity to reinforcer devaluation when compared to LD rats. After SIP, we hypothesized that HD rats will be more vulnerable to increases in CORT levels due to the high drinking rates in SIP, but that no differences in serum sodium levels would be evident between the groups.

Methods

Animals

30 Adult male rats from Janvier (France) weighed approximately 300–400 g at the beginning of the experiment. The animals were housed three/cage or four/cage (57 x 35 x 20 cm) at 22 °C with 08:00–20:00-h dark-light cycle, with food and water available ad libitum. Before the behavioral trainings and after 10 days of habituation to the vivarium conditions, the rats were weighed and handled daily. They were gradually reduced to 85% of their free-feeding body weight by controlled feeding and then maintained at this level of deprivation throughout the experiment. Food was made available by daily feeding of lab chow approximately 30 min after each experimental session. Water was always available in the home cages. All testing was performed between 9:00 and 17:00h.

All procedures were conducted in accordance with the Spanish Royal Decree 53/2013 on the protection of experimental animals, with the European Community Council Directives (2010/63/EU) and with the University of Almería Animal Research Committee approval.

Apparatus

We conducted the tasks in 13 standard operant conditioning chambers (MED Associates) that were 32 cm long x 25 cm wide x 34 cm high, with stainless steel grid floors. A detailed description of the apparatus has been provided previously (Cardona et al., 2006; López-Grancha et al., 2008; Moreno et al., 2010). The SIP and reinforcer devaluation tasks were conducted in 8 chambers with two

retractable levels at both sides of the pellet dispenser. For the reversal-learning task, 6 chambers with an array of five contiguous square holes (2.5 cm), 2.2 cm deep and 2 cm above floor level at the back panel were used. The three inner apertures were blocked using metal tape, so only the two outermost holes remained unobstructed. The scheduling and recording of experimental events were controlled by Med PC IV computer and commercial software (Cibertec SA, Spain).

Experimental Procedures

The experimental design is depicted in Figure 1. The order of testing and screening was as follows: reversal-learning task; reinforcer devaluation task; and the SIP task. The first blood glucose and plasma CORT measurements were performed 24 h before the first SIP session. The second blood glucose and plasma CORT measurements, together with serum electrolytes sodium, potassium, and chloride samples, were obtained 24 h after the final SIP session.

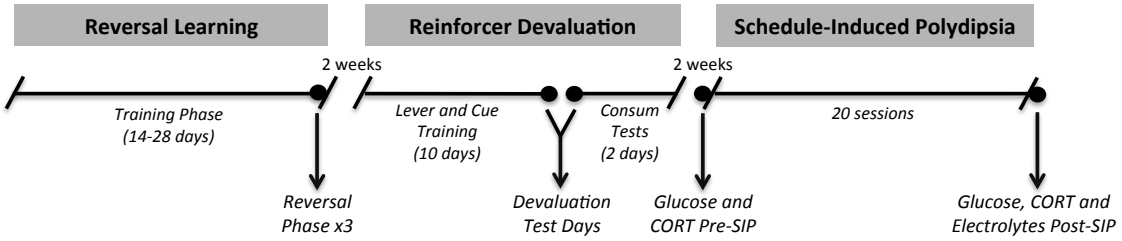

Figure 1. Experimental procedure illustrated in a timetable.

Spatial-Discrimination Serial Reversal Learning

Behavioral training was modified from a previously described task (Barlow et al., 2015), and is depicted in Figure 2. Rats were habituated to the apparatus over a two-day period for 20 min/session. In these sessions, the house light was switched on and 20 food pellets were placed in the magazine and two in the holes. The animals were then trained to nose-poke in the magazine to trigger the illumination of the stimulus lights into the holes and to respond with a nose-poke

in the holes for food delivery within a 30-s limited hold (LH) period. This phase of training occurred successively in each hole under a fixed ratio-1 schedule of reinforcement (FR1) to a criterion of 50 correct trials in 20 min and, thereafter, under FR2 and FR3 schedules based on the same criterion. Responses in the unrewarded hole were not punished but omission errors (failure to respond to the stimulus within the LH) resulted in a 5-s time-out period, in which all lights were extinguished. An inter-trial interval of 5 s was introduced when responses had stabilized under a FR3 schedule. For the acquisition of spatial discrimination, subjects were trained on a two-hole discrimination task. A nose-poke in the food magazine triggered the illumination of both stimulus lights. A sequence of three nose-pokes in one of the holes resulted in reward and was considered a "correct trial." Three nose-pokes in the "incorrect" hole resulted in a time-out without reward and was considered an incorrect trial. Rats were trained across sessions until they achieved a criterion of 9 correct trials across the previous 10 trials. "Correct'" and "incorrect" holes were designated randomly and counterbalanced across subjects.

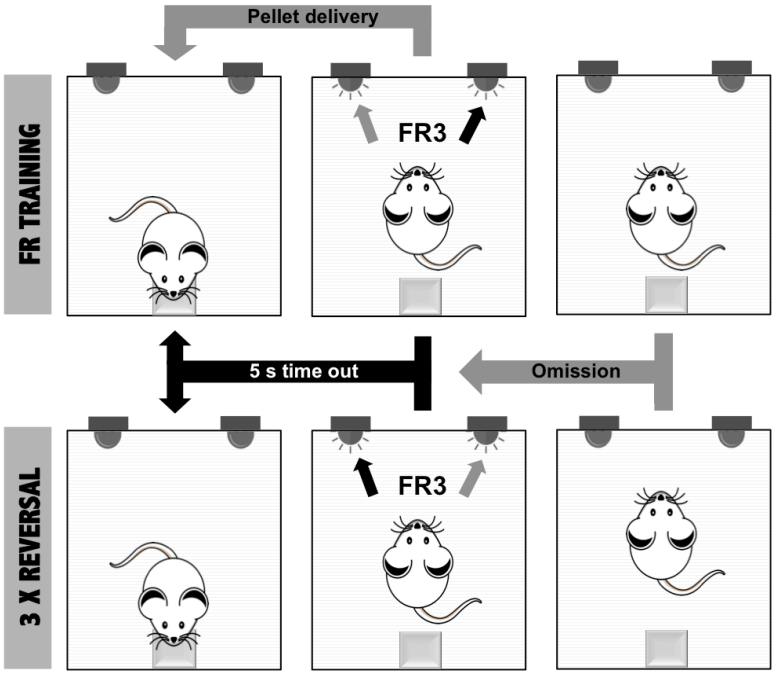

Figure 2. Schematic illustration of the spatial-discrimination serial reversal-learning task.

For the within-session reversal learning, “correct” and “incorrect” holes were kept the same as those experienced in the acquisition of spatial discrimination. After the rats had reached the criterion on this retention phase, the “correct” and “incorrect’” holes were reversed, such that the previously rewarded response now resulted in a time-out period, and the previously unrewarded response resulted in the delivery of a food pellet. Subjects completed three reversals during the 1 h session. The variables measured were: the number of trials to reach the criterion; number of incorrect responses; and perseverative responses and omissions. Perseverative responses were identified when ≥7 consecutive incorrect responses were made in blocks of 10 trials.

Reinforcer Devaluation task

Two weeks after the spatial-discrimination serial reversal-learning task, the animals were subjected to the reinforcer devaluation task (Figure 1). Behavioral training was modified from a previously described task (West et al., 2016), and is depicted in Figure 3. First, the rats were trained to press two spatially distinct levers for a food pellet, until reaching stable levels on an FR5 schedule. Next, during cue training, a cue light was illuminated over one of the two levers and, after 5 s, the lever under that cue light was extended into the chamber (the other cue light/lever remained off/retracted). The rats were trained to press the active lever to receive a specific reinforcer on an FR5 schedule within a 30 s LH period until they obtained 90% correct responses (at least 45 of 50 trials) in two consecutive daily test sessions. The position of the active cue light/lever alternated for each trial pseudorandomly. Once the rats reached this criterion, they were moved to a variable-ratio (VR9) schedule, in which they had to press the active lever, on average, 9 times to receive a reinforcer (VR range 4–13) to obtain 90% of correct responses in two consecutive daily test sessions. The inter-trial interval was 5 s for all cue-training sessions. On every cue training session, rats also received an equal amount of a second (i.e., different) reinforcer in an empty standard rat cage at least 2 h after they completed the cue training for that

day. This ensured that all rats had equal experience with both reinforcers. Reinforcers were counterbalanced across rats such that one-half of the rats received chocolate-flavored pellets (TestDiet®, Purina, USA) during training (and sucrose-flavored pellets in individual standard rat cages; TSE systems, Germany) and the other one-half received sucrose-flavored pellets during training (and chocolate-flavored pellets in individual standard rat cages). Previously, rats have exhibited similar preference for both chocolate and sugar pellets under an instrumental preference task (West et al., 2011).

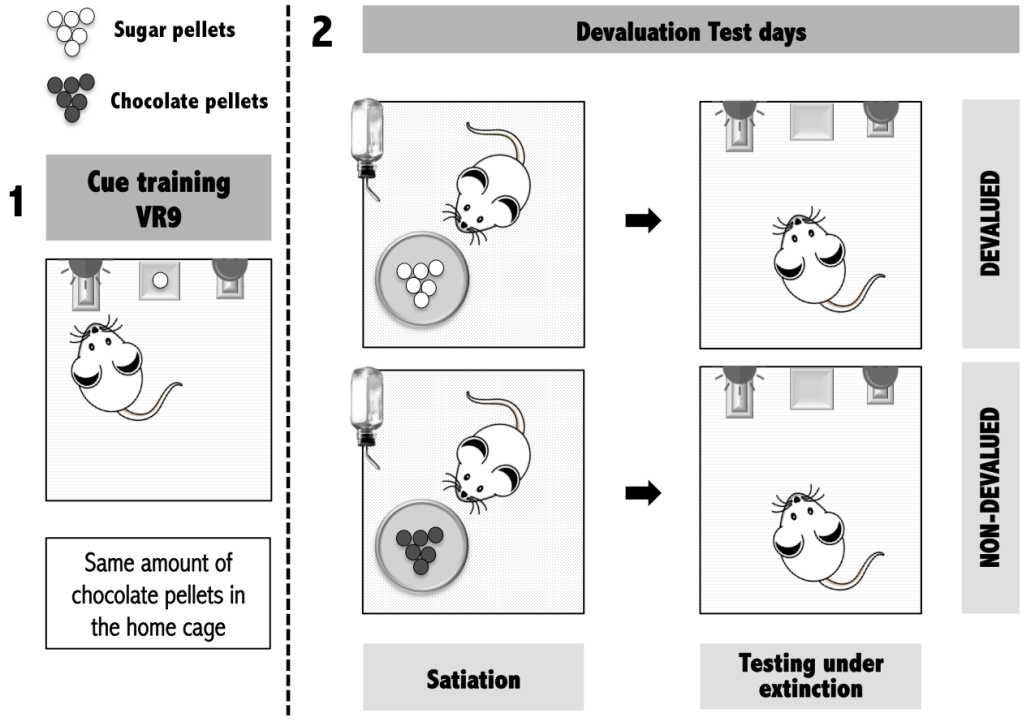

Figure 3. Schematic illustration of the reinforcer devaluation procedure.

The devaluation test days were divided in two distinct phases: satiation and testing under extinction. In the satiation phase, rats received ad libitum access for 25 min of either the same reinforcer received during training (devalued) or the other reinforcer (non-devalued) immediately before testing to achieve outcome-selective satiation. In the second phase (testing under extinction), rats were permitted to lever press for 30 trials using the same cues as training except no

reinforcers were delivered. Rats were tested on an FR9 schedule to ensure that each trial would contain the same number of lever presses before the lever retracted. After 48 h and one cue training reminder session, the same test was repeated, except that the other reinforcer was consumed before testing. The order of reinforcer was counterbalanced so that one-half of the rats were sated on the different reinforcer (non-devalued) and the other one-half were sated on the same reinforcer (devalued).

A consummatory test for evaluating devaluation of the reinforcer was performed two days after the final test day of testing under extinction in the operant chambers. Rats were given 25 min to eat one of the foods (training reinforcer or different reinforcer) ad libitum in an empty standard rat cage. After selective satiation, rats were given access to both reinforcers for 20 min, and the amount of each reinforcer consumed was recorded. At least 48 h later, the same test was repeated but the other reinforcer was devalued. The food consumption data of the same condition (devalued or non-devalued) were averaged.

The variables measured included the number of lever presses under extinction and amount of food consumed (in grams) in the consummatory test.

SIP

Two weeks after the reinforcer devaluation task, the animals were exposed to the SIP protocol (Figure 1). Over two successive days, a water-ingestion test was administered (baseline). Sixty pellets were placed together, and the amount of water each rat consumed in 60 min was measured. After one adaptation day, the animals were exposed to a fixed time 60s (FT-60s) schedule of food pellet presentation during 60 min sessions (Figure 4). Water bottles containing 100 ml of fresh water were provided immediately before each session. After 20 daily sessions, the average total licks for each animal was calculated based on the last three SIP sessions. Following the protocol described by Moreno et al. (2012), rats were classified as HD (n=14) or LD (n=14) if their average water intake was above or below the group median, respectively. Two rats were not included in the

statistical analysis due to inadequate drinking behavior (spilling water from the water bottle during the SIP sessions). The following measures were recorded for each rat: total amount of water (ml) removed from the bottle; total number of licks; and total number of nose-pokes (for more details, see López-Grancha et al., 2008).

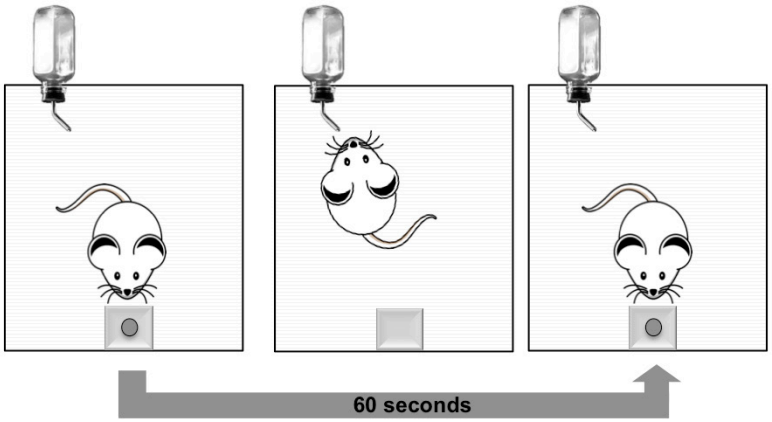

Figure 4. Schematic illustration of the schedule-induced polydipsia procedure.

Plasma CORT Levels

The rats were anesthetized using isoflurane and blood samples were collected from the lateral tail vein in EDTA-coated capillary vials (Microvette CB300, Sarstedt S.A.U, Barcelona, Spain). Plasma was separated by centrifuging (Sigma 3-18KS, Germany) the blood samples at 3,000 rpm (800 g) for 20 min at 4ºC and stored at -80ºC until assay. CORT levels were determined using DetectX® enzyme immunoassay kit (K014-H1, DetectX®, Arbor Assays™, Ann Arbor, USA).

Blood Glucose Levels

Fasting blood glucose level was measured using a glucometer (Accu-Chek Performa®, Roche Diagnostics, Germany) between 09:00 am and 12:00 pm. The blood drop was rapidly obtained through lateral tail vein puncture using a sterile

needle. To minimize discomfort/pain, a eutectic mixture of local anesthetics (lignocaine base 2.5% and prilocaine base 2.5%) cream was applied to the tail 30 min before vein puncture (EMLA 25 mg/g + 25 mg/g, AstraZeneca Farmacéutica Spain SA, Spain).

Serum Electrolytes Levels

The rats were anesthetized using isoflurane (5%) and euthanized by decapitation. Trunk blood samples were collected into serum vacuum tubes with clot activator and gel separator (BD vacutainer® SST ™ II Advance, Plymouth, United Kingdom). Blood samples were mixed by six complete inversions and were permitted to clot for 1 h at room temperature (20ºC). Sera were obtained by centrifuging (CENCOM, J.P. Selecta, Barcelona, Spain) at 3,600 rpm (1,450 g) for 30 min at 24ºC and stored at -20ºC. Levels of sodium (Na+), potassium (K+) and chloride (Cl-) were estimated using an ion-selective electrode method in an automated chemistry analyzer (ADVIA Chemistry XPT system, Siemens Healthineers, Erlangen, Germany).

Statistical Analysis

Behavioral data of the reversal-learning task were analyzed by one-way repeated measures analysis of variance (ANOVA) with “group” (HD and LD) as the between-subject factor and "phase" (1 retention phase and/or reversal 1, reversal 2 and reversal 3) as the within-subject factor. The number of lever presses under extinction in the reinforcer devaluation task was examined by two-way repeated measures ANOVA with two between-subject factors, “group” (HD and LD) and "condition" (Devalued and Non-devalued), and "devaluation test days" (2 devaluation test days) as within-subject factor. The amount of food consumed in the consummatory test was examined by two-way ANOVA with “group” (HD and LD) and "condition" (Devalued and Non-devalued) as between-subject factors. SIP measures were analyzed using one-way repeated measures ANOVA, with “group” (HD and LD) as the between-subject factor and “sessions” (20

levels) as the within-subject factor. CORT, glucose levels and body weight were analyzed using one-way repeated measures ANOVA, with "group" (HD and LD) as the between-subject factor and "days" (Pre-SIP and Post-SIP) as the within-subject factor. Electrolytes levels were analyzed by Student's t-tests, with "group" (HD and LD) as the between-subject factor. The results are expressed as the mean ± standard error of the mean (SEM). When appropriate, *post hoc* comparisons were made using the Sidak's test. Partial eta-squared values (η^2_p) are reported as a measure of the effect size, for which values of .01, .06, and .14 are considered to reflect small, medium, and large effects, respectively (Cohen, 1973). All analyses were computed using the SPSS 22.0 software package. Statistical significance was set at $p<0.05$.

Results

Reversal Learning

The mean trials to criterion are shown in Figure 5A. Repeated measures ANOVA did not reveal differences between HD and LD rats across the reversal-learning phases (phases x group effect $F_{3,72}=0.536$; $p=0.659$) nor a main effect of group ($F_{1,24}=0.042$; $p=0.840$). When comparing reversal-learning phases, significant differences in trials to criterion were observed (phases effect $F_{3,72}=50.222$; $p<0.001$; $\eta^2_p=0.68$). *Post-hoc* analysis revealed a higher number of trials to criterion in reversal 1 than in the other phase ($p<0.001$). The number of trials to criterion was higher in reversal 1 and reversal 3 ($p<0.001$) than in the retention phase, but reversal 2 did not show any such difference ($p=1.00$).

The mean omissions are shown in Figure 5B. HD and LD rats had a similar number of omissions across the reversal-learning phases (phases x group effect $F_{3,72}=0.536$; $p=0.659$). The main effect of group was not significant ($F_{1,24}=0.155$; $p=0.697$). Differences in omissions between the reversal-learning phases were observed (phases effect $F_{3,72}= 8.389$; $p<0.001$; $\eta^2_p=0.26$), and the *post-hoc* analysis indicated a higher number of omissions in reversal 1 than in the retention phase ($p<0.05$).

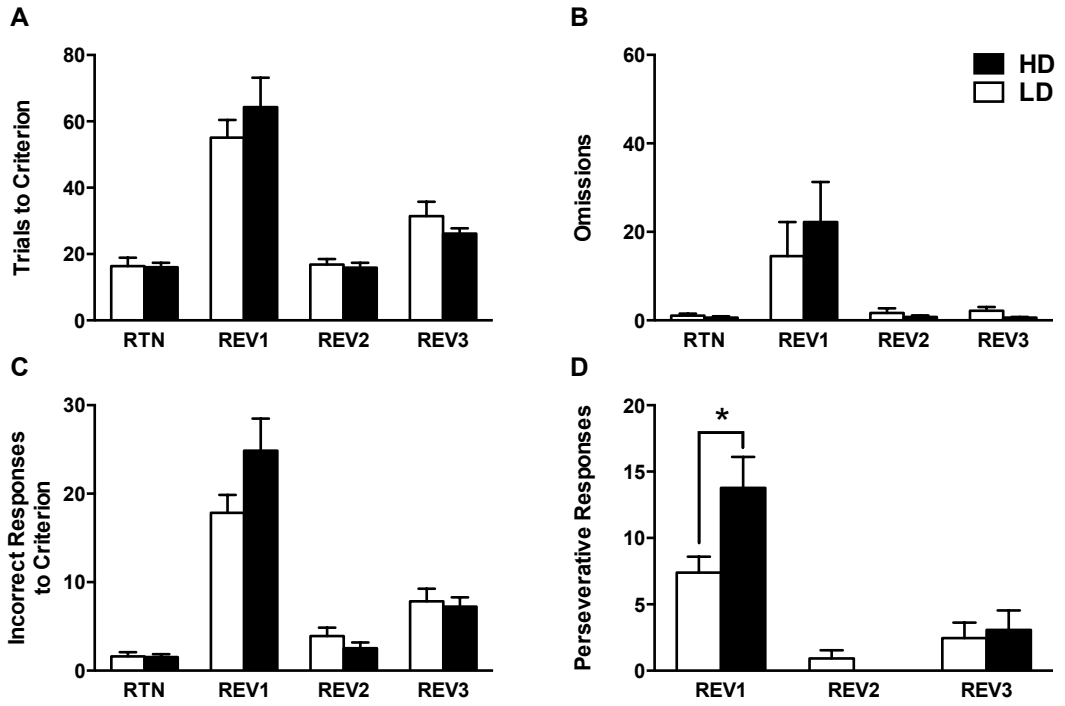

Figure 5. Mean (± SEM) trials to criterion (A), omissions (B), incorrect responses to criterion (C) and perseverative responses (D) in the retention (RTN), reversal 1 (REV1), reversal 2 (REV2) and reversal 3 (REV3) phases of the spatial-discrimination serial reversal-learning task for High drinker (HD, n=13) and Low drinker (LD, n=13) rats. *p<0.05 indicates significant differences between HD and LD rats.

The mean incorrect responses to criterion are shown in Figure 5C. Although a significant interaction effect was found (phases x group effect $F_{3,72}$=2.947; p<0.05; η^2_p=0.11), the *post-hoc* analysis did not reveal differences between HD and LD rats in any reversal-learning phase (not even in reversal 1 [p=0.105]). Furthermore, no significant main effect of group was found ($F_{1,24}$= 0.042; p=0.840). When comparing reversal-learning phases, significant differences in incorrect responses to criterion were observed (phases effect $F_{3,72}$=62,956; p<0.001; η^2_p=0.72). *Post-hoc* analysis revealed a higher number of incorrect responses to criterion in reversal 1 than in the other phases (p<0.001). The number of incorrect responses to criterion was higher in reversal 1 and reversal 3 (p<0.001) than in the retention phase, but reversal 2 showed no such difference (p=0.112).

The mean perseverative responses are shown in Figure 5D. Repeated measures ANOVA revealed differences between HD and LD rats across the reversal-learning phases (phases x group effect $F_{2,48}$=5.466; p<0.01; η^2_p=0.19). *Post-hoc* analysis revealed that HD had more perseverative responses than LD rats in reversal 1 (p<0.05). No significant main effect of group was found ($F_{1,24}$=2.288; p=0.143). When comparing reversal-learning phases, significant differences in perseverative responses were observed (phases effect $F_{2,48}$=41.399; p<0.001; η^2_p=0.63). *Post-hoc* analysis revealed a higher number of perseverative responses in reversal 1 than in reversals 2 and 3 (p<0.001). Moreover, this value was higher in reversal 3 than in reversal 2 (p<0.05).

Reinforcer Devaluation

A two-way repeated measures ANOVA revealed a statistically significant interaction in the number of lever presses under extinction after the consumption of either a different reinforcer (non-devalued) or the same reinforcer (devalued) performed in two different devaluation test days by HD and LD rats (days x condition x group effect $F_{1,24}$=4.625; p<0.05; η^2_p=0.16). In fact, significant differences between the two devaluation test days were found (days effect $F_{1,24}$=132.369; p<0.001; η^2_p=0.85). *Post-hoc* analysis indicated that lever presses were greater on the first devaluation test day than on the second devaluation test day (p<0.001). The mean (± SEM) data values were 170.52 ± 9.82 and 57.46 ± 5.48, respectively. The mean number of lever presses on the first devaluation test day is presented in Figure 6A. *Post-hoc* analysis revealed reduced lever pressing in the LD group (p<0.05) that were sated on the same reinforcer (devalued) than the LD counterparts sated on the different reinforcer (non-devalued). Furthermore, significant differences were found between HD and LD rats in the devalued condition (p<0.05). HD rats lever-pressed similarly in the devalued compared with the non-devalued condition (p=0.521). Additionally, a group x days interaction was observed ($F_{1,24}$=7.238; p<0.05; η^2_p=0.23), and *post-hoc* analysis indicated greater lever pressing in LD (p<0.01) than in HD rats in the

second devaluation test day. The mean (± SEM) number of lever presses performed by HD and LD were 41.79±7.70 and 73.13±7.78, respectively (data not shown).

Figure 6B shows the mean amount of food consumed for either a different reinforcer (non-devalued) or the same reinforcer (devalued) that was previously eaten by the same HD and LD groups from the first devaluation test days. Statistically significant differences were not found between HD and LD rats in the devalued and non-devalued conditions (condition x group effect $F_{1,27}$=0.514; p=0.481), nor in food consumption between HD and LD rats (group effect $F_{1,27}$= 1.832; p=0.189). However, the differences between the devalued and non-devalued conditions were confirmed (condition effect $F_{1,27}$= 49.340; p<0.001; η^2_p=0.67). *Post-hoc* analysis revealed significantly lower food consumption in the devalued condition than in the non-devalued condition (p<0.001).

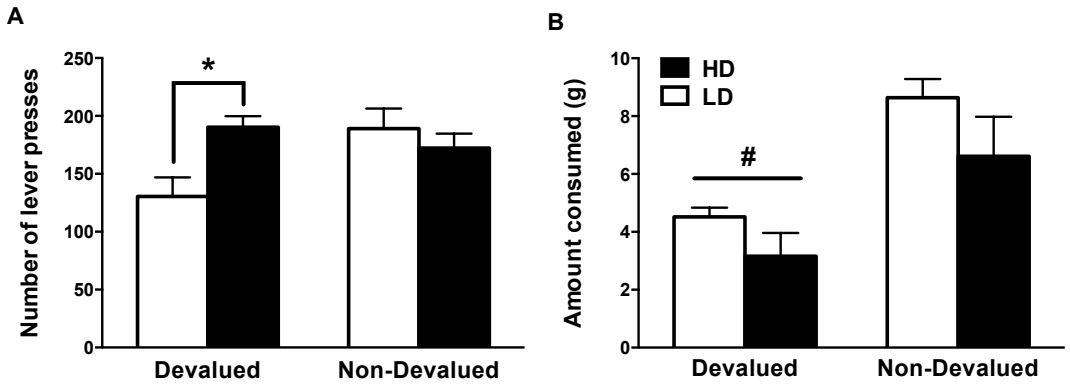

Figure 6. Behavioral responses after devaluation of a specific reinforcer in an operant test on the first devaluation testing day (A) and the consummatory test (B). The mean (± SEM) total number of lever presses that rats made under extinction after the consumption of either a different reinforcer (Non-Devalued) or the same reinforcer (Devalued) received during testing (A), and the amount of food consumed of either a different reinforcer (Non-devalued) or the same reinforcer (Devalued) that was previously eaten (B). The groups were: High drinker (HD, Devalued n=7 and Non-Devalued n=7) and Low drinker rats (LD, Devalued n=6 and Non-Devalued n=7). *p<0.05 indicate significant differences between HD and LD rats. #p<0.001 indicate significant differences between Devalued and Non-Devalued conditions.

SIP

The mean total licks, water intake, and total magazine entries in HD and LD rats in SIP are shown in Figure 7. HD rats showed more total licks (sessions x group effect $F_{19,494}$=2.842; p<0.001; η^2_p=0.09) and a higher water intake (sessions x group effect $F_{19,494}$=2.204; p<0.01; η^2_p=0.08) across SIP sessions than did LD rats (Fig. 7A and 7B). The main effects of group were found in total licks ($F_{1, 26}$=57.982; p<0.001; η^2_p=0.69) and water intake ($F_{1, 26}$=40.74; p<0.001; η^2_p=0.61). *Post hoc* analysis indicated that HD rats showed significantly more total licks and higher water intake from session 2 onward than did LD rats (p<0.05 and p<0.01). Furthermore, HD rats significantly increased their total licks and water intake from session 2 (p<0.05 and p<0.01) to the end of training, reaching stable levels at sessions 3 and 2, respectively. LD rats did not exhibit acquisition of total licks and water intake across SIP sessions. No interaction effect was found in magazine entries (sessions x group effect $F_{19,494}$=0.483; p<0.969) (Fig. 7C).

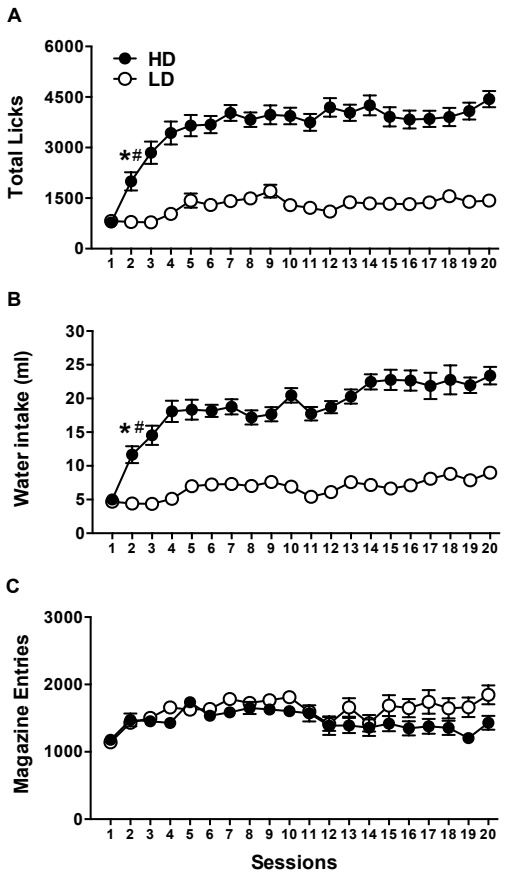

Figure 7. Mean (± SEM) total licks (A), water intake (B) and total magazine entries (C) in FT-60s across 20 sessions of schedule-induced polydipsia (SIP) in High drinker (HD, n=14) and Low drinker (LD, n=14) rats. *p<0.05 indicates significant differences between HD and LD rats from that session onward. #p<0.05 indicates significant differences compared with session 1 in the same group.

Plasma CORT levels

Mean plasma CORT levels (ng/ml) 24 h before the first SIP session (Pre-SIP) and 24 h after the final SIP session (Post-SIP) in the HD and LD groups are shown in Figure 8A. Repeated measures ANOVA revealed a significant interaction in plasma CORT levels between HD and LD rats on the Pre-SIP and Post-SIP days (days x group $F_{1,24}$=5.616; p<0.05; η^2_p=0.19). The main effects of days ($F_{1,24}$=0.303; p=0.587) and group ($F_{1,24}$=0.170; p=0.684) were not significant. *Post-hoc* analysis indicated that HD rats had higher plasma CORT levels on the Post-SIP day than on the Pre-SIP day (p<0.05). Additionally, a trend toward significance was observed when comparing groups, indicating that HD had higher plasma CORT levels than LD rats on the Post-SIP day (p=0.086).

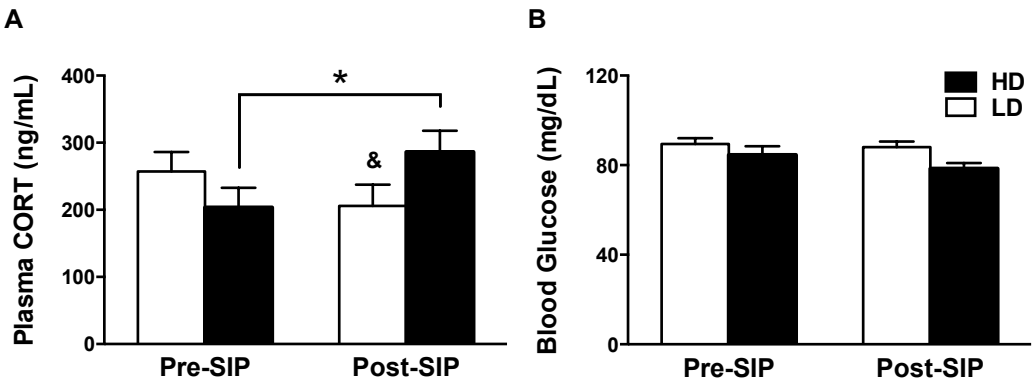

Figure 8. Mean (± SEM) plasma corticosterone (A) and blood glucose (B) levels 24-h before the first session of schedule-induced Polydipsia (Pre-SIP) and 24-h after the final SIP session (Post-SIP) in the High drinker (HD, n=13) and Low drinker (LD, n=13-14) groups. ***p<0.05 indicates significant differences between Pre-SIP and Post-SIP in the HD group. **&**Trend towards a significant difference between HD and LD rats (p=0.086).

Blood Glucose Levels

Mean blood glucose levels (mg/dL) 24 h before the first SIP session (Pre-SIP) and 24 h after the final SIP session (Post-SIP) in the HD and LD groups are shown in Figure 8B. Repeated measures ANOVA did not reveal statistically significant differences in blood glucose levels between HD and LD rats on the Pre-SIP and Post-SIP days (days x group $F_{1,25}$=0.762; p=0.391). A main effect of

group was found ($F_{1,25}$=5.148; p<0.05; η^2_p=0.17) and *post-hoc* analysis indicated that HD rats had lower blood glucose levels than did LD rats (p<0.05). The main effect of days ($F_{1,25}$=1.987; p=0.171) was not significant. Body weight was similar on Pre-SIP and Post-SIP days between HD and LD rats (days x group $F_{1,25}$=0.244; p=0.625; group effect $F_{1,25}$=2.413; p=0.133) (data not shown).

Serum Electrolyte Levels

Mean serum electrolyte levels for sodium, potassium, and chloride (mEq/L) in the HD and LD groups 24 h after the final SIP session are summarized in Table 1. Student's t-tests did not reveal statistically significant differences between HD and LD rats in serum sodium (t_{26}=0.503; p=0.619), potassium (t_{26}=0.608; p=0.548) or chloride levels (t_{26}=0.398; p=0.694).

Table 1. Serum electrolyte levels of sodium, potassium and chloride (mEq/L) in High Drinker (HD, n=14) and Low Drinker (LD, n=14) rats 24 h after the final SIP session.

	HD (n=14)	**LD (n=14)**	**t - tests (p value)**
Na+	135.82 ± 0.83	135.26 ± 0.73	0.619
K+	5.57 ± 0.10	5.66 ± 0.11	0.548
Cl-	100.57 ± 0.71	100.93 ± 0.55	0.694

Data are mean ± SEM. *Na*+ Sodium, *K*+ Potassium, *Cl*- Chloride.

Discussion

In the present study, we demonstrated that before the acquisition of SIP, HD rats exhibited increased perseverative responses in a reversal-learning task and were insensitive to devaluation in a reinforcer devaluation task when compared with LD rats. These results confirm that behavioral inflexibility and excessive habit formation are behavioral traits present in HD rats before SIP exposure, and thus candidates for predicting compulsive drinking in SIP. Before SIP, HD rats did not exhibit increased HPA axis activity or metabolic deficits measured according

to plasma CORT and blood glucose levels, respectively. However, plasma CORT levels increased only in HD rats after 20 daily SIP sessions, which may indicate that the SIP protocol provides a stressful situation for vulnerable individuals. In addition, HD rats did not exhibit altered sodium levels that could indicate hyponatremia after 20 daily SIP sessions.

In the reversal-learning task, we found that HD compulsive rats exhibited behavioral inflexibility as reflected by increased perseverative responses in reversal 1 when compared with LD rats. In contrast, HD and LD rats did not differ statistically in trials to criterion, incorrect responses to criterion, and omissions in any of the reversal phases. Moreover, the finding that significant differences were found only during reversal 1 are in accordance with previous literature (Boulougoris et al., 2009, 2008, 2007; Navarro et al., 2017). Previous studies have reported similar results regarding a lack of behavioral flexibility in compulsive rats in SIP (Navarro et al., 2017). Regarding the reinforcer devaluation task, we observed an important decrease of lever pressing in the second devaluation test day in both groups that may be relevant for further studies on habit formation. A previous study documented a similar reduction of lever pressing in a second extinction under selective satiation even after being exposed to 15 daily training sessions of recovery (Iguchi et al., 2017). In fact, the decrease in responding was more pronounced in HD rats, indicating that this group may be more sensitive to the extinction procedure. In the first devaluation test day, we found that HD compulsive rats exhibited insensitivity of the response to motivational changes measured by similar lever pressing under extinction after the consumption of either a different reinforcer or the same reinforcer received during testing when compared with LD rats. Moreover, HD and LD rats did not differ significantly in terms of performance on the consummatory test, suggesting that the food was effectively devalued following selective satiation. This is the first SIP study to demonstrate deficits in the reinforcer devaluation paradigm, which identifies deficits in the neurobehavioural mechanism mediating habit formation, and provides another biomarker for predicting compulsive drinking in SIP. In support of our findings,

previous studies have shown that compulsive rats in SIP exhibit altered habit formation measured by resistance to extinction in the 5-CSRT task (Moreno et al., 2012), increased lever pressing under a VI-60s schedule of reinforcement (Navarro et al., 2017), and a preference for response-learning strategies in a Y-maze (Gregory et al., 2015). It is important to note that the present study evaluated behavioral flexibility and habit formation before SIP exposure, while previous studies have tested the performance in behavioral tasks after SIP exposure and classification of HD and LD rats (Cardona et al., 2011; Merchán et al., 2017; Moreno et al., 2012, 2010; Navarro et al., 2017; López-Grancha et al., 2008). This protocol challenges the argument that SIP exposure may alter brain circuits modulating behavioral flexibility and sensitivity to reinforcer devaluation in HD rats, and confirms that these behavioral markers may contribute to the development of compulsive drinking in SIP. Moreover, these results support the sensitivity of the SIP model for identifying individuals with deficits in behavioral flexibility and habit formation.

The neural substrate of reversal learning has been shown to include the orbitofrontal cortex in animal studies (Boulougouris et al., 2007; Clarke et al., 2007, 2005; Dias et al., 1996; McAlonan & Brown, 2003), as well as in humans (Cools et al., 2002; Fellows & Farah, 2003; Hampshire & Owen, 2006). Furthermore, serotonin has been implicated in the modulation of reversal learning in the orbital frontal cortex (OFC) (Clarke et al., 2007, 2005; Lapiz-Bluhm & Morilak, 2010; Lapiz-Bluhm et al., 2009). Moreover, mounting evidence from rodent (Corbit & Balleine, 2003; Gremel & Costa, 2013; Yin et al., 2004; Faure et al., 2005) and human studies (de Wit et al., 2012, 2009; Gillan et al., 2015; Tricomi et al., 2009; Liljeholm et al., 2015; Valentin et al., 2007; Voon et al., 2015) has highlighted the importance of the caudate nucleus (dorsomedial striatum in rodents) and medial OFC for the goal-directed system, and the putamen for the habit system. In fact, compulsivity across different psychiatric disorders has been related to an impairment in goal-directed learning, leading to excessive habit formation (Gillan et al., 2016). In SIP studies, compulsive drinker rats exhibited hyperactivity in the OFC and prefrontal areas

(Gregory et al., 2015; Merchán et al. 2018; Pellón et al., 2011), increase in the spine density of dorsolateral striatum neurons (Íbias *et al.* 2015), and abnormalities in the basolateral amygdala (Merchán et al., 2018; Moreno et al., 2012; Navarro et al., 2017; Pellón et al., 2011), a brain area that appears to be implicated in the acquisition of goal-directed actions (Balleine, 2005). Moreover, the activation of the serotonin 2A (5-HT_{2A}) receptor in the OFC by selective serotonin reuptake inhibitors (SSRIs) has been proposed as a mediator of behavioral flexibility (Barlow et al., 2015; Boulougouris et al. 2008; Furr et al., 2012) and goal-directed control (Gillan et al., 2016) in the therapeutic effect in OCD patients. Consistent with this, HD rats exhibited increased compulsive drinking after chronic tryptophan depletion by diet (Merchán et al., 2017), reduced 5-HT_{2A} receptor binding, and alterations in 5-HT tone in the frontal cortex when compared with LD rats (Mora et al., in press). Moreover, the 5-HT_{2A} receptor mediated the anti-compulsive effect of 5-$HT_{2A/C}$ receptor agonist DOI in SIP (Navarro et al., 2015). Collectively, these results suggest that HD rats may have alterations in the brain circuits mediating behavioral flexibility and habit formation, providing supportive evidence that SIP is a reliable animal model for studying compulsive behavior.

With respect to the HPA axis, HD and LD rats did not exhibit different basal CORT levels before SIP, which may indicate that individual differences do not depend on previous HPA axis activity. In fact, SIP studies have not found differences between HD and LD rats in anxiety measured according to the percentage of time spent on open arms or in the percentage of open-arm entries (López-Grancha et al., 2008). However, HD rats exhibited increased basal CORT levels 24 h after the final SIP session—an increment that was not observed in LD rats. A previous SIP study evaluating corticosterone levels under conditions without water access found that this stress hormone increased in rats exposed to food-reinforcement schedules that have been found to produce high drinking rates (López-Grancha et al., 2006). In the present study, we demonstrated that the increase in HPA axis activity after repetitive SIP exposure is observed only in the vulnerable HD population. This result does not support the hypothesis of SIP

drinking as coping and anxiolytic behavior (Brett & Levine, 1981, 1979), which is based on studies reporting lower plasma CORT levels in rats exhibiting excessive drinking in SIP than in animals with lower drinking rates or without a bottle in the operant chamber (Brett & Levine, 1981, 1979; Dantzer et al., 1988). In fact, other studies reported similar or higher CORT levels in rats acquiring SIP than in rats under conditions without a water bottle or without a schedule of intermittent reinforcement (Mittleman et al., 1988; Tazi et al., 1986; Wallace et al., 1986). Moreover, the intracerebroventricular administration of corticotropin-releasing hormone decreased SIP drinking instead of increasing it (Cole & Koop, 1994). From a pharmacological perspective, there appears to be no evidence of such anxiolytic function in SIP as well because the administration of low-dose diazepam increases SIP drinking (López-Grancha et al., 2008; Mittleman et al., 1988). In fact, OCD patients do not respond to many common anxiety disorder treatments such as benzodiazepines (Bartz & Hollander, 2006). In human studies, findings regarding the relationship between HPA axis functioning and OCD are controversial. Several studies have reported hyperactivity of the HPA axis in OCD patients. For example, OCD patients exhibited higher plasma cortisol levels (Catapano et al., 1994, 1992; Erbay & Kartalci, 2015; Gustafsson et al., 2008; Monteleone et al., 1994), increased nocturnal secretion of adrenocorticotropic hormone (Kluge et al., 2007) and increased urinary cortisol levels (Gehris et al., 1990). Additionally, increased HPA axis activity in OCD has been reflected by elevated levels of corticotrophin-releasing hormone in the cerebrospinal fluid (Altemus et al., 1992) and a lack of inhibition of cortisol secretion after a dexamethasone suppression test (Catapano et al., 1990; Cottraux et al., 1984). However, these results could not be confirmed by others (Chappell et al., 1996; Coryell et al., 1989; Jenike et al., 1987; Lieberman et al., 1985; Lucey et al., 1992; Vallejo et al., 1988). Hyperactivity of the HPA axis in OCD patients may be a consequence of stressful events or, conversely, may be involved in the pathophysiology of OCD (Faravelli et al., 2012). Our results do not support the hypothesis that compulsive drinking in SIP is driven by altered HPA axis activity. Alternatively, high drinking in SIP may be a rigid excessive habit

developed by inflexible vulnerable individuals under a stressful situation induced by the reinforcement-food schedules.

Regarding the metabolic system, HD rats did not exhibit altered levels of blood glucose before and after SIP exposure when compared with LD rats. The glucose levels in HD and LD rats were approximately 80 mg/dL, which is within the normal value for fasting whole blood glucose (70-110 mg/dL) (Estridge et al., 2000). The results do not indicate that compulsive drinker rats are vulnerable to develop type 2 diabetes mellitus. A study involving OCD patients found that 21.1% of the sample exhibited metabolic and cardiovascular complications, of whom 4.8% had hyperglycemia (Albert et al., 2013). In a recent study involving a wider sample, OCD patients had increased risk for obesity, type 2 diabetes mellitus, and circulatory system diseases (Isomura et al., 2018). Interestingly, patients taking SSRIs had significantly lower risks, which may reflect the indirect effects of reliable and sustained symptom reduction associated with evidence-based treatment in general and lifestyle changes. In fact, factors related to lifestyle choices, as well as the prolonged use of psychiatric medication, may be underlying the vulnerabilities for developing type 2 diabetes mellitus or other metabolic abnormalities observed in OCD patients (Isomura et al., 2018). However, further research should investigate the possible vulnerabilities of OCD patients in terms of biological markers for compulsivity and its prevention.

Clinically significant hyponatremia, defined as serum sodium concentration <135 mmol/L, is estimated to occur in approximately 29% of psychiatric patients exhibiting psychogenic polydipsia (de Leon et al., 1994). Serum electrolyte levels of sodium, potassium, and chloride in HD and LD rats were within the normal range according to Estridge et al. (2000) (sodium, 135-148 mEq/L; potassium, 3.5-5.5 mEq/L; and chloride, 98-108 mEq/L), and the values were not statistically different between HD and LD rats. These results indicate that the SIP protocol (20 sessions with FT-60s) does not lead to hypernatremia in HD rats. In fact, this finding is consistent with previous studies indicating that symptoms associated with hyponatremia, such as disorientation, ataxia, or

seizures, have not been observed among compulsive rats in SIP (Navarro et al., 2017). Moreover, the regulatory levels of drinking are not different between HD and LD rats in the home-cage (Flores et al., 2014). The present study was the first to demonstrate that the SIP exposure neither alter brain circuits modulating behavioral flexibility and sensitivity to reinforcer devaluation nor produce brain damage associated with hyponatremia in compulsive rats and, therefore, supports previous studies reporting behavioral, neurochemical and neuroanatomical markers for compulsive drinking in SIP (Flores et al., 2014; Moreno & Flores, 2012).

In conclusion, the present study demonstrated that HD rats exhibit deficits in the neurobehavioural mechanisms mediating behavioral flexibility and habit formation before the acquisition of compulsive drinking in SIP. Additionally, HPA axis activity in HD rats increased after SIP exposure, indicating that compulsive drinking may be driven by a stressful situation specifically in vulnerable individuals. Our data confirm previous findings that suggest behavioral inflexibility and excessive habit formation as behavioral markers for predicting compulsive activity in a broad range of compulsive disorders, and point toward SIP as a suitable animal model for investigating the complex neuropathology of compulsive behavior.

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5

Excessive habit formation in schedule-induced polydipsia: Microstructural analysis of licking among rat strains and involvement of the orbitofrontal cortex

Ana Merchán, Santiago Mora, Belén Gago, Elisa Rodríguez-Ortega, Albert Fernández-Teruel, Jorge López Puga, Fernando Sánchez-Santed, Margarita Moreno and Pilar Flores

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Experimental Series II:

Serotonergic involvement in compulsive drinking in SIP and gut microbiota

6

Tryptophan depletion affects compulsive behaviour in rats: strain dependent effects and associated neuromechanisms

Ana Merchán, Silvia Navarro, Anders B. Klein, Susana Aznar, Leticia Campa, Cristina Suñol, Margarita Moreno and Pilar Flores

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ORIGINAL INVESTIGATION

Tryptophan depletion affects compulsive behaviour in rats: strain dependent effects and associated neuromechanisms

A. Merchán[1] · S. V. Navarro[1] · A. B. Klein[2] · S. Aznar[3] · L. Campa[4] · C. Suñol[4] · M. Moreno[1] · P. Flores[1]

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Abstract
Rationale Compulsive behaviour, present in different psychiatric disorders, such as obsessive-compulsive disorder, schizophrenia and drug abuse, is associated with altered levels of monoamines, particularly serotonin (5-hydroxytryptamine) and its receptor system.
Objectives The present study investigated whether 5-HT manipulation, through a tryptophan (TRP) depletion by diet in Wistar and Lister Hooded rats, modulates compulsive drinking in schedule-induced polydipsia (SIP) and locomotor activity in the open-field test. The levels of dopamine, noradrenaline, serotonin and its metabolite were evaluated, as well as the 5-HT_{2A} and 5-HT_{1A} receptor binding, in different brain regions.
Methods Wistar rats were selected as high (HD) or low (LD) drinkers according to their SIP behaviour, while Lister hooded rats did not show SIP acquisition. Both strains were fed for 14 days with either a TRP-free diet (T−) or a TRP-supplemented diet (T+)
Results The TRP depletion diet effectively reduced 5-HT levels in the frontal cortex, amygdala and hippocampus in both strains of rats. The TRP-depleted HD Wistar rats were more sensitive to 5-HT manipulation, exhibiting more licks on SIP than did the non-depleted HD Wistar rats, while the LD Wistar and the Lister Hooded rats did not exhibit differences in SIP. In contrast, the TRP-depleted Lister Hooded rats increased locomotor activity compared to the non-depleted rats, while no differences were found in the Wistar rats. Serotonin 2A receptor binding in the striatum was significantly reduced in the TRP-depleted HD Wistar rats.
Conclusions These results suggest that alterations of the serotonergic system could be involved in compulsive behaviour in vulnerable populations.

Keywords Compulsivity · Inhibitory control · Chronic tryptophan depletion · Schedule-induced polydipsia · Monoamines · 5-HT_{2A} binding

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Introduction

Compulsivity represents the performance of repetitive and functionally impairing overt or covert behaviours without adaptive function, performed in a habitual or stereotyped fashion, either according to rigid rules or as a means of avoiding perceived negative consequences (Fineberg et al. 2014). Neuropsychiatric disorders characterized by compulsivity are included in the newly created Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5) "obsessive-compulsive and related disorders" (OCRDs) cluster, such as obsessive-compulsive disorder (OCD), body dysmorphic disorder, trichotillomania (repetitive hair pulling), hoarding disorder and excoriation (skin-picking) (American Psychiatric Association 2013). Moreover, this behaviour is also present across different disorders, such as schizophrenia, attention-deficit hyperactivity disorder (ADHD), pathological gambling, eating disorders, depression or substance addiction (Skodol and Oldham 1996).

Dysfunctions in cortico-limbic-striatal circuits, involving areas such as mOFC, caudate-putamen, amygdala and hippocampus, have been associated with the symptomatology in OCD (Gillan and Robbins 2014; Rădulescu and Marra 2016).

Evidence from animal and human studies implicates the serotonin 5-hydroxytryptamine (5-HT) system in impulsivity and compulsivity (Eagle and Baunez 2010; Fineberg et al. 2010). Mice lacking the gene encoding brain tryptophan hydroxylase 2 (Tph2−/−), the initial and rate-limiting enzyme in 5-HT synthesis, showed intense impulsive and compulsive behaviours to include extreme aggression (Angoa-Pérez et al. 2012). Moreover, studies on 5-HT depletion by excitotoxic lesions in rats have revealed an increase of perseverative responding in the five-choice serial reaction time task (Winstanley et al. 2004), impairment of behavioural flexibility measured through the reversal learning task (Bari et al. 2010; Lapiz-Bluhm et al. 2009; Wallace et al. 2014) and an increment of compulsive cocaine seeking under punishment (Pelloux et al. 2012).

A non-invasive and more naturalistic method to reduce central 5-HT is through nutritional depletion of the 5-HT precursor tryptophan (TRP). Under normal physiological conditions, the biosynthesis of 5-HT is limited by the availability of the essential amino acid TRP (Fernstrom 1983; Gessa et al. 1974). Rats receiving a TRP-free diet reduced the 5-HT synthesis, content (Gessa et al. 1974) and release (Stancampiano et al. 1997a, b). While acute tryptophan depletion by diet (ATD) produced a moderate serotonergic reduction in adult rats (Brown et al. 1998; Lieben et al. 2004), chronic tryptophan depletions (CTD) have shown stronger effects, reducing 5-HT brain levels to 35–40% at 14 days (Fadda et al. 2000) and to 75% at 5-week exposures (Vergnes and Kempf 1981). Moreover, long-term TRP-depleting diets lead to changes in serotonergic receptors in animals, increasing serotonin 5-HT_{2A} receptor density but having no effect on serotonin 5-HT_{1A} receptor (Cahir et al. 2007; Franklin et al. 1999). Behavioural studies in rodents have demonstrated that a TRP depletion by diet increased aggressiveness (Vergnes and Kempf 1981), locomotor activity (Vergnes and Kempf 1981) and sexual behaviour (Fratta et al. 1977). The increase of these behaviours may suggest a lack of inhibitory control leading to compulsive behaviour. However, only one study has evaluated the effect of acute TRP depletion on some facets of compulsivity, showing no effects on behavioural flexibility in reversal learning or in an extinction paradigm (Van der Plasse and Feenstra 2008). Thus, the effects of chronic TRP depletion by diet on compulsivity remain unknown.

Because of its characteristics of "excessiveness" and "persistence", schedule-induced polydipsia (SIP) is a useful model to study neuropsychiatric disorders characterized by the presence of compulsive behaviour (Ford 2014; Gilpin et al. 2008; Hawken et al. 2011; Hawken and Beninger 2014; for review, see Moreno and Flores 2012). The SIP model is characterized by the development of excessive drinking in food-deprived animals exposed to intermittent food reinforcement schedules (Falk 1961, 1971). Important differences between individuals in the amount of fluid intake and licks support the differentiation of two phenotypes of rats, one with high or excessive drinking (HD) and a second group with low or not SIP acquisition (LD) (López-Grancha et al. 2008). Recent data have shown that HD animals present increased levels of 5-HT and metabolites in the amygdala compared to the LD group (Moreno et al. 2012). In addiction, the systemic administration of citalopram and the serotonin 5-$HT_{2A/C}$ receptor agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) reduced dose-dependent compulsive drinking in HD rats, without affecting the drinking behaviour of LD rats (Navarro et al. 2015). All those data point out the involvement of 5-HT system in the vulnerability to the development of compulsive drinking between HD and LD rats.

In our laboratory, Wistar rats are commonly used in SIP, showing individual differences between HD and LD phenotypes. However, no previous studies have tested the strain-dependent differences between Wistar and Lister Hooded rats in SIP acquisition. In fact, behavioural differences in inhibitory control have been observed between these two strains of rats. Wistar rats compared to Lister Hooded rats show more anticipatory responses in a three-choice serial reaction time task and more food hoarding behaviour (Broersen and Uylings 1999), indicating a poorer inhibitory control of this strain and a higher vulnerability to develop compulsive drinking on SIP.

We hypothesised that a reduction of 5-HT through a chronic TRP depletion by diet will increase the compulsive behaviour in vulnerable populations such as the HD Wistar rats compared to non-vulnerable populations such as LD Wistar rats and Lister Hooded rats and that could be accompanied by changes in the serotonin 5-HT_{2A} receptor, a serotonin receptor subtype proposed as a candidate for mediating compulsive behaviour (Aznar and Hervig 2016; Aznar and Klein 2013; Fineberg et al. 2010, 2011; Navarro et al. 2015). To test the previous hypothesis, we screened high and low drinking rates during SIP in both strains. Next, we produced a dysfunction of the 5-HT system in the brain by a chronic TRP depletion by diet and evaluated possible motor disruptions or hyperactivity in an open-field test and the effect on compulsive drinking on SIP. Then, brain monoamine levels and serotonin 5-HT_{2A} and 5-HT_{1A} receptor densities were measured in different brain regions of the cortico-limbic-striatal circuits associated with compulsivity.

Methods

Subjects

Two strains of rats were used for this experiment: adult male Lister Hooded rats from Charles River (Barcelona, Spain) and adult male Wistar rats from Harlan Iberica (Barcelona, Spain).

Both strains of rats weighed approximately 300–400 g at the beginning of the experiment. The animals were housed three/cage or two/cage (57 × 35 × 20 cm) at 22 °C with 08:00–20:00-h light-dark cycle, with food and water available ad libitum. Before the SIP training and after 10 days of habituation to the vivarium conditions, the rats were weighed and handled daily. They were gradually reduced to 80–85% of their free-feeding body weight by controlled feeding and then maintained at this level of deprivation throughout the experiment. Food was made available by daily feeding of lab chow approximately 30 min after each experimental session. Water was always available in the home cages.

Rats were assigned to each experimental group taking into consideration the amount of water consumed in the previous experimental SIP, in order to match all groups. The Wistar rats were split as following: high drinkers receiving a TRP-free diet (n = 7), high drinkers receiving a control diet (n = 7), low drinkers with a TRP-free diet (n = 7) and low drinkers with a control diet (n = 7). The Lister Hooded rats, as they did not show differences in SIP (water intake and licks), were divided into two groups depending on the diet: One group (n = 8) received a TRP-free diet, while the other group (n = 9) received a control diet. Once the animals started the specific diets, they were housed in cages individually (50 × 25 × 18 cm) to control body weight.

All procedures were conducted in accordance with the Spanish Royal Decree 53/2013 on the protection of experimental animals, with the European Community Council Directives (86/609/EEC) and with the University of Almería Animal Research Committee approval.

Experimental design

The experiment was developed in two phases. One previous phase consisted of screening the acquisition of SIP in the Lister Hooded and Wistar rats. Once the rats were identified as high drinkers or low drinkers by their SIP behaviour, they were divided into groups depending on the given diet. After 14 days of TRP depletion by diet, the rats were exposed to different behavioural tasks. The order of presentation was as follows: one session of the open-field test and six sessions of SIP (see Fig. 1 for the entire experimental design).

Apparatus and behavioural procedures

Schedule-induced polydipsia

We conducted the tests in ten standard operant conditioning chambers (MED Associates) that were 32 cm long × 25 cm wide × 34 cm high, with stainless steel grid floors. A detailed description of the apparatus has been provided previously for the SIP (López-Grancha et al. 2008; Moreno et al. 2012). The scheduling and recording of experimental events were controlled by a Med PC computer and commercial software (Cibertec SA, Spain).

Baseline consumption All rats were individually housed in plastic cages containing a dish with the same amount of food to be delivered in the experimental sessions and the same water bottle used in the operant chambers. Over two successive days, 60 food pellets were placed together in a dish, and the amount of water consumed by each rat in 60 min was measured.

Magazine habituation The day after the first baseline consumption sessions, rats were habituated to the test chambers for 60 min and were given 30 food pellets placed in the food magazine.

Schedule-induced polydipsia pre-treatment After the magazine habituation, the animals were exposed to a fixed time 60-s (FT-60s) schedule of food pellet presentation during 60-min sessions. Water bottles containing 100 ml of fresh water were provided immediately before each session. After 20 daily sessions, the average water drinking for each animal was calculated based on the last three SIP sessions. Following Moreno et al.'s protocol (2012), rats are classified as high drinkers (HD) and low drinkers (LD) if their average water intake was above or below the group median, respectively. The following measures were recorded for each rat (a) total number of licks, (b) total amount of water (ml) removed from the bottle, (c) total number of magazine entries and (d) licking efficiency, which was calculated as the total number of licks/ by the total solution consumed. Lick efficiency detects possible fine motor impairments or changes in the stereotypic manner of licking, which indicates with higher score values that the animal needs more total number of licks to obtain the same amount of target solution (Escher and Mittleman 2006).

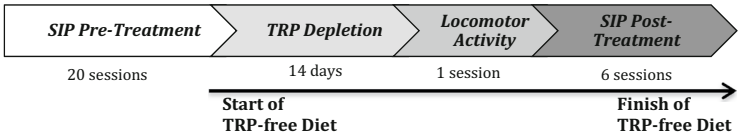

Fig. 1 Experimental procedure illustrated in a timetable

Schedule-induced polydipsia post-treatment After 14 days of the TRP depletion diet, the animals were exposed again to a FT-60s schedule of food pellet presentation during 60-min sessions. Water bottles with fresh water were available.

Spontaneous locomotor activity

The test was an open-field test, performed in eight Plexiglas activity cages (measuring 39 × 39 × 15 cm) equipped with photocell beams (16 × 16 × 16) interfaced to a microcomputer VersaMax Animal Activity Monitoring System (AccuScan Instruments Inc., USA). Spontaneous locomotor activity was measured as the number of photocell beam breaks due to the movements of the animals. TRP-depleted and TRP-non-depleted rats were tested for their locomotor responses to a novel environment in the activity cages. Rats were not habituated to the activity cages prior to this test. Spontaneous locomotor behaviour was quantified in 5-min blocks over a 60-min period following placement into the test cage. We measured total distance, counted as the number of centimetres travelled by the animal (an indicator of ambulatory activity).

Tryptophan depletion diet

The TRP-free diet (TD08126, Harlan Laboratories S.A., Barcelona, Spain) had a standard nutritional value, but with a complete lack of TRP. The control groups were fed a similar diet, containing a standard amount of TRP (1.8 g/kg diet) (TD99366, Harlan Laboratories S.A., Barcelona Spain). A chronic TRP-free diet exposure of 14 days was given before the behavioural tasks as previous studies have established (Bortolato et al. 2008; Franklin et al. 2012; Stancampiano et al. 2013), and the amount of food was controlled in order to maintain the body weight at 80–85% of their free-feeding body weight.

Brain analyses

The day after the SIP post-treatment, the rats were rapidly decapitated using a guillotine. Brains were quickly removed, frozen and stored at −80 °C. The cerebral hemispheres were separated, and each half was used either for measuring monoamines or for measuring serotonin receptor binding. The hemispheres were counterbalanced.

Brain monoamine analyses

For brain tissue preparation, the samples were thawed sufficiently to enable dissection of the prefrontal cortex, striatum, nucleus accumbens, hippocampus and amygdala (Moreno et al. 2012). These were weighed and homogenized in 0.4 N perchloric acid with 0.1 metabisulfite, 0.01% EDTA and 1 ng/ml cysteine. The homogenates were centrifuged at 15,000 rpm for 20 min at 4 °C, and supernatants were collected and frozen at −80 °C until biochemical analyses for determining the levels of norepinephrine (NE), dopamine (DA), serotonin (5-HT) and 5-hydroxy-3-indolacetic acid (5-HIAA), which were measured using reverse-phase high-performance liquid chromatography with electrochemical detection (+0.7 V). The mobile phase, containing 100 mM KH_2PO_4,0.1 mM Na_2-EDTA, 2.06 mM PICB8 and 16% methanol, adjusted to pH 2.65 with orthophosphoric acid, was delivered at 1 ml/ min. Monoamines were separated on a 5-mm particle size column at 30 °C (Phenomenex C25 10 × 0.46 cm, Micron Analitica SA, Spain).

Autoradiography

To determine 5-HT_{1A}R and 5-HT_{2A}R binding in the Wistar and Lister Hooded rats, their brains were cut in 10-μm sections, mounted on Super Frost slides and stored at −80 °C. The protocol was modified from Klein et al. (2014). The 5-HT_{2A} autoradiography protocol was performed using ^{3}H-MDL100907 [R(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorphenyl)-ethyl]-4-piperidin-methanol] (specific activity, 2.8 TBq/mmol, Novandi Chemistry, Sweden; and non-specific binding was determined using 10 μM ketanserin tartrate (3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]-ethyl]-2,4[1H,3H] quinazolinedione tartrate) (Tocris Cookson Ltd., Bristol, UK). For 5-HT_{1A} autoradiography, we used ^{3}H-WAY100635 (specific activity, 2.9 TBq/mmol, Novandi Chemistry) and measured non-specific binding with 10 μM 5-HT (Sigma–Aldrich, Copenhagen, Denmark). Briefly, the sections were allowed to thaw for 1 h at room temperature (RT) and then pre-incubated with 50 mM Tris–HCl (Sigma), pH 7.4, containing 0.01% ascorbic acid (Sigma) and 10 μM pargyline hydrochloride (*N*-methyl-*N*-2-propynylbenzylamine hydrochloride) (Research Biochemicals International, MA, USA) for 30 min at RT under constant gentle shaking. Sections were then incubated for 60 min at RT using the same buffer containing 2 nM of ^{3}H-MDL100907 (1.5 nM of ^{3}H-WAY100635 for 5-HT_{1A} binding). Following incubation, the slides were washed 2 × 2 min in ice-cold 50 mM Tris–HCl, pH 7.4, and 1 × 20 s in ice-cold dH_2O and dried for 1 h under a gentle stream of air.

All sections were placed at 4 °C in a fixator containing paraformaldehyde vapour and later placed in an exicator box for 3 h before the slides, and the ^{3}H-microscales (GE Healthcare, UK) were exposed to a BAS-TR2040 Imaging Plate (Science Imaging Scandinavia AB, Nacka, Sweden) for 3–14 days at 4 °C. Finally, the imaging plate was scanned on a CR-35 scanner (Raytest, Straubenhardt, Germany) and specific and non-specific binding was determined in the frontal cortex, striatum and hippocampus using the AIDA 5.0 software (Raytest) and expressed as femtomole per milligramme tissue equivalents (TE).

Data analyses

Data analyses of the different strains were performed separately, due to the complexity of the groups. Analyses of variance (ANOVAs) were performed with two between-subject factors for the Wistar strain data, group (HD and LD) and treatment (T+ and T−) and one between-subject factor for the Lister Hooded strain data (treatment, T+ and T−). The within-subject factors were sessions of SIP, bins on the locomotor activity, body weight, brain monoamine data and receptor binding. Lick efficiency was analysed by repeated measures ANOVA, with treatment as between-subject and sessions as within-subject factors, in HD and LD rats. When appropriate, post hoc comparisons were made using the Newman-Keuls test. The significance level was set at $p \leq 0.05$. All statistics were two-tailed.

Results

Body weight measure

Figure 2 shows body weight during 14 days of TRP depletion by diet and 6 days of SIP post-treatment in Wistar and Lister Hooded rats. No differences in body weight between T+ and T − groups were found neither in Wistar (treatment effect $F_{1,24} = 0.09$; $p = 0.761$) nor in Lister Hooded rats (treatment effect $F_{1,15} = 0.496$; $p = 0.492$).

Schedule-induced polydipsia pre-treatment

Figure 3 shows the mean total licks, water intake and total magazine entries in high-drinker Wistar rats (WHD), low-drinker Wistar rats (WLD) and Lister Hooded (LH) rats on the SIP pre-treatment FT-60s schedule of food presentation. ANOVA revealed significant differences on SIP acquisition between WHD and WLD in total licks (group effect $F_{1,26} = 71.5$; $p < 0.001$) and water intake (group effect $F_{1,26} = 44.18$; $p < 0.001$). SIP session effects were significant in both measures: total licks ($F_{19,494} = 23.6$; $p < 0.001$) and water intake ($F_{19,494} = 13.03$; $p < 0.001$). Interaction between sessions and group was also significant in total licks ($F_{19,494} = 15.5$; $p < 0.001$) and water intake ($F_{19,494} = 9.24$; $p < 0.001$). Post hoc analysis indicated that the FT-60s schedule of food delivery induced different drinking rates across the 20 test sessions in both groups. Differences in total licks between WHD and WLD animals were evident from session 3 ($p = 0.031$) and from session 3 in water intake ($p = 0.039$). WHD animals significantly increased their consumption of water from session 3 ($p = 0.002$) to the end of training, reaching stable levels from session 10. WLD animals did not show a significant increase in their consumption of water across SIP sessions. No interaction effect was found in magazine entries ($F_{19,494} = 0.56$; $p = 0.933$) (Fig. 3e).

No significant differences were found between LH and WLD rats in water intake (strain effect $F_{1,29} = 1.76$; $p = 0.195$), total licks (strain effect $F_{1,29} = 2.26$; $p = 0.143$) and magazine entries (strain effect $F_{1,29} = 1.56$; $p = 0.696$).

Schedule-induced polydipsia post-treatment

Figure 3 shows the effects of the chronic TRP depletion by diet on Wistar and LH rats on SIP. The TRP depletion in WHD T− rats increased the total number of licks over the days (see Fig. 3b; group × treatment × session effect $F_{5,120} = 2.46$; $p = 0.037$) but did not affect water intake on SIP (see Fig. 3d; group × treatment × session effect $F_{5,120} = 1.06$; $p = 0.387$). Post hoc analysis indicated that the differences on licks between WHD T+ and WHD T− occur from session 3 ($p = 0.007$). WHD T− animals significantly increased their licks rate from session 3 ($p = 0.021$). Differences between WHD and WLD rats remained stable in water intake (group effect $F_{1,24} = 55.33$; $p < 0.001$) and total licks (group effect $F_{1,24} = 32.44$; $p < 0.001$). An increase of total licks was

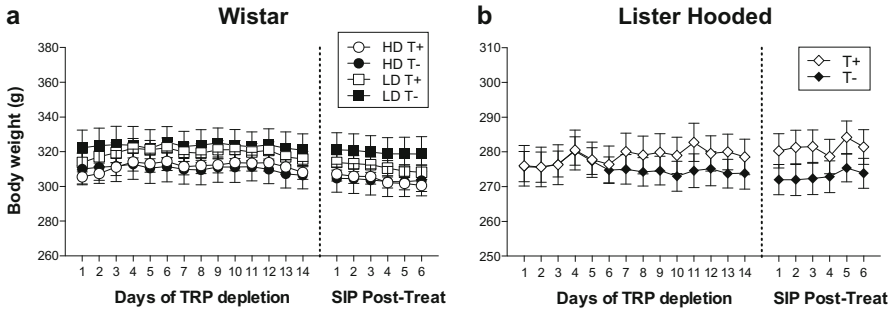

Fig. 2 Body weight during 14 days of TRP depletion by diet and six sessions of schedule-induced polydipsia post-treatment for Wistar (**a**) and Lister Hooded rats (**b**). Wistar rats are grouped as TRP non-depleted high drinkers (HD T+), TRP-depleted high drinkers (HD T−), TRP non-depleted low drinkers (LD T+) and TRP-depleted low drinkers (LD T −). Lister Hooded rats are grouped as TRP non-depleted (T+) and TRP-depleted rats (T−). Data are means ± SEM

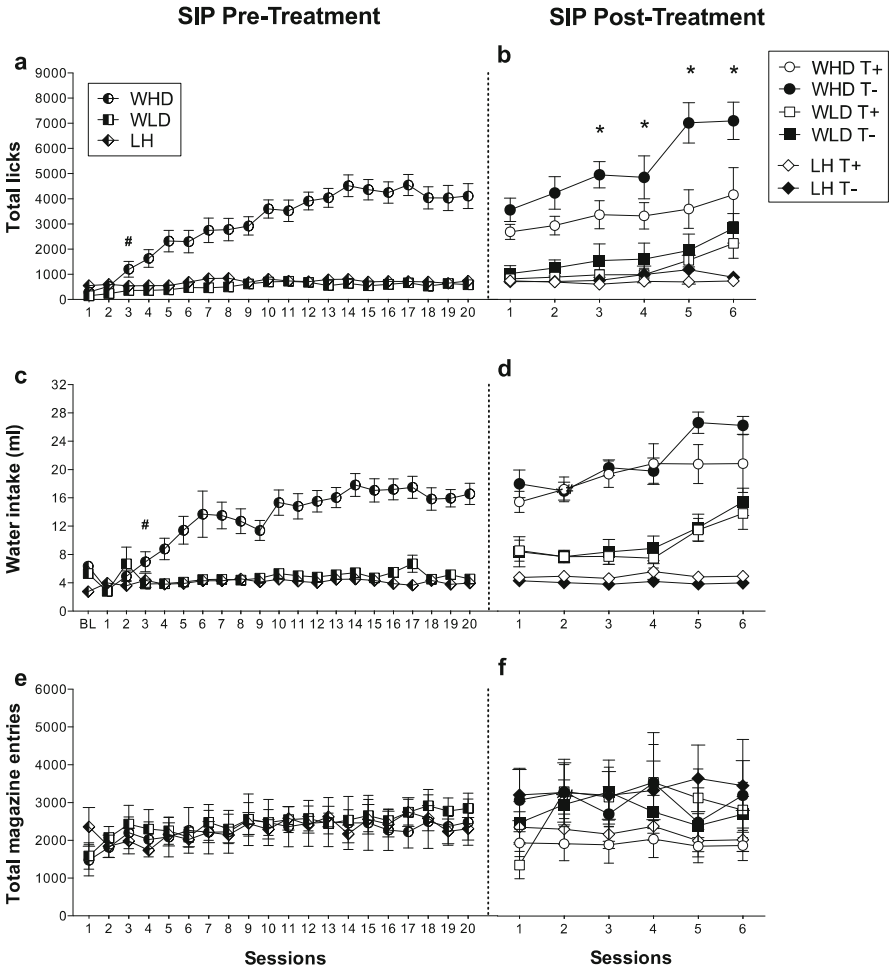

Fig. 3 The mean (±SEM) total licks (**a**, **b**), water intake (**c**, **d**) and total magazine entries (**e**, **f**) in FT-60s across 20 sessions of SIP pre-treatment and six sessions of SIP post-treatment for both Wistar and Lister Hooded rats. Rats are grouped in the SIP pre-treatment as high-drinker Wistar rats (WHD), low-drinker Wistar rats (WLD) and Lister Hooded rats (LH). Wistar rats are grouped in the SIP post-treatment as TRP non-depleted high drinkers (WHD T+), TRP-depleted high drinkers (WHD T−), TRP non-depleted low drinkers (WLD T+) and TRP-depleted low drinkers (WLD T−). Lister Hooded rats are grouped in the SIP post-treatment as TRP non-depleted (LH T+) and TRP-depleted rats (LH T−). *Asterisks*: statistical analyses indicate significant differences between HD T+ and HD T−. *Number sign*: statistical analyses indicate significant differences between WHD and WLD from that session onward

observed in WHD T+ ($p = 0.020$) and WLD T− ($p = 0.002$) on session 6, but these groups remain statistically different from each other ($p = 0.040$). To understand the discrepancy of finding increments in total licks not observed in water intake, we explored licking efficiency in WHD and WLD rats during the six sessions of SIP. Interestingly, we found statistical increases of licking efficiency in WHD T− compared to WHD T+ (treatment × session $F_{5,60} = 3.283$; $p = 0.011$), but no differences were found in WLD T− compared to WLD T+ (treatment × session $F_{5,60} = 0.205$; $p = 0.959$) (data not shown). Post hoc analysis revealed increments of licking efficiency in WHD T− compared to WHD T+ from session 2 onwards ($p < 0.001$).

LH T+ and LH T− did not differ in water intake (Fig. 3d; treatment × session effect $F_{5,75} = 0.353$; $p = 0.879$), total number of licks (Fig. 3b; treatment × session effect $F_{5,75} = 1.013$; $p = 0.416$) and licking efficiency (treatment × session $F_{5,75} = 0.790$; $p = 0.560$) (data not shown). TRP depletion by diet did not alter total number of magazine entries neither in the Wistar rats (group × treatment × session: $F_{5,120} = 1.02$; $p = 0.410$) nor in the LH rats (group × treatment × session: $F_{5,75} = 2.076$; $p = 0.078$) (Fig. 3f).

Spontaneous locomotor activity

Figure 4 shows locomotor response measured as total distance (cm) in four blocks of 15 min for Wistar and LH rats. TRP depletion by diet increased locomotor response only in the LH rats (treatment × blocks $F_{3,45} = 3.08$; $p = 0.037$). Post hoc analyses revealed that LH T− showed an increased locomotor activity in the first 15 min ($p = 0.015$) and the second 15 min ($p = 0.008$) of the 60-min session compared to LH T+ (Fig. 4b). No effects of the TRP depletion by diet were found in locomotor response in the Wistar rats (treatment × blocks $F_{3,72} = 1.30$; $p = 0.280$), not even considering groups of HD and LD rats (see Fig. 4a; group × treatment × blocks $F_{3,72} = 0.78$; $p = 0.508$). Wistar ($F_{3,72} = 79.91$; $p < 0.001$) and LH rats ($F_{3,45} = 81.16$; $p < 0.001$) decreased the activity over the session significantly.

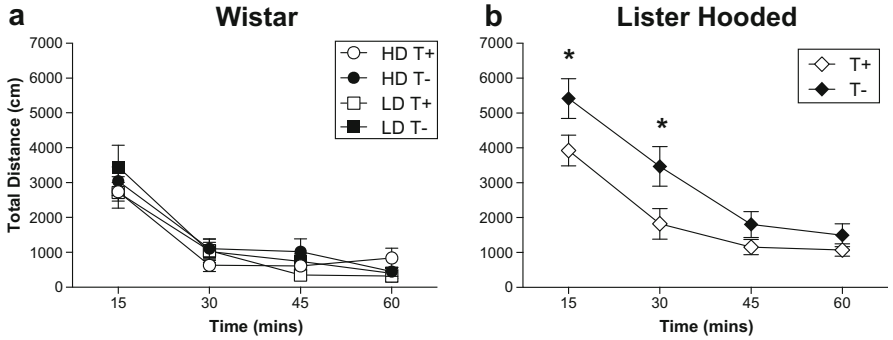

Fig. 4 Total distance in four blocks of 15 min for Wistar (**a**) and Lister Hooded rats (**b**). Wistar rats are grouped as TRP non-depleted high drinkers (HD T+), TRP-depleted high drinkers (HD T−), TRP non-depleted low drinkers (LD T+) and TRP-depleted low drinkers (LD T−). Lister Hooded rats are grouped as TRP non-depleted (T+) and TRP-depleted rats (T−). Data are means ± SEM. *Asterisks*: statistical analyses indicate significant differences between T+ and T−

Serotonin receptor binding

Table 1 shows mean ± SEM ^{3}H-MDL100907 and ^{3}H-WAY100635 binding for groups of depleted and non-depleted Wistar and LH groups of rats. For the 5-HT$_{2A}$ receptor in Wistar rats, there was a group × treatment interaction in the striatum for ^{3}H-MDL100907 binding ($F_{1,23}$ = 8.648; p = 0.007) (see Fig. 5a). Post hoc analyses revealed a reduction of 5-HT$_{2A}$ receptor density in WHD T− rats compared to WHD T+ rats (p = 0.014). In the frontal cortex, we observed a 10% reduction of 5-HT$_{2A}$ binding in the HD T− compared to HD T+ rats; however, the statistical analysis did not detect statistical differences (group × treatment $F_{1,24}$ = 0.990; p = 0.330). TRP depletion by diet did not alter 5-HT$_{2A}$ levels of LH T− compared to LH T+ neither in the frontal cortex ($F_{1,16}$ = 0.117; p = 0.737) nor in the striatum ($F_{1,16}$ = 0.066; p = 0.801).

The 5-HT$_{1A}$ receptor density in the frontal cortex showed a reduction in LH T− rats compared to LH T+ in terms of ^{3}H-WAY100635 binding ($F_{1,16}$ = 19.091; p = 0.001) (see Fig. 5b), but no differences in density were found in the striatum ($F_{1,16}$ = 1.80; p = 0.677) and the hippocampus ($F_{1,16}$ = 2.157; p = 0.163). The effect of TRP depletion by diet in the 5-HT$_{1A}$ receptor density observed in LH T− did not occur in the depleted Wistar rats (group × treatment $F_{1,24}$ = 0.240; p = 0.629). Also, 5-HT$_{1A}$ density was unaffected in the striatum (group × treatment $F_{1,23}$ = 0.138; p = 0.714) and hippocampus (group × treatment $F_{1,25}$ = 1.672; p = 0.209) in the depleted Wistar rats.

Monoamine concentration levels

The chronic TRP depletion by diet significantly reduced 5-HT, 5-HIAA and 5-HIAA/5-HT turnover ratio in the prefrontal cortex (PFC), amygdala and hippocampus in both strains of rats. No interaction effects of group and treatment were found in monoamine concentration levels in the different areas for Wistar rats. In the depleted groups of Wistar rats, 5-HT levels

Table 1 ^{3}H-MDL100907 and ^{3}H-WAY100635 binding (fmol/mg TE) in the frontal cortex, striatum and hippocampus in all groups of Wistar and Lister Hooded rats

		Wistar				Lister Hooded	
		HD		LD			
		T+	T−	T+	T−	T+	T−
FC	5-HT$_{2A}$	73.16 ± 4.68	65.44 ± 2.87	73.90 ± 4.86	76.39 ± 7.17	102.70 ± 4.59	105.7 ± 7.85
	5-HT$_{1A}$	27.13 ± 0.95	29.07 ± 1.84	26.57 ± 2.07	26.49 ± 2.90	45.44 ± 1.57	*37.07 ± 1.00***
Striat	5-HT$_{2A}$	19.13 ± 0.75	*16.41 ± 0.49**	16.91 ± 0.73	18.34 ± 0.80	25.51 ± 0.80	25.23 ± 0.70
	5-HT$_{1A}$	10.48 ± 0.33	10.38 ± 0.41	10.03 ± 0.30	9.68 ± 0.28	14.75 ± 0.26	14.41 ± 0.79
Hippo	5-HT$_{1A}$	121.23 ± 4.02	116.6 ± 3.88	111.43 ± 7.42	117.54 ± 8.69	110.73 ± 7.29	96.36 ± 6.33

Data are mean ± SEM. Significant differences between T+ and T− (*p < 0.05, **p < 0.01)

FC frontal cortex, *Striat* striatum, *Hippo* hippocampus

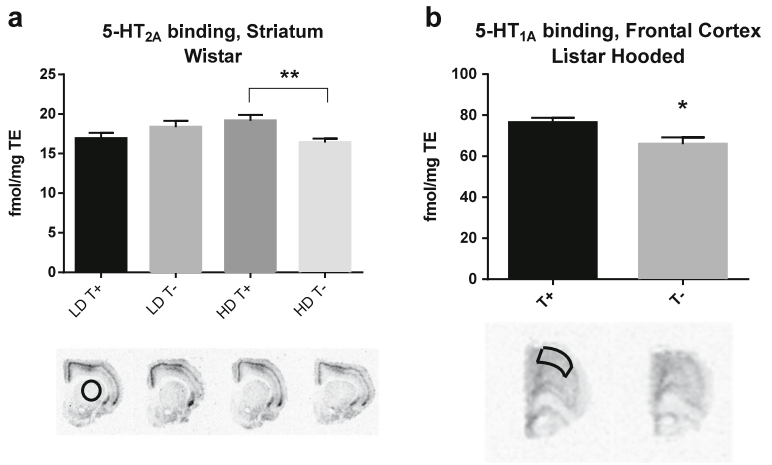

Fig. 5 The mean (±SEM) 5-HT_{2A} receptor binding of striatum slices in the Wistar rats (**a**) and 5-HT_{1A} receptor binding of frontal cortex slices in the Lister Hooded rats (**b**). 5-HT_{2A} receptor binding was detected by [^{3}H]MDL100907, and 5-HT_{1A} binding was detected by [^{3}H]WAY100635. Wistar rats are grouped as TRP non-depleted high drinkers (HD T+), TRP-depleted high drinkers (HD T−), TRP non-depleted low drinkers (LD T+), and TRP-depleted low drinkers (LD T−). Lister Hooded rats are grouped as TRP non-depleted (T+) and TRP-depleted rats (T−). *Asterisks*: statistical analyses indicate significant differences between T+ and T− (*$p < 0.05$, **$p < 0.01$)

were reduced in the PFC ($F_{1,23} = 20.86$; $p < 0.001$) and hippocampus ($F_{1,24} = 5.89$; $p < 0.023$) (see Table 2), and there was a tendency toward significance in the amygdala ($F_{1,24} = 3.92$; $p = 0.059$). 5-HIAA levels were decreased in the PFC ($F_{1,23} = 29.52$; $p < 0.001$), striatum ($F_{1,22} = 4.79$; $p = 0.040$), amygdala ($F_{1,24} = 29.79$; $p < 0.001$) and hippocampus ($F_{1,24} = 19.837$; $p < 0.001$). In addition, a decreased 5-HIAA/5-HT turnover ratio in all areas was found: PFC ($F_{1,23} = 16.13$; $p < 0.001$), striatum ($F_{1,22} = 51.90$; $p < 0.001$), amygdala ($F_{1,24} = 52.97$; $p < 0.001$), nucleus accumbens ($F_{1,19} = 19.67$; $p < 0.001$) and hippocampus ($F_{1,24} = 64.45$; $p < 0.001$). No significant changes in levels of 5-HT and 5-HIAA were observed in the nucleus accumbens. NE and DA were not significantly affected in any brain regions.

In the depleted group of LH rats, 5-HT levels were reduced in the PFC ($F_{1,15} = 33.43$; $p < 0.001$), striatum ($F_{1,14} = 16.82$; $p < 0.001$), amygdala ($F_{1,14} = 6.63$; $p = 0.022$) and hippocampus ($F_{1,15} = 9.10$; $p = 0.009$) (see Table 3). 5-HIAA levels and 5-HIAA/5-HT turnover ratio decreased in the PFC ($F_{1,13} = 38.89$; $p < 0.001$; $F_{1,13} = 10.25$; $p = 0.007$), striatum ($F_{1,14} = 21.82$; $p < 0.001$; $F_{1,14} = 14.45$; $p = 0.002$), amygdala ($F_{1,14} = 31.85$; $p < 0.001$; $F_{1,14} = 40.83$; $p < 0.001$), nucleus accumbens ($F_{1,11} = 17.65$; $p < 0.001$; $F_{1,11} = 20.56$; $p < 0.001$) and hippocampus ($F_{1,15} = 32.38$; $p < 0.001$; $F_{1,15} = 56.49$; $p < 0.001$). Besides, there was a compensatory increase in DA in nucleus accumbens ($F_{1,11} = 8.28$; $p = 0.015$) and a decrease in NE in PFC ($F_{1,15} = 6.42$; $p = 0.023$); DA and NE were not affected in other areas.

Table 2 Monoamine concentration levels (picomole/milligramme of tissue) in the prefrontal cortex, striatum, amygdala, nucleus accumbens and hippocampus in T+ and T− for Wistar rats ($n = 11–14$)

		5-HT	5-HIAA	5-HIAA/5-HT ratio	NE	DA
PFC	T+	1.67 ± 0.15	1.16 ± 0.14	0.70 ± 0.06	1.86 ± 0.14	1.68 ± 0.46
	T−	*0.86 ± 0.09***	*0.36 ± 0.03***	*0.44 ± 0.03***	1.72 ± 0.13	2.87 ± 0.77
Striat	T+	3.56 ± 0.81	4.13 ± 0.88	1.17 ± 0.07	0.14 ± 0.03	130.64 ± 27.47
	T−	3.08 ± 0.47	*2.08 ± 0.37**	*0.65 ± 0.03***	0.22 ± 0.08	146.89 ± 23.26
Amyg	T+	2.93 ± 0.32	2.36 ± 0.22	0.83 ± 0.04	1.10 ± 0.21	18.89 ± 3.57
	T−	*2.16 ± 0.21*$^{\#}$	*1.01 ± 0.09***	*0.48 ± 0.02***	1.47 ± 0.25	20.23 ± 2.83
NAc	T+	2.78 ± 0.75	2.50 ± 0.69	0.90 ± 0.02	2.50 ± 0.55	41.04 ± 13.00
	T−	2.61 ± 1.22	1.45 ± 0.59	*0.62 ± 0.06***	5.73 ± 2.58	44.67 ± 20.58
Hippo	T+	1.23 ± 0.15	1.70 ± 0.22	1.38 ± 0.03	1.97 ± 0.20	1.11 ± 0.19
	T−	*0.78 ± 0.10**	*0.67 ± 0.05***	*0.91 ± 0.04***	1.56 ± 0.11	1.72 ± 0.25

Data are mean ± SEM. Significant differences between T+ and T− (*$p < 0.05$, **$p < 0.01$, $^{\#}p = 0.059$)

PFC prefrontal cortex, *NAc* nucleus accumbens, *Amyg* amygdala, *Striat* striatum, *Hippo* hippocampus

Table 3 Monoamine concentration levels (picomole/milligramme of tissue) in the prefrontal cortex, striatum, amygdala, nucleus accumbens and hippocampus in T+ and T− for Lister Hooded rats (n = 4–9)

		5-HT	5-HIAA	5-HIAA/5-HT ratio	NE	DA
PFC	T+	1.77 ± 0.09	1.02 ± 0.09	0.56 ± 0.04	1.62 ± 0.09	3.89 ± 1.08
	T−	*0.99 ± 0.11***	*0.04 ± 0.07***	*0.35 ± 0.07***	*1.31 ± 0.08**	2.34 ± 0.72
Striat	T+	2.20 ± 0.27	1.98 ± 0.31	0.88 ± 0.04	0.25 ± 0.07	79.35 ± 11.61
	T−	*0.92 ± 0.15***	*0.52 ± 0.06***	*0.60 ± 0.06***	0.22 ± 0.08	83.19 ± 7.21
Amyg	T+	2.81 ± 0.32	2.12 ± 0.21	0.78 ± 0.05	1.38 ± 0.28	12.94 ± 2.56
	T−	*1.75 ± 0.21**	*0.68 ± 0.09***	*0.39 ± 0.02***	1.13 ± 0.34	20.18 ± 3.28
NAc	T+	2.74 ± 0.31	2.34 ± 0.19	0.90 ± 0.06	8.01 ± 1.98	23.14 ± 4.36
	T−	2.09 ± 0.22	*1.01 ± 0.15***	*0.48 ± 0.02***	3.40 ± 1.96	*48.99 ± 9.52**
Hippo	T+	1,45 ± 0.19	2.17 ± 0.24	1.54 ± 0.07	2.60 ± 0.23	4.95 ± 1.71
	T−	*0.79 ± 0.07***	*0.64 ± 0.07***	*0.81 ± 0.06***	3.05 ± 0.62	9.45 ± 3.46

Data are mean ± SEM. Significant differences between T+ and T− (*$p < 0.05$, **$p < 0.01$)

PFC prefrontal cortex, *NAc* nucleus accumbens, *Amyg* amygdala, *Striat* striatum, *Hippo* hippocampus

Discussion

The present study has shown the effects of chronic TRP depletion by diet in two strains of rats: Wistar and LH. Before TRP depletion by diet, we examined between-strain differences in the model of compulsive behaviour, SIP, and we found in the Wistar strain two groups of rats based on their drinking behaviour, HD and LD, while the LH strain did not show acquisition of compulsive drinking. After the chronic TRP depletion by diet, the TRP-depleted HD group of Wistar rats increased their compulsive drinking based on the total number of licks, but no changes in drinking behaviour were observed in either the LD Wistar or LH rats. Conversely, TRP depletion produced an increase in spontaneous locomotor activity only in LH rats, while the Wistar rats were unaffected. A reduction of striatal 5-HT_{2A} receptor binding was observed in depleted HD Wistar rats compared to non-depleted HD Wistar rats, while depleted LD Wistar rats were not affected by the TRP manipulation. On the contrary, depleted LH rats showed reduced binding of the 5-HT_{1A} receptor in the frontal cortex. Monoamine measures confirmed that 5-HT, 5-HIAA and the 5-HIAA/5-HT ratio were depleted in different brain regions in both Wistar and LH rats. These results will be further discussed in terms of the relationship between serotonin and inhibitory control.

Acquisition of schedule-induced polydipsia and strain differences

In the SIP procedure, the exposure of the Wistar strain to an FT-60s schedule of food delivery differentiated two populations based on the amount of drinking: high and low drinkers. The HD Wistar rats showed an increased volume of water intake and number of licks from session 3 compared to the LD Wistar rats. These results confirm previous studies in which consistent individual differences are found (for review, see Moreno and Flores 2012). However, the LH rats did not show an acquisition of SIP. This study is the first to evaluate strain differences between Wistar and LH rats in SIP acquisition and the development of SIP drinking. Regarding strain differences, LH rats compared to Wistar rats show a higher inhibitory control measured by less anticipatory responses in the 3-CSRT task and less food hoarding behaviour (Broersen and Uylings 1999). Other strains exhibiting inhibitory control deficits have shown increased SIP behaviour. For instance, spontaneously hypertensive rats, characterized as hyperactive and impulsive in terms of exacerbated sensitivity to delay of reinforcement, displayed increased drinking in SIP compared to Wistar–Kyoto rats (Ibias and Pellón 2011), as well as two rat lines selectively bred for high ethanol preference compared to their non-preferring counterparts (Gilpin et al. 2008). Moreover, the selective breeding of Roman high- (RHA) and low-avoidance (RLA) rats for rapid vs. extremely poor acquisition of active avoidance behaviour in a shuttle box resulted in two phenotypes that present differences in SIP acquisition (Moreno et al. 2010). RHA rats, which show traits such as higher novelty seeking, susceptibility to addictive drugs and impulse behaviours in the delay-discounting task and five-choice serial reaction time (5-CSRT) task (Escorihuela et al. 1999; Fattore et al. 2009; Moreno et al. 2010), also display increased SIP acquisition compared to RLA rats. Thus, SIP seems to be sensitive in distinguishing phenotypes of rats that have shown deficits in inhibitory control responses in different tasks of impulsivity/compulsivity, indicating a lack of inhibitory control as the main characteristic involved in the compulsive drinking in SIP (Moreno and Flores 2012).

Effect of chronic tryptophan-deficient diet on schedule-induced polydipsia and possible mechanisms

Chronic TRP-deficient diet exposure increased the total number of licks in the HD Wistar rats without affecting the amount of water drunk on SIP. We found similar observations in our

laboratory of an increase in total licks on SIP after 6 months of an acute chlorpyrifos (CPF) administration (Cardona et al. 2006, 2011). There is evidence that long-term CPF intoxication affects the serotonergic system (Chen et al. 2011; Moreno et al. 2008), possibly by inducing TRP hydroxylase, the rate-limiting enzyme for 5-HT biosynthesis, and suppressing expression of 5-HT transporter genes (Slotkin and Seidler 2008). Therefore, a disruption in serotonin levels may be the underlying mechanism for the increased total licks observed on SIP. On the other hand, the increase in licks is task-dependent because groups differ from session 3 onward and not from session 1. The effect of increasing the number of licks without affecting the amount of water intake, also observed by Cardona et al. (2006, 2011), may suggest a change of the drinking behaviour understood as an expression of compulsivity. In this sense, lick efficiency analyses showed differences in HD Wistar rats due to the TRP depletion by diet. This result could be interpreted as an increase in the stereotypic/compulsive manner of drinking by depleted HD rats, and this increase is not due to motor problems since depleted LD Wistar and LH rats did not differ in lick efficiency. The specific effect of the chronic TRP depletion increasing total licks and licking efficiency in HD Wistar rats but not in LD Wistar rats indicates a vulnerability of the HD group to compulsive symptoms and an implication of the serotonergic system mediating this vulnerability.

Only a few studies have tested the effect of acute TRP depletion in OCD patients, showing not significant increases of obsessions or compulsions according to the scores of the Yale Brown Obsessive Compulsive Scale at rest or following symptom provocation (Barr et al. 1994; Berney et al. 2006). However, taking into account studies using neuropsychological tasks instead of questionnaires, patients with psychopathologies from the impulsive-compulsive spectrum seem to aggravate their symptoms when exposed to ATD. For instance, ATD increased omissions in the continuous performance task (Mette et al. 2013; Zepf et al. 2010) and aggressive behaviour in ADHD patients (Kötting et al. 2013; Stadler et al. 2007; Zepf et al. 2008, 2009; Zimmermann et al. 2012). Interestingly, ATD impaired go/no-go performance (LeMarquand et al. 1999) and stop signal reaction time (Crean et al. 2002) in healthy men with family history of alcoholism and also increased commission errors in the go/no-go task in an aggressive subgroup of people with ADHD (LeMarquand et al. 1998). These findings suggest that ATD may reveal vulnerable 5-HT systems in certain populations at risk of impulse control disorders (Faulkner and Deakin 2014), though it is still unknown precisely which receptor subtypes may lay behind this vulnerability.

In our study, we found a reduction of striatal 5-HT_{2A} receptor density in TRP-depleted HD Wistar rats compared to non-depleted HD Wistar rats. No differences were obtained in the LD Wistar or LH rats. Alterations of 5-HT_{2A} receptor levels in 5-HT depletion studies are controversial. Several studies report upregulation of this receptor subtype in the hippocampus and frontal cortex (Franklin et al. 2012; Heal et al. 1985; Seeman et al. 1980), while other studies do not observe any difference (Blackshear et al. 1981; Conn and Sanders-Bush 1986; Fischette et al. 1987). In support of our findings, Licht et al. (2009) found that 5-HT_{2A} receptor binding was markedly reduced in striatum and prefrontal cortex regions after 5-HT depletion. Barlow et al. (2015) had similar findings regarding the 5-HT_{2A} receptor reductions and low levels of 5-HT in the orbitofrontal cortex in perseverative rats in the reversal learning task. They furthermore reported differences in gene expression levels of the MAO-A and MAO-B enzymes. They suggest that decreased MAO activity in the DRN resulted in reduced 5-HT breakdown and consequently increased autoinhibition of 5-HT neurons by somatodendritic 5-HT receptors (Barlow et al. 2015; Liu et al. 2005).

The specific downregulation of the striatal 5HT_{2A} receptor in HD but not LD rats by manipulation of the central 5-HT system reveals a specific role of the 5-HT_{2A} receptor system in the observed increase in compulsive drinking on SIP. Evidence from animal and human studies underlies a key role of the 5-$HT_{2A/C}$ receptors in compulsive symptoms (Fineberg et al. 2010, 2011). Activation of prefrontal 5-HT_{2A} receptors has been proposed to underpin the anticompulsive effect of SSRIs (Dannon et al. 2000; for a review, see El Mansari and Blier 2006; Westenberg et al. 2007). Second-generation antipsychotics may exacerbate compulsive behaviours in patients with schizophrenia and proposed to be through the potent 5-HT_{2A} antagonism (Poyurovsky et al. 2008). In fact, activation of the 5-$HT_{2A/C}$ by DOI reduces compulsive drinking on SIP, and this reduction is blocked by the 5-HT_{2A} receptor antagonists ketanserin and M100907, but not by the 5-HT_{2C} receptor antagonist SB242084, indicating that the 5-HT_{2A} receptor mediates the anticompulsive effect of DOI on SIP (Navarro et al. 2015). Moreover, systemic administration of M100907 in rats impairs spatial reversal learning, increasing perseverative responses (Boulougouris et al. 2008). Alterations in 5-HT_{2A} receptor levels have also been observed in Roman high-avoidance (RHA) rats (Klein et al. 2014), an inbred strain characterized by a compulsive drinking profile on SIP, impulsivity on the delay-discounting task and poor inhibitory control in the 5-CSRT task (Moreno et al. 2010). In humans, neuroimaging studies have also linked differences in 5-HT_{2A} receptor levels to the development of compulsive spectrum disorders. Positron emission tomography (PET) studies in drug-naive OCD patients reveal a reduction in frontal cortex serotonin 5-HT_{2A} receptor availability (Perani et al. 2008), with specific correlations between serotonin 5-HT_{2A} receptor availability in the orbitofrontal cortex and age of onset of the disorder (Simpson et al. 2011).

Little is known regarding the contribution of the striatal serotonin receptor subtypes to cognitive function. In the

striatum, 5-HT receptors modulate the activity of DA, GABA and glutamate neurotransmission and output regions of the basal ganglia (Nicholson and Brotchie 2002), suggesting a role of the 5-HT system in regulating action selection and motor control (Di Matteo et al. 2008). More studies are needed to evaluate the role of the striatal 5-HT_{2A} receptor in the impulsive-compulsive spectrum disorders.

Effect of chronic tryptophan-deficient diet on spontaneous locomotor activity and possible mechanisms

The diet-induced chronic TRP depletion resulted in the LH strain in an increase in spontaneous locomotor activity not observed in the Wistar strain. Contrary to our results, previous studies with Wistar rats reported increases in locomotor activity after a 5-week exposure to a TRP-deficient diet (Vergnes and Kempf 1981). Possibly, the effect of TRP depletion by diet on locomotor activity depends on the severity of 5-HT reductions, based on the observations of greater global reductions of 5-HT and its metabolite in LH but not in Wistar rats in our study. In fact, the increases of locomotor activity in Vergnes and Kempf's study (1981) were found after a period of 5-week exposure to a TRP-free diet, in which 75% of reductions in brain 5-HT levels were observed. However, we have carried out an exposure of 2 weeks that is similar to the chronic TRP treatment in Fadda et al.'s study (2000), in which 35–45% of 5-HT reductions were found. Moreover, central 5-HT depletion by administration of 5,7-dihydroxytryptamine, an invasive neurotoxic method that drastically reduces 5-HT levels, reported an increase in locomotor activity in LH rats (Eagle et al. 2008). On the other hand, our results confirm previous observations of no differences in locomotor activity between the HD and LD Wistar rats (Moreno et al. 2010).

Besides this effect, depleted LH rats showed a downregulation of prefrontal 5-HT_{1A} receptor that was not observed in the depleted Wistar rats. This is interesting as 5-HT_{1A} receptors seem to be less susceptible to changes in serotonergic tonus compared to other 5-HT receptor subtypes, based on several studies of 5,7-DHT lesions (Berendsen et al. 1991; Frazer and Hensler 1990; Hensler et al. 1991; Miquel et al. 1992; Verge et al. 1986). However, Kawai et al. (1994) proposed a downregulation of 5-HT_{1A} receptors in the frontal cortex as a homeostatic adaptive change in response to chronic TRP deprivation.

Effect of chronic tryptophan-deficient diet on monoaminergic concentration levels

Chronic TRP depletion was effective in reducing the levels of 5-HIAA/5-HT turnover ratio in prefrontal cortex, striatum, amygdala, nucleus accumbens and hippocampus in both strains of rats. In addition, the serotonin metabolite 5-HIAA was reduced in all cases with the exception of nucleus accumbens of the Wistar rats. More variability was found when exploring significant reductions of 5-HT, which was effectively decreased in all areas but not in the striatum of the Wistar rats and the nucleus accumbens of both strains. Previous studies have reported similar findings regarding significant reductions of serotonin and its metabolite in prefrontal cortex and hippocampus when challenging rats to a TRP depletion by diet (Cahir et al. 2007; Franklin et al. 2012; Koot et al. 2012). However, there were strain differences, as the LH rats showed additional changes in other monoamines such as DA, which was increased in the nucleus accumbens, and NE, which was decreased in the prefrontal cortex, not observed in the Wistar rats. Alterations of DA and NA when depleting chronically TRP by diet were also observed by Koot et al. (2012) in Wistar rats, indicating that this non-invasive depleting method of 5-HT may possibly lead to alterations in other brain neurotransmitter systems.

In conclusion, the primary findings of the present study highlight the contribution of serotonergic mechanisms in the compulsive drinking behaviour of rats on SIP, in which the serotonin depletion by chronic exposure to a TRP-free diet increased compulsive licking in HD Wistar rats compared to LD Wistar and LH rats on SIP. Moreover, the TRP depletion by diet produced a modulation of 5-HT_{1A} and 5-HT_{2A} receptor subtypes. The depleted HD Wistar rats showed 5-HT_{2A} receptor reductions in the striatum, which may underlie the increases in licking on SIP. Changes in the 5-HT_{2A} receptor subtype may represent a good potential biomarker for the vulnerability to compulsive spectrum disorders and a new target in the development of new therapeutic strategies.

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Conflicts of interest The authors declare that they have no conflict of interest.

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7

Dietary tryptophan depletion alters the faecal bacterial community structure of compulsive drinker rats in schedule-induced polydipsia

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Submitted

Abstract

Compulsive behaviour, present in different psychiatric disorders such as obsessive-compulsive disorder, schizophrenia and drug abuse, is associated with altered levels of serotonin (5-hydroxytryptamine, 5-HT), and the gut microbiota may be implicated. The present study investigated whether chronic tryptophan (TRP) depletion by diet alters the faecal bacterial community profiles of compulsive versus non-compulsive rats in schedule-induced polydipsia (SIP). Peripheral plasma 5-HT and brain-derived neurotrophic factor (BDNF) levels were evaluated. Wistar rats were selected as High Drinkers (HD) or Low Drinkers (LD) according to their SIP behaviour and were fed for 14 days with either a TRP-free diet (T-) or a TRP-supplemented diet (T+). The faecal bacterial community structure was investigated with 16S rDNA gene-targeted denaturing gradient gel electrophoresis (DGGE) fingerprinting analysis. Compulsive HD rats showed a general lower bacterial diversity than LD rats, irrespectively of the diet. The TRP-depleted HD rats, the only group increasing compulsive licking in SIP, showed a reduction of bacterial evenness and a highly functionally organized community compared with the other groups, indicating that this bacterial community is more fragile to external changes due to the dominance of a low number of species. The chronic TRP depletion by diet effectively reduced peripheral plasma 5-HT levels in both HD and LD rats, while plasma BDNF levels were not altered. These results highlight the possible implication of reduced microbial diversity in compulsive behavior and the involvement of the serotonergic system in modulating the gut brain-axis in compulsive spectrum disorders.

Introduction

Compulsions are repetitive mental or overt acts that are experienced as urge-driven, either in response to an obsession or according to a rule that must be rigidly applied, and are aimed at reducing anxiety or distress, or preventing a feared event from occurring (Gillan et al., 2017). The presence of this symptom is characteristic of various psychiatric disorders, such as obsessive-compulsive disorder (OCD), body dysmorphic disorder, hoarding disorder, hair-pulling disorder, and skin-picking disorder, which comprise the newly created Obsessive Compulsive and Related Disorders cluster in the *Diagnostic and Statistical Manual of Mental Disorders*, 5th Edition (American Psychiatric Association, 2013). Among the potential mechanisms underlying compulsive behaviours, pharmacological treatments in clinical and pre-clinical observations in OCD suggest the involvement of serotonergic dysfunctions (Angoa-Pérez et al., 2012; Goddard et al., 2008; Robbins & Crockett, 2010).

Emerging data implicates the gut microbiota (GM) in the regulation of brain function, behaviour and mental health by immune, endocrine and neural pathways of the *brain-gut axis*, which is the bi-directional system of communication between the central nervous system and the gastrointestinal tract (Foster et al., 2016). In particular, the GM regulates tryptophan (TRP) metabolism and may affect global serotonin (5-hydroxytryptamine, 5-HT) synthesis in the enteric and central nervous systems, pointing toward the GM as a therapeutic target for serotonin-related brain-gut axis disorders (O'Mahony et al., 2015). In fact, central serotonin production represents just 5% of total serotonin synthesis, with the vast majority of serotonin made in the periphery (Jenkins et al., 2016). Specific bacterial strains can produce serotonin from tryptophan (Ozogul, 2004; Ozogul et al., 2012; Shishov et al., 2009) and are susceptible to the effects of serotonergic drugs administered to the host such as selective serotonin reuptake inhibitors (SSRIs) (Munoz-Bellido et al., 2000). Recent evidence from germ-free (GF) animals found that these animals had increased plasma TRP (Clarke et al., 2013), increased hippocampal and striatal 5-HT

(Clarke et al., 2013; Diaz-Heijtz et al., 2011), and decreased plasma 5-HT levels (Wikoff et al., 2009). When these animals have tryptophan-metabolising bacteria introduced to their gut, circulating levels of TRP fall, with this alteration accompanying a sex-specific effect on hippocampal 5-HT concentrations in male germ-free animals (Clarke et al., 2013). Moreover, these animals also displayed a reduction in central brain-derived neurotrophic factor (BDNF), a neurotrophin that is involved in the synaptic plasticity In fact, reduced peripheral BDNF levels have been linked to the pathogenesis of several neuropsychiatric disorders such as major depressive disorder (Sen et al., 2008), schizophrenia (Ikeda et al., 2008), bipolar disorder (Machado-Vieira et al., 2007), eating disorders (Nakazato et al., 2003) or autism (Hashimoto et al., 2006). Moreover, a recent meta-analysis found that the decreased BDNF levels observed in patients with anxiety disorders were mostly due to the effects found in those suffering from OCD (Suliman et al., 2013). Therefore, peripheral BDNF levels have been proposed as a biomarker for OCD (Hall et al., 2003; Hemmings et al., 2008; Katerberg et al., 2009; Suliman et al., 2013) and could be modulated by altered GM in this population.

Although no research has been coducted on the GM of OCD patients to date, several researchers have hypothesized possible alterations based on several observations (Bastiaanssen et al., 2018; Rees, 2014; Turna et al., 2016). First, it has been noted that many of the risk factors for the onset of OCD are also known to disrupt the GM including stress, pregnancy, and antibiotic use (Rees, 2014). Second, there is preclinical evidence that compulsive behaviour in rodents (frequently based on performance on the marble burying test) can be modified by microbial treatments, including germ-free environments and probiotic treatments (Nishino et al., 2013; Kantak et al., 2014; Savignac et al., 2014).

Schedule-induced polydipsia (SIP), a phenomenon characterized by the development of excessive drinking in food-deprived animals exposed to intermittent food reinforcement schedules (Falk 1961, 1971), has been proposed as a useful model to study neuropsychiatric disorders distinguished by the presence of compulsive behaviour (Flores et al., 2014; Ford, 2014; Gilpin et al.,

2008; Hawken et al., 2011; Hawken & Beninger, 2014; Merchán et al., 2018; Moreno & Flores, 2012). Important differences among individual subjects in the amount of fluid intake and licks support the differentiation of two phenotypes of rats, one with high or excessive drinking (High Drinkers-HD), and a second group with low or no SIP acquisition (Low Drinkers- LD) (López-Grancha et al., 2008). Previous SIP studies have found alterations of the serotonergic system in HD rats, such as increased 5-HT levels in the medial prefrontal cortex and amygdala (Mora et al., 2018; Moreno et al., 2012) and reduced 5-HT_{2A} receptor binding in the frontal cortex, compared with LD rats (Mora et al., 2018). Therefore, the HD rats in SIP represent a suitable model of compulsivity for studying serotonergic vulnerabilities.

The biology of the serotonergic system is often studied by using protocols that deplete the precursor TRP. The dietary manipulation of TRP is a non-invasive and naturalistic method that is able to reduce the 5-HT synthesis, content (Gessa et al., 1974) and release (Stancampiano et al., 1997a, b). Acute TRP depletion (ATD) has been shown to produce a moderate serotonergic reduction in rats (Brown et al., 1998; Lieben et al., 2004), while chronic TRP depletions had stronger effects, reducing brain 5-HT levels to 35-40% at 14 days (Fadda et al., 2000) and to 75% at 5-week exposures (Vergnes & Kempf, 1981). A recent study found that HD rats increased compulsive drinking after chronic dietary TRP depletion (Merchán et al., 2017), and this increment was accompanied by a reduction in striatal 5-HT_{2A} receptors. However, little is known about the consequences of TRP depletion for serotonergic functions outside the central nervous system, particularly in the gut microbiota.

We propose that compulsive HD rats will be more vulnerable to GM alterations following chronic dietary TRP depletion, and we will expect to find differences in GM between non-depleted HD and LD rats in SIP. To test this hypothesis, we studied the faecal bacterial community profiles of HD and LD rats in SIP, fed with either a TRP-free diet (T−) or a TRP supplemented diet (T+), by 16S rRNA gene-targeted denaturing gradient gel electrophoresis

(DGGE) fingerprinting analysis, and expressed by Shannon-Weaver diversity index, evenness index, functional diversity (Fo) and clustering analyses. Additionally, plasma 5-HT and BDNF levels were measured in depleted and non-depleted HD and LD rats.

Methods

Subjects

Twenty-eight adult male Wistar rats from Harlan Iberica (Barcelona, Spain), weighing approximately 300-400 grams at the beginning of the experiment, were housed three/cage or two/cage (57×35×20 cm cm) at 22ºC with 08:00-20:00 hours light-dark cycle, with food and water available ad libitum. The animals were gradually reduced to 80%-85% of their free-feeding body weight by controlled feeding and then maintained at this level of deprivation throughout the experiment. Food was made available by daily feeding of lab chow approximately 30 minutes after each experimental session. Water was always available in the home cages.

These animals also participated in a previous study (Merchán et al., 2017). Rats were classified as High Drinkers and Low Drinkers according to their total number of licks in the previous experimental SIP. The rats were divided as follows: High Drinkers receiving a TRP-free diet (HD T-, *n*=7), High Drinkers receiving a control diet (HD T+, *n*=7), Low Drinkers with a TRP-free diet (LD T-, *n*=7) and Low Drinkers with a control diet (LD T+, *n*=7). Once the animals had begun the specific diets, they were housed in cages individually (50x25x18 cm) to prevent the ingestion of faecal samples from other animals. All procedures were conducted in accordance with the Spanish Royal Decree 53/2013 on the protection of experimental animals, the European Community Council Directives (86/609/EEC) and approval was obtained from the University of Almería Animal Research Committee.

Schedule-induced polydipsia

Apparatus. We conducted the tests in ten standard operant-conditioning chambers (MED Associates, Inc., Cibertec, Madrid, Spain) that were 32-cm long ×25-cm wide ×34-cm high, with stainless-steel grid floors. A detailed description of the apparatus has been provided previously for the SIP (López-Grancha et al., 2008; Moreno et al., 2012). The scheduling and recording of experimental events were controlled by a Med PC computer and commercial software (Cibertec SA, Madrid, Spain).

Behavioural Procedure. Following one day of adaptation, the animals were exposed to a fixed-time 60s (FT-60s) schedule of food pellet presentation throughout 60 min sessions. Water bottles with fresh water were available. After 20 daily sessions, the average total licks for each animal was calculated based on the last three SIP sessions. Rats were classified as high drinkers (HD) and low drinkers (LD) if their average total licks were above or below the group median, respectively. After 14 days of exposure to a TRP-free diet, the animals were again subjected to a FT-60s schedule of food pellet presentations in 60-min sessions. The following measures were shown in the present study: (a) total number of licks and (b) total number of magazine entries. A full description of the SIP results was reported in Merchán et al. (2017).

Tryptophan depletion diet

The TRP-free diet (TD08126, Harlan Laboratories S.A., Barcelona, Spain) has a standard nutritional value, but with a complete lack of TRP (T- groups). The control groups (T+ groups) were fed a similar diet containing a standard amount of TRP (1.8 g/Kg diet) (TD99366, Harlan Laboratories S.A., Barcelona Spain). The rats received chronic exposure to a TRP-free diet for 14 days before the behavioural tasks, in accordance with previous studies (Bortolato et al., 2008; Franklin et al., 2012; Stancampiano et al., 2013), and this was maintained until the end of the experiment.

Faeces–DNA extraction and PCR–DGGE analysis

Faecal samples were collected from the gut after sacrifice and stored at -80ºC. The sacrifice was conducted 21 days after the last SIP post-treatment session to prevent the effects of high fluid intake in the faecal bacterial community structure. The total DNA was extracted from 250 mg faeces by the bead-beating method, following the manufacturer's instructions (MoBio UltraClean Soil DNA Isolation kit, MoBio Laboratories Inc., Solana Beach, CA, USA). For the analysis of the bacterial community, the amplification of the variable region V3–V5 of 16S rDNA was carried out using the primers 341F (CCTACGGGAGGCAGCAG) and 907R (CCGTCAATTCCTTTGAGTTT). The primer 341F had at its 5′ end an additional 40-nucleotide GC-rich tail (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′) (Muyzer et al., 1993). The total reaction mixture of the PCR consisted of 25 µL with the following ingredients: 3 µl of extracted DNA, (0.75µL) 1 mM primer 341F-GC, (0.75µL) 1 mM primer 907R, 4.5 µl AptaTaq Fast PCR (Roche, Mannheim, Germany) and sterile Milli-Q water to a final volume. The PCR was conducted as follows: 7-min initial denaturation of DNA at 94 °C, followed by 35 cycles of 0.45-min denaturation at 94 °C, 0.45-min annealing at 49 °C, and 1.50-min extension at 72 °C. Amplification was completed by a final extension step at 72 °C for 30 min. PCR products were first visualized in a 1.5% (w/v) agarose gel in TBE 1× buffer by ethidium bromide staining and then purified using filters Diffinity Rapid Tip (Sigma-Aldrich, St. Louis, MO, USA). The DNA samples were checked for concentration and quality using the NanoDrop1 ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). DGGE of the amplified 16S rRNA gene sequences was carried out using the Dcode System (Universal Mutation Detection System, Bio-Rad Laboratories Inc., Hercules, CA, USA). DGGE analyses were conducted using 30 µl (300 ng) of PCR product loaded into a 40–70% urea-formamide–polyacrylamide gel. The run was performed in 1 x Tris-acetate-EDTA buffer at 60 °C and a constant voltage of 70 V for 16 h to separate the fragments. The gels were ethidium

bromide stained and photographed under UV light (l = 254 nm) using a Gel DocTM XR (Bio-Rad Laboratories Inc., Hercules, CA, USA). Samples were run to obtain at least three profiles.

The DGGE band patterns in different lanes were compared with the Gelanalyzer2010a (http://www.gelanalyzer.com/). The DGGE data were the means of three replicates (n = 3) selected randomly from each group of rats. The lanes were normalized to contain the same amount of total signal after background subtraction and the gel images were straightened and aligned to give a densitometric curve. Band positions were converted to Rf values between 0 and 1. The DGGE profiles obtained were analysed considering each band as a species or individual operational taxonomic unit (OTU) having 16S rDNA sequences with similar melting behaviour, while the band intensity indicated the relative abundance of the species. Processing of the DGGE data resulted in a square matrix containing the presence and abundance of DGGE band types per sample. The Shannon–Weaver (H', diversity), and evenness (E′) indices (Shannon & Weaver, 1963; Magurran, 2013) were calculated for each DGGE lane. Shannon-Weaver diversity index (H′) was calculated with the formula, $H' = \sum p_i \ln(p_i)$ where p_i is the band intensity of the ith band divided by the sum of all band intensities in a DGGE lane (Magurran, 2013). Evenness index (E) was calculated as E = H′/ln S, where S is the number of bands detected in a DGGE lane. Evenness index can range from near 0, indicating pronounced dominance, to near 1, indicating complete evenness, i.e. equal abundance of all species. The functional organization (*Fo*) of the community was analyzed by using the Pareto-Lorenz (PL) distribution curves that represent the evenness of the bacterial community. The *Fo* is the ability of the community to organize into an adequate distribution of dominant and resilient microorganisms, a condition that should ensure the potentiality of counteracting the effect of a sudden exposure to stress. For this measurement, the bands in each DGGE lane were ranked from high to low, based on their intensity levels. The cumulative normalized numbers of bands were sequentially represented on the x-axis, and their respective cumulative normalized intensity on the y-axis (Marzorati et al., 2008). Mathematically, this

yields a convex curve. The more the PL curve deviates from the theoretically perfect evenness line (i.e., the 45° diagonal), the less evenness can be observed in the structure of the studied community. To numerically interpret the PL curves, the *y*-axis projection of their respective intercepts with the vertical 20% *x*-axis line is scored (Wittebolle et al., 2008).

Plasma 5-HT and BDNF analyses

The day after the SIP post-treatment, the rats were rapidly euthanized by decapitation. Trunk blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes. Plasma was separated by centrifuging (Sigma 3-18KS, Germany) the blood samples at 3,000 rpm (800 g) for 20 min at 4ºC and stored at -80ºC until assay. 5-HT levels were determined using a commercial ELISA kit (RE59121, IBL, Hamburg, Germany), previously described in Sánchez-Mateos et al. (2008). BDNF levels were determined using an Ultrasensitive ELISA kit (SK00752-02, Aviscera-Bioscience, Santa Clara, CA, USA). All samples were assayed in duplicate on each plate. Protocols were performed according to the manufacturer's instructions. The optical density of each well was measured using an automated microplate reader (DTX-880, Beckman Coulter, Inc., USA). Plasma 5-HT levels are expressed in nanogram/millilitre (ng/mL), and plasma BDNF levels in picogram/millilitre (pg/mL).

Statistical analysis

Analyses of variance (ANOVAs) were conducted with two between-subject factors, "group" (HD and LD) and "treatment" (T+ and T−). Repeated measures ANOVAs were conducted with "SIP sessions" as the within-subject factor. The *Fo* of the community was analysed by using the PL distribution curves. The similarity between the communities was calculated using the Bray–Curtis cluster analysis. A Pearson's correlation analysis was used to assess the possible relationship between plasma 5-HT and BDNF levels. When appropriate, *post hoc* comparisons were made using the Newman-Keuls test. Partial eta-squared values

(η^2_p) are reported as a measure of the effect size, for which values of .01, .06, and .14 are considered to reflect small, medium, and large effects, respectively (Cohen, 1973). Statistical significance was set at $p<0.05$. All statistics were two-tailed.

Results

Schedule-induced Polydipsia pre-treatment and post-treatment

Figure 1 shows the mean total licks and total magazine entries in high-drinker (HD) and low drinker rats (LD) on the SIP pre-treatment and post-treatment FT-60s schedule of food presentation. A repeated measures ANOVA revealed significant differences in total licks from Session 1 to session 20 between HD and LD rats (Fig. 1a; group x session effect $F_{1, 26}=43.595$; $p<0.001$; $\eta^2_p=0.63$). Further, significant main effects of group ($F_{1, 26}=48.016$; $p<0.001$; $\eta^2_p=0.65$) and sessions ($F_{1, 26}=69.264$; $p<0.001$; $\eta^2_p=0.73$) were observed. *Post-hoc* analyses indicated that HD increased total licks from Session 1 to Session 20 ($p<0.001$), showing an increased number of total licks compared with LD rats on Session 20 ($p<0.001$). LD rats did not show increments in total licks from Session 1 to 20 ($p=0.455$). No interaction effect was found for magazine entries (Fig. 1c*;* $F_{1, 26}=0.478$; $p<0.495$).

The TRP depletion by diet increased the total number of licks in HD T− rats over the course of the sessions (Fig. 1b; group x treatment x session effect $F_{5,120}=2.529$; $p<0.05$; $\eta^2_p=0.095$), whereas magazine entries were unaffected (Fig. 1d; group x treatment x session effect $F_{5,120}=1.018$; $p=0.410$). *Post hoc* analysis indicated that the differences in total licks between HD T+ and HD T− occur from Session 3 ($p<0.01$). HD T− animals significantly increased their lick rate from Session 3 onwards ($p<0.05$). Although an increase in total licks was observed in HD T+ ($p<0.05$) and LD T− ($p<0.01$) rats on Session 6 compared with Session 1, these groups remain statistically different from each other ($p<0.05$). In fact, a significant main effect of group was found (group effect

$F_{1,24}$= 32.36; p<0.001), indicating that the differences between HD and LD rats remained stable in terms of total licks.

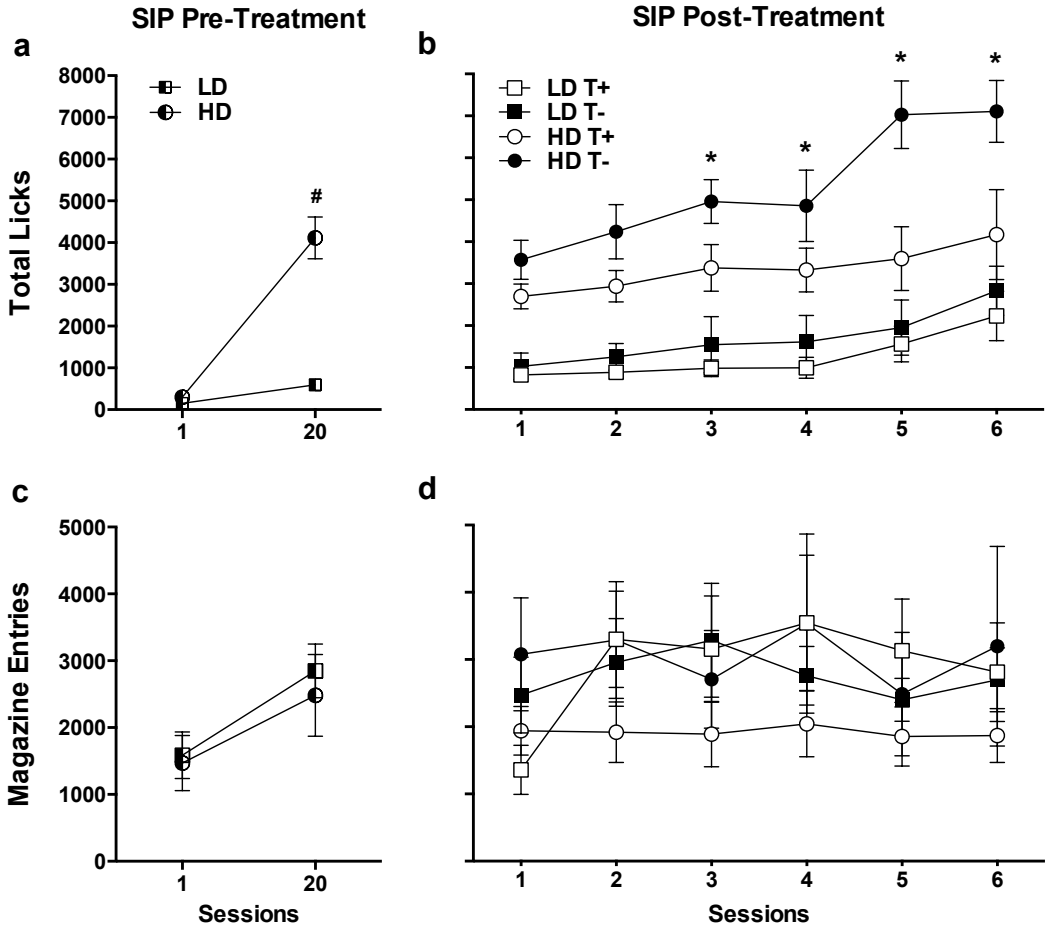

Fig. 1 The mean (±SEM) total licks (**a**, **b**) and magazine entries (**c**, **d**) on the SIP pre-treatment and SIP post-treatment FT-60s sessions. Rats are grouped in the SIP pre-treatment as High Drinkers (HD, n=14) and Low Drinkers (LD, n=14). Rats are grouped in the SIP post-treatment as TRP non-depleted High Drinkers (HD T+, n=7), TRP depleted High Drinkers (HD T-, n=7), TRP non-depleted Low Drinkers (LD T+, n=7) and TRP depleted Low Drinkers (LD T-, n=7). Number sign indicates significant differences between HD and LD (#p<0.001). Asterisks indicate significant differences between HD T+ and HD T− (*p<0.05).

Shannon-Weaver diversity and Evenness indices

The Shannon-Weaver diversity and evenness indices of the faecal bacterial community from HD T+, HD T-, LD T+ and LD T- rats, calculated from DGGE profiles of PCR-rDNA fragments, are reported in Figure 2. Chronic dietary TRP depletion significantly altered the bacterial diversity of HD and LD rats (Fig. 2a;

treatment x group $F_{1, 8}$=8.148; p<0.05; η^2_p=0.51). *Post-hoc* analysis revealed that chronic TRP depletion caused a reduction in bacterial diversity of LD T- compared with LD T+ rats (p<0.05). Nonetheless, the bacterial diversity of LD T- rats was greater than that for HD T- (p<0.05) and HD T+ rats (p<0.01). HD T+ and HD T- rats had similar diversity values (p=0.202). In non-depleted groups, HD rats showed a significant decrease in bacterial diversity compared with LD rats (p<0.001). Moreover, a main effect of group was found in the diversity index (group effect $F_{1, 8}$=46.587; p<0.001; η^2_p=0.85), and *post-hoc* analysis revealed that HD rats had lower bacterial diversity than LD rats, irrespective of diet (p<0.001).

Chronic TRP depletion also altered the evenness index in HD rats (Fig. 2b; treatment x group $F_{1,8}$=10.596; p<0.05; η^2_p=0.57). *Post-hoc* analysis indicated that HD T- rats showed significantly lower evenness than the LD T- group (p<0.05). Additionally, a trend towards significance was observed between HD T- and HD T+ rats (p=0.08). No significant differences were found between HD T+ and LD T+ rats (p=0.339).

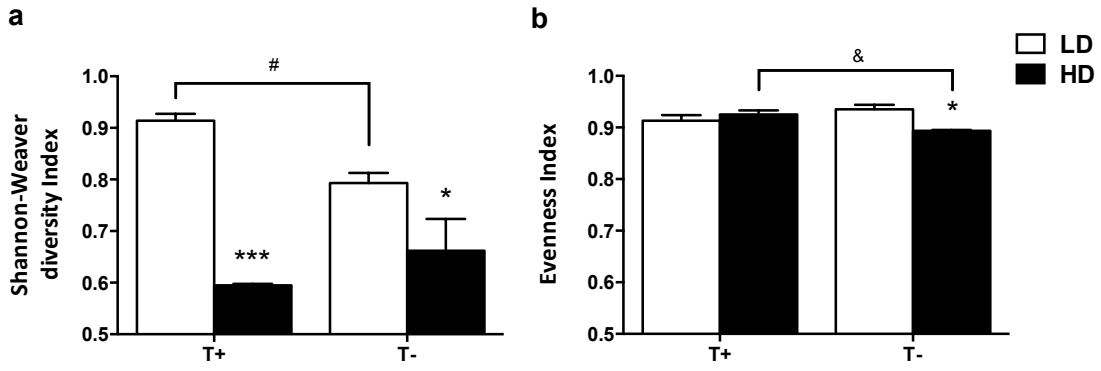

Fig. 2 The mean (±SEM) Shannon-Weaver diversity (**a**) and Evenness (**b**) indices calculated from DGGE profiles of the faecal bacterial community of TRP non-depleted High Drinkers (HD T+, n=3), TRP depleted High Drinkers (HD T-, n=3), TRP non-depleted Low Drinkers (LD T+, n=3) and TRP depleted Low Drinkers (LD T-, n=3). Asterisks indicate statistical differences between HD and LD groups (*p<0.05; ***p<0.001). Number sign indicates a significant difference between LD T+ and LD T- (#p<0.05). Ampersand indicates a trend toward a significant difference between HD T+ and HD T- (&p=0.08).

Functional organization

In order to graphically represent the evenness of the bacterial community (species distribution), PL curves were constructed based on the DGGE profiles (Fig. 3). The samples analysed in this study showed *Fo* values ranging from 53% to 66%. The curves of HD T+ and LD T+ rats were positioned similarly (*Fo*=62%), indicating similar species evenness. However, chronic dietary TRP depletion caused significant differences in functional organization depending on the group. The most specialized community (in which a small number of the species is dominant and all the others are present in low numbers) was found in HDT- rats, which indicates a more functionally organized community (high *Fo*, 66%), which implies that it is more vulnerable to external changes than communities having lower *Fo*. In contrast, LD T- rats had a more balanced community (lower Fo values, 56%), even when compared with non-depleted groups.

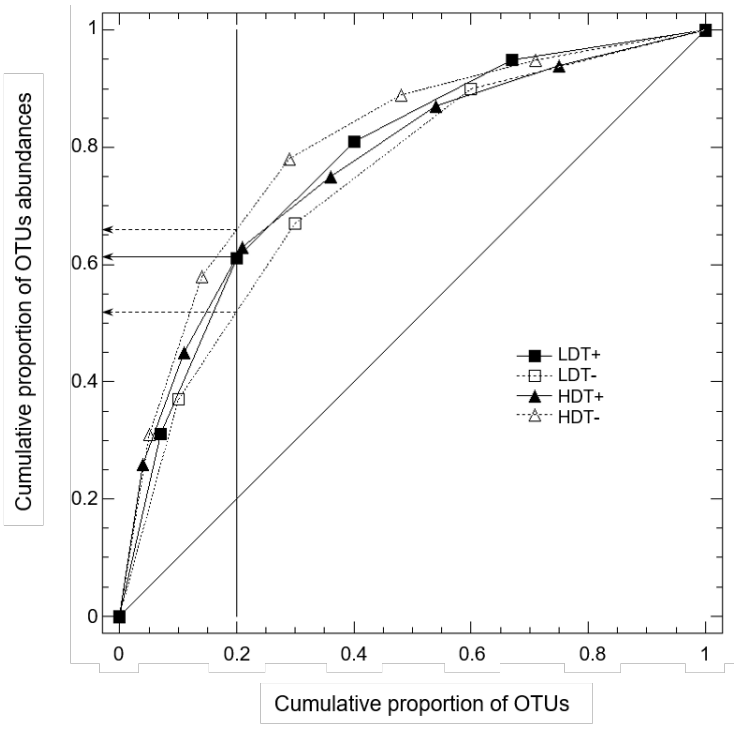

Fig. 3 Pareto-Lorenz distribution curves based on the DGGE profiles of the faecal bacterial community of TRP non-depleted High Drinkers (HD T+, *n*=3), TRP depleted High Drinkers (HD T-, *n*=3), TRP non-depleted Low Drinkers (LD T+, *n*=3) and TRP depleted Low Drinkers (LD T-, *n*=3). The dashed vertical line at the 20% x-axis level is plotted to evaluate the range of the Pareto values. The 45° diagonal represents the perfect evenness of a community. The y-axis projection of their respective intercepts with the vertical 20% x-axis line is scored.

To better visualize the relationships among samples, the binary matrix based on the presence/absence of bands and their quantity was analysed using the Bray–Curtis correlation, a distance matrix was calculated, and a cluster analysis was conducted which resulted in a dendrogram (Fig. 4). The generated dendrogram revealed two clear clusters. The first one exclusively included HD T- rats which clustered separately from the remaining samples. The remaining samples were grouped into two sub-clusters, one for LD T+ and LD T-, and another for HD T+ rats.

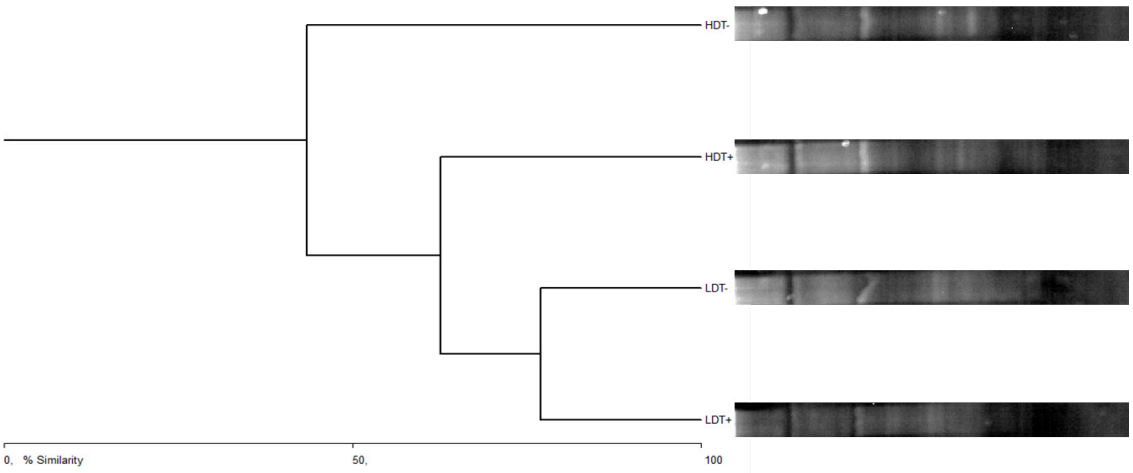

Fig. 4 Dendrogram obtained from the Bray–Curtis cluster analysis based on the DGGE profiles of the faecal bacterial community of TRP non-depleted High Drinkers (HD T+, n=3), TRP depleted High Drinkers (HD T-, n=3), TRP non-depleted Low Drinkers (LD T+, n=3) and TRP depleted Low Drinkers (LD T-, n=3).

Plasma 5-HT and BDNF levels

The mean plasma 5-HT (ng/mL) and BDNF levels (pg/mL) in HD T+, HD T-, LD T+ and LD T- rats are depicted in Figure 5. The chronic TRP depletion by diet altered plasma 5-HT levels (Fig. 5a; treatment effect $F_{1, 24}$=10.754; p<0.01; η^2_p=0.31), but no significant differences were observed between HD and LD rats (group x treatment effect $F_{1, 24}$=0.041; p<0.842; group effect $F_{1, 24}$=1.522; p<0.229). *Post-hoc* analysis revealed that the depleted groups had lower plasma 5-HT levels than the non-depleted groups (p<0.01). Plasma BDNF levels were

not altered by the serotonergic manipulation between HD and LD rats (Fig. 5b; group x treatment effect $F_{1, 23}$=1.062; p<0.313). Moreover, the main effects of treatment ($F_{1, 23}$=0.894; p<0.354) and group ($F_{1, 23}$=2.572; p<0.122) were not significant. However, a positive Pearson's correlation was found between plasma 5-HT and BDNF levels (Fig. 5c; r=+0.514, p<0.01, n=27).

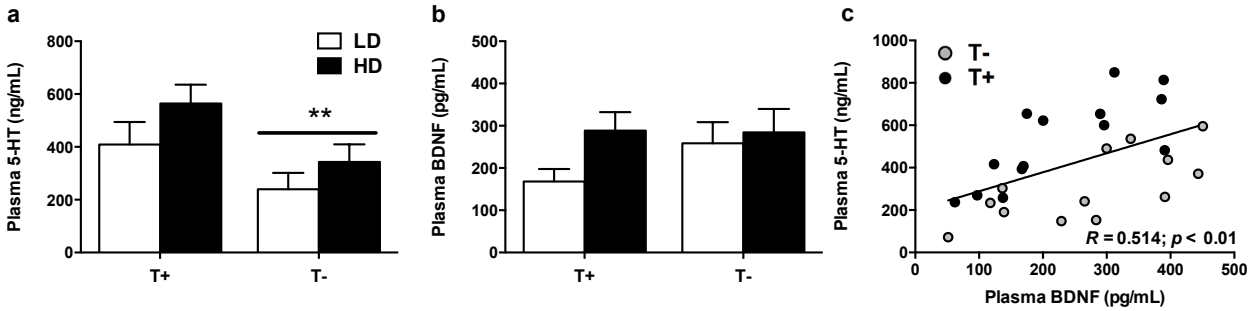

Fig. 5 The mean (±SEM) plasma 5-HT (**a**) and BDNF levels (**b**) in TRP non-depleted High Drinkers (HD T+, n=7), TRP depleted High Drinkers (HD T-, n=7), TRP non-depleted Low Drinkers (LD T+, n=7) and TRP depleted Low Drinkers (LD T-, n=6-7), and correlation between plasma 5-HT and BDNF levels (n=27) (**c**). Asterisks indicate significant differences between TRP non-depleted and depleted rats (**p<0.01).

Discussion

The present study has shown different faecal bacterial community profiles in HD and LD rats in SIP, and the specific effects of chronic dietary TRP depletion on these bacterial communities. We have observed that HD rats had lower bacterial diversity than LD rats, suggesting a possible altered gut microbiota in these vulnerable population to compulsivity. Moreover, TRP-depleted HD rats, the only group that exhibited an increase in compulsive licking in SIP, showed a reduction of bacterial evenness values and a highly functionally organized community compared with the other groups, indicating that this bacterial community is more vulnerable to external changes due to the dominance of a low number of species. Despite the fact that the TRP-depleted LD rats showed a reduction in bacterial diversity, the values were greater that those from TRP-

depleted HD rats. In fact, the reduction of bacterial diversity observed in TRP-depleted LD rats was not sufficient to alter the functional organization of the bacterial community, with these rats showing the most balanced microbiota according to the PL curves. In addition, chronic dietary TRP depletion effectively reduced peripheral plasma 5-HT levels in depleted HD and LD rats, while plasma BDNF levels were unaffected.

As previously reported by Merchán et al. (2017), chronic TRP depletion produced an increase of compulsive licking in HD, which was not observed in LD rats, and a reduction of striatal 5-HT_{2A} receptor binding, a serotonin receptor subtype that has been proposed as a candidate for mediating compulsive behaviour (Aznar & Hervig, 2016; Aznar & Klein, 2013; Fineberg et al., 2010, 2011). This receptor subtype was found to mediate the anti-compulsive effect of the serotonin 5-$HT_{2A/C}$ receptor agonist DOI in SIP (Mora et al., 2018; Navarro et al., 2015). Moreover, previous studies have shown that vulnerable HD rats exhibited increased 5-HT levels in the medial prefrontal cortex and amygdala (Mora et al., 2018; Moreno et al., 2012), and reduced 5-HT_{2A} binding in the frontal cortex (Mora et al., 2018). Collectively, these results point to the involvement of the 5-HT system in the development of compulsive drinking in SIP. Additionally, the present study has explored differences in the faecal bacterial community structure of HD and LD rats. Interestingly, compulsive HD rats have shown a general reduction in bacterial diversity compared with LD rats. The faecal samples were collected 3 weeks after the last post-treatment SIP sessions, thus we consider that the lower bacterial diversity observed in HD rats is unlikely to be a consequence of the high fluid intake (i.e. diarrhea). Previous studies with rodents have found that maternal separation (O'Mahony et al., 2009), prolonged restrain stressors (Bangsgaard Bendtsen et al. 2012), and social stressors (Bailey et al., 2011) altered the microbiota composition and reduced the abundance of species. However, few studies have explored the relationship between compulsive behaviour and gut microbiota. For instance, GF mice have been shown to spend an increased amount of time on self-grooming (Desbonnet et al., 2013) and had a higher number of buried marbles compared with controls

(Nishino et al., 2013). In innately anxious male BALB/c mice, the number of marbles buried was reduced by probiotics in a manner similar to the SSRI escitalopram (Savignac et al., 2014). In line with this, a study in mice showed that 2 weeks of pre-treatment with probiotics reduced compulsive behaviours such as perseverative open-field locomotion, stereotypic turning and marble burying, induced by the acute 5-HT1A/1B agonist RU24969, in a similar way to mice pre-treated for 4-weeks with fluoxetine, a first-line treatment for OCD (Kantak et al., 2014). Although no research has been conducted to explore the GM of OCD patients, a study in healthy humans found that probiotics administrated daily for 30 days reduced "obsessive-compulsive" sub-scores on the Hopkins symptoms checklist (Messaoudi et al., 2011). Taken together, the findings in the literature suggest that the modulation of the gut microbiota by probiotics may be beneficial in reducing compulsive behaviour and, therefore, an altered microbiota-gut-brain system may be implicated in the pathogenesis of such behaviour.

Moreover, chronic dietary TRP depletion had different effects in the faecal bacterial community of LD and HD rats. TRP-depleted LD rats showed a reduction of faecal bacterial diversity, without affecting the evenness of the community. In spite of this, the bacterial diversity values of TRP-depleted LD were greater that those from TRP-depleted HD rats. In fact, this reduction in bacterial diversity was not sufficient for altering the functional organization of the bacterial community of TRP-depleted LD rats, showing the most balanced microbiota according to the PL curves, whilst not increasing their rates of compulsive licking in SIP. In contrast, TRP-depleted HD rats showed an increase in compulsive licking in SIP along with a bacterial community structure that was different from the remaining groups according to the cluster analysis. In this vulnerable group, the chronic TRP depletion reduced the evenness of species, which, together with a lower bacterial diversity observed in the HD groups, resulted in a highly functionally organized community. In support of our findings, a previous study found that mice with dietary TRP insufficiency had an altered gut microbial composition, and these effects were reversed by a TRP

supplemented diet (Hashimoto et al., 2012). The alteration of the bacterial community structure after TRP deficiency confirms the modulating role of TRP in the GM. Although most microorganisms can synthesize their own tryptophan, some depend on an exogenous source of amino acids (Zouali, 2009). In fact, microorganisms are sensitive to the activity of the Indoleamine 2,3 dioxygenase-1 enzyme (IDO1), the first and rate-limiting step in TRP catabolism along the kynurenine pathway in the gut (Gao et al., 2018; Ciorba, 2014). The IDO1 enzyme has been shown to play an essential role in maintaining microbial diversity (Le Floc'h et al., 2011). For instance, IDOI1-Knockout mice exhibit increased production of bacterial TRP metabolites (Zelante et al., 2013). Similarly, IDOI1 activation leading to host TRP depletion can reduce microbial proliferation, possibly by microbial amino acid deprivation and immune tolerance (Gao et al., 2008). Therefore, the reduction of bacterial diversity observed in LD rats may be a consequence of microbial TRP deprivation. However, the mechanisms though which the TRP-deficient diets had a more marked effect on the vulnerable compulsive HD rats in terms of gut microbiota remains unknown. Further studies should investigate the mechanisms underlying the serotonergic modulation of the microbiota-gut-brain axis in vulnerable populations to compulsivity.

On the other hand, chronic dietary TRP depletion significantly reduced plasma 5-HT levels, independently of the SIP groups. Previous studies in rodents have shown that chronic TRP depletion by diet effectively reduced plasma TRP, brain 5-HT levels and its metabolite (Cahir et al., 2007; Franklin et al., 1995, 1999, 2012; Koot et al., 2012; Merchán et al., 2017; Vergnes & Kempf, 1981) and altered 5-HT_{2A} receptor levels (Franklin et al., 2012; Merchán et al., 2017). This is the first study that measures plasma 5-HT levels following chronic TRP depletion by diet, which may serve as an indirect measure of 5-HT depletion in the peripheral nervous system. Similarly, a previous study reported increments in plasma 5-HT levels after 7 days of oral TRP administration in rats (Sánchez-Mateos et al., 2008). Moreover, acute intra-gastric TRP administration increased 5-HT levels in intestinal tissue (Teff & Young, 1988), indicating that intestinal 5-

HT could be altered by dietary intake (Biggio et al., 1977). Although we did not find differences in plasma 5-HT levels between HD and LD groups after dietary TRP depletion, HD rats seems to have a vulnerable serotonergic system mediating the specific alteration of the faecal bacterial community structure. In fact, SSRIs, which are effective in reducing OCD symptoms, are also capable of decreasing pain and other symptoms associated with chronic gastro-intestinal disorders (Vanuytsel et al., 2014). Specifically, irritable bowel syndrome has been associated with small intestinal bacterial overgrowth (Stern & Brenner, 2018), and the criteria for this syndrome are met in 35,1% of patients with OCD in contrast to 2.5% of controls (Masand et al., 2006).

Regarding the plasma BDNF levels, HD and LD rats showed similar levels of this protein despite of the chronic TRP depletion. In support of our findings, previous ATD studies in rats found no alterations in plasma and central BDNF levels (Cahir et al., 2008; Van Donkelaar et al., 2009). Moreover, serotonin depletion by para-chloroamphetamine administration or 5,7-dihydroxytryptamine injections into the dorsal and median raphe nuclei did not change hippocampal BDNF levels compared with non-depleted rats (Hamani et al., 2012; Zhou et al., 2008). Similarly, ATD in depressive patients did not change plasma BDNF levels, but produced a temporary compensatory increase in healthy controls. With respect to the modulating role of GM in BDNF levels, previous studies found that GF mice or healthy mice with altered GM by antibiotic treatment or by infection with a non-invasive parasite exhibited reduced BDNF levels in different brain areas (Bercik et al., 2010, 2011; Clarke et al., 2013; Desbonnet et al., 2015; Diaz-Heijtz et al., 2011; Gareau et al., 2011; Sudo et al., 2004), which were normalized by probiotic administration (Bercik et al., 2010; Liang et al., 2015; O'Sullivan et al., 2011; Sudo et al., 2004). However, the altered bacterial community structure observed in TRP-depleted HD rats did not change the plasma BDNF levels, which possibly indicates that the chronic dietary TRP depletion may not be strong enough to produce a dysbiosis in depleted HD rats leading to plasma BDNF alterations. Moreover, non-depleted HD rats did not exhibit reduced plasma BDNF levels compared with non-depleted LD rats,

which does not support the notion that peripheral BDNF is a biomarker for compulsive behaviour in the SIP model. Nonetheless, a positive correlation between plasma 5-HT and BDNF levels was observed in the present study, which is in accordance with previous studies showing positive correlations between TRP and BDNF levels in the hippocampus and prefrontal cortex in rats (Van Donkelaar et al., 2009) and SERT availability and BDNF levels in humans (Chan et al., 2018; Chou et al., 2013). Therefore, our data confirm the relationship between serotonergic transmission and BDNF expression that has been reported by previous authors (Martinowich & Lou, 2008; Sen et al., 2008). One limitation of the present study is that the approach used does not allow for identifying the specific bacterial strains that were reduced in diversity and evenness in the TRP-depleted HD group. Nonetheless, from a more general perspective, the data provided from the DGGE profiles showed a clear cluster of the microbiota from depleted HD rats, which is not evident in their non-depleted HD counterparts, suggesting that the serotonin manipulation specifically affected the microbiota of HD rats vulnerable to compulsivity. Further studies should investigate the influence of neurotransmitters on the gut microbiota of subjects vulnerable to compulsive spectrum disorders, and explore possible alterations in the gut-brain axis that could underlie the vulnerability to compulsive behaviour.

In conclusion, the primary findings of the present study highlight the involvement of reduced microbial diversity in the vulnerability to develop compulsive drinking in SIP, and the role of the serotonergic system in the alteration of the gut microbiota of vulnerable populations to compulsivity. In the TRP-depleted HD rats, the chronic dietary TRP depletion increased compulsive licking in SIP and produced a reduction of species evenness that, together with a lower microbial diversity, resulted in a higher specialized bacterial community compared with the other groups, indicating an unbalanced, less adaptive and more vulnerable bacterial community structure. Further research should aim to establish the underlying mechanisms regulating the microbiota-gut-brain axis in compulsive spectrum disorders.

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General Discussion

Growing evidence suggests that alterations of the neurobehavioural mechanisms mediating cognitive flexibility and habit formation contribute to the vulnerability to compulsivity (Fineberg et al., 2018; Robbins et al., 2012). In line with this, previous studies found that compulsive HD rats had increased perseverative responding in the reversal-learning task (Navarro et al., 2017) and under extinction in the 5-choice serial reaction time task, compared with LD rats (Moreno et al., 2012). The mentioned SIP studies have tested the performance of HD and LD in behavioural tasks after the SIP exposure and classification in HD and LD, which might lead uncertainty over whether the SIP procedure alters the brain circuits modulating the neurobehavioural traits. In **chapter 4,** we tested the animals before the SIP exposure, to evaluate whether behavioural inflexibility and excessive habit formation predict high drinking in SIP. Indeed, before the SIP exposure, we found that HD rats exhibited higher behavioural inflexibility by showing increased perseverative responses in the reversal-learning task and insensitivity to reinforcer devaluation during extinction under selective satiation, compared with LD rats. These results support previous studies that indicate that HD rats have behavioural inflexibility (Moreno et al., 2012; Navarro et al., 2017) and tendencies to engage in habitual behaviour measured by increased lever pressing under a variable interval 60s schedule of reinforcement (Navarro et al., 2017) and a preference for response-learning strategies in a Y-maze (Gregory et al., 2015), compared with LD rats. Moreover, the present Doctoral Thesis provides the first evidence of insensitivity to outcome devaluation in HD rats. Thus, we confirm that behavioural inflexibility and excessive habit formation are behavioural traits present in HD rats before the SIP exposure, and may represent behavioural markers for predicting compulsive drinking in SIP.

Additionally, we found that only HD rats increased plasma corticosterone levels after the SIP exposure. A previous study found that rats exposed to SIP without water access exhibited increased corticosterone levels compared with rats non-exposed to the intermittent food-reinforcement schedule (López-Grancha et al., 2006), indicating that the SIP context represent a stressful

situation regardless of the acquisition of high drinking. In **chapter 4**, we found that only HD rats increased plasma corticosterone levels, which indicates several things: first, the SIP drinking is not a coping and anxiolytic behaviour; second, the SIP protocol represent a stressful situation for vulnerable individuals, since LD rats did not increase the plasma levels of this stress hormone. Further, in the same study, we found that HD rats after 20 daily SIP sessions had serum sodium levels within the normal range, indicating that the high fluid intake displayed by HD rats in SIP does not lead to hyponatremia and brain damage associated to this electrolyte imbalance (Penders et al., 2015). Therefore, the lack of hyponatremia in HD rats supports previous studies showing behavioural, neurochemical and neuroanatomical markers for compulsivity in this population in SIP (Flores et al. 2014; Moreno & Flores, 2012). Although some authors have speculated that OCD patients may have higher risk for developing metabolic syndromes such as type 2 diabetes mellitus (Albert et al., 2013; Isomura et al., 2018) or increased hypothalamic-pituitary-adrenal axis activity (Faravelli et al., 2012), we did not observed differences between HD and LD rats before the SIP exposure.

In line with previous findings on habit formation, the main objectives of **chapter 5** was to investigate excessive habit formation by exploring SIP microstructural variables among different rats strains, and to compared the brain areas activated during SIP in rat populations selected by cluster analysis of the pattern of licking. Within this chapter, the *first experiment* revealed specific patterns of licking among Wistar (WIST), Long Evans (LE), Roman-high (RHA) and low-avoidance (RLA) rat strains. We found that WIST and RHA showed higher frequency of licking than LE and RLA, which we related to the concept of habitual behaviour, and this result is in accordance with previous studies showing deficits in inhibitory control in both rat strains (Entlerova et al., 2013; Fattore et al., 2009; Giorgi et al., 2007; Klein et al., 2014; Kumar et al., 2015; Moreno et al., 2010). However, the WIST strain was the only one that showed both a high frequency and intensity of licking, indicating possible tendencies in engaging in excessive habitual behaviour. In order to study individual differences within each

rat strain, we clustered rats according to the frequency and intensity of their licking, which were the best SIP microstructural measures characterizing drinking among rat strains, and we obtained three populations that exhibited: high intensity and frequent licking (compulsive drinkers), low intensity but frequent licking (habitual drinkers) and low intensity and low-frequent licking (low drinkers). The WIST strain had the largest group of compulsive drinker rats when compared with the other strains, revealing that this strain is the most suitable for studying compulsive behaviour, as previously suggested by Moreno and Flores (2012).

Following the findings of the first experiment, we conducted a *second experiment* with a larger group of WIST rats to investigate the acquisition of the microstructural SIP measures and to analyse the neuronal activity in different brain areas related to compulsivity among the clusters mentioned above during SIP. Although we found that compulsive and habitual drinkers had a similar acquisition of licking frequency, the intensity of licking was the behaviour that best distinguished compulsive drinkers, showing an early and pronounced increment of intensity of licking across the SIP sessions. As we have seen in the **chapter 4**, rats developing high drinking rates in SIP had insensitivity to outcome devaluation. Thus, we suggest that the excessive licking in compulsive drinkers may be due to a lack of inhibition to exert control over habitual processes. Moreover, the quantification of neuronal activity, measured by c-Fos expression, in different brain areas of the prefrontal cortex and the amygdala during SIP revealed hyperactivity of the lateral orbitofrontal (OFC) cortex and a trend towards a significant increase in the basolateral amygdala (BLA) in compulsive rats compared with low drinker rats. These results are in line with fMRI studies showing increased activity of the OFC and amygdala in OCD patients (Adler et al., 2000; Banca et al., 2015; de Wit et al., 2015; Gillan et al., 2015; Simon et al., 2010, 2014; van den Heuvel, 2004). These brain areas, that are anatomically interconnected (Holland & Gallagher, 2004), have been related not only to goal-directed control over actions (Corbit & Balleine, 2005;

Lichtenberg et al., 2017; Valentin et al., 2007; de Wit et al., 2009), but also to behavioural flexibility (Bisonnette et al., 2008; Boulougouris et al., 2007; Chudasama & Robbins, 2003; Dias et al., 1996; Izquierdo et al., 2004; McAlonan & Brown, 2003; Rudebeck & Murray, 2008; Schoenbaum et al., 2008; Stalnaker et al., 2007). Moreover, recent studies have suggested that the BLA projections to the OFC facilitate flexible or goal-directed behaviour by enabling the OFC to use environmental stimuli to generate expectations of potential future rewarding events (Lichtenberg et al., 2017). Since we have observed increased hyperactivity of the OFC and BLA amygdala in compulsive rats in SIP, we may hypothesize a disruption of the flexibility to disengage from habitual behaviours in these animals.

The fact that compulsive drinkers in SIP exhibited both behavioural inflexibility and excessive habit formation, and that the brain circuits involved in these neurobehavioural processes are overlapped (OFC-BLA), lead us to a question that should be further investigated: Are behavioural flexibility and goal-directed control similar constructs? Behavioural inflexibility relates to the inability to disengage from a behaviour that was previously reinforced (thus it may be habitual), and later it is punished (e.g. by a time-out period in the reversal-learning task). On the other hand, failure in goal-directed learning (leading to excessive habits) refers to behaviours that are strongly associated with the stimuli and are displayed regardless of the outcome desirability (e.g. by satiation of the reinforcer). The main difference between both is the motivational state for the reinforcer, which is devalued in the reinforcer devaluation paradigm, but still both have in common a lack of ability to change a behaviour that has been previously reinforced over the time. In the SIP context, the excessive drinking in SIP, which could have been reinforced over the time according to Killeen & Pellón's theory (2013), may represent an inflexible, habitual and excessive behaviour that is develop by vulnerable rats to habitual tendencies in a stressful situation.

Once we have characterized compulsive drinker rats as inflexible and excessively engaged in habitual behaviours, with altered activity of the brain areas related to behavioural flexibility and goal-directed control, we explored in **chapter 6** the effects of central serotonin (5-HT) reductions by chronic dietary tryptophan (TRP) depletion in vulnerable Wistar HD rats compared with non-vulnerable Wistar LD and Lister Hooded rats on compulsive behaviour in SIP, spontaneous locomotor activity, and the 5-HT_{2A} and 5-HT_{1A} receptor binding in frontal cortex, striatum and hippocampus. We observed that the TRP-depleted HD group of Wistar rats increased compulsive licking in SIP, without showing increments in spontaneous locomotor activity, and showed reduced striatal 5-HT_{2A} receptor binding, compared with LD Wistar and Lister Hooded rats. These results confirm the modulating role of the 5-HT_{2A} receptor in compulsive behaviour (Aznar & Hervig, 2016; Aznar & Klein, 2013; Fineberg et al., 2011, 2010; Westenberg et al., 2007) and provide new insights regarding the 5-HT function in the striatum. Conversely, Lister Hooded rats, which did not show an acquisition of SIP, increased spontaneous locomotor activity and showed a reduced binding of the 5-HT_{1A} receptor in the frontal cortex after the chronic TRP depletion. We hypothesize that the increments in spontaneous locomotor activity may be due to the unexpected alterations of the dopamine and noradrenaline levels in the nucleus accumbens and prefrontal cortex, respectively. Despite that, the dietary TRP depletion was effective in reducing the levels of 5-HIAA/5-HT turnover ratio in prefrontal cortex, striatum, amygdala, nucleus accumbens and hippocampus in both rat strains, without altering the dopamine and noradrenaline levels in the Wistar strain, as previously reported (Fadda et al., 2000; Vergnes & Kempf, 1981).

Although a growing interest has emerged in the literature regarding the implication of the microbiota-gut-brain axis in neuropsychiatric disorders (Foster et al., 2016), the studies investigating the link between this axis and compulsivity are limited (Rees, 2014; Turna et al., 2016). The **chapter 7** shows the first findings about the gut microbiota of a vulnerable population to compulsivity.

Interestingly, we found that HD rats had a marked reduction of bacterial diversity compared with LD rats, indicating a possible involvement of reduced microbial diversity in compulsive behaviour in SIP. Further, we investigated whether chronic dietary TRP depletion alters the faecal bacterial community structure of TRP-depleted HD rats, the only group that exhibited an increase in compulsive licking in SIP, compared with non-depleted HD and LD groups of rats. We observed that the vulnerable HD rats were more affected by the chronic dietary TRP depletion, showing a reduction of the bacterial evenness and a highly functionally organized community compared with the other groups. The specialization of the bacterial community in TRP-depleted HD rats indicated an unbalance, less adaptive and more vulnerable microbiota to external changes due to the dominance of a low number of species. Moreover, these results are supported by cluster analysis, revealing that TRP-depleted HD had a different bacterial community structure compared with the remaining rat groups. Contrary, TRP-depleted LD rats reduced the diversity values, without affecting the evenness of the community. Nonetheless, the bacterial diversity values of TRP-depleted LD rats were greater than those from TRP-depleted HD rats, and exhibited the most balanced microbiota according to the functional organization of the bacterial community, indicating that this group did not show an unbalanced microbiota regardless of the reduction of bacterial diversity. We hypothesize that this reduction may be a consequence of microbial TRP deprivation. In fact, a previous study found that mice with dietary TRP insufficiency had an altered gut microbial composition (Hashimoto et al., 2012), indicating a possible modulatory role of the serotonergic system in the gut microbiota. However, the mechanisms through which the TRP-deficient diet had a more marked effect on the gut microbiota of the vulnerable compulsive HD rats remains unknown. On the other hand, the chronic dietary TRP depletion was effective in reducing plasma 5-HT levels in HD and LD rats, showing both groups similar reductions of peripheral 5-HT. However, we did not find differences between HD and LD rats in plasma brain-derived neurotrophic factor

levels, which has been proposed as a biomarker for OCD (Suliman et al., 2013), indicating that this protein does not predict compulsive drinking in SIP.

Taking together all the results from the present Doctoral Thesis, we can conclude that SIP is a sensitive model for identifying neurobehavioural alterations that may be underlying the neuropathology of compulsive behaviour. We demonstrated that compulsive drinker rats in SIP exhibited behavioural inflexibility and excessive habit formation before the SIP acquisition, indicating that these traits are candidates for predicting compulsive behaviour. In fact, several biomarkers for compulsivity have emerged from our results, such as the hyperactivity of the lateral orbitofrontal cortex and the basolateral amygdala, reduced serotonin 2A receptor subtype in the striatum, and reduced bacterial diversity of the gut microbiota. Furthermore, we provided evidence of the relationship between compulsivity and the serotonergic system, showing that serotonin reductions increased compulsive behaviour and unbalanced the gut microbiota in HD rats. Future research on compulsivity should investigate whether behavioural inflexibility and excessive habit formation are both related to either habit learning or the core executive function flexibility for improving our knowledge of the transdiagnostic domains involved in compulsivity. Moreover, further studies should elucidate the serotonergic mechanisms underlying these altered neurobehavioural domains, and the possible role of the gut microbiota in compulsive spectrum disorders.

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9

Conclusions

According to the results obtained, the conclusions of the present Doctoral thesis are:

1. Behavioural inflexibility and excessive habit formation were behavioural markers for predicting compulsive behaviour in schedule-induced polydipsia (SIP):

1.1. Before the SIP exposure, High Drinker (HD) rats showed higher behavioural inflexibility evidenced by an increase of perseverative responses in the reversal condition compared with Low Drinker (LD) rats.

1.2. Before the SIP exposure, HD rats exhibited insensitivity to reinforcer devaluation compared with LD rats.

1.3. Plasma corticosterone and blood glucose levels were similar between HD and LD before the SIP exposure. However, HD rats increased plasma corticosterone levels after 20 SIP sessions, indicating that SIP might be a stressful situation for vulnerable individuals.

1.4. After the SIP exposure, HD rats did not show hyponatremia, or low serum sodium levels, despite the high fluid intake, suggesting that the behavioural, neurochemical and neuroanatomical markers for compulsivity in SIP are not as a consequence of brain damage associated to this electrolyte imbalance.

2. The microstructural analysis of SIP allowed to differentiate vulnerability to excessive habitual licking in rat strains and identify the brain areas activated in compulsive rats during SIP:

2.1. Strain-dependent differences in the pattern of licking were observed in Wistar, Long Evans (LE), Roman high-avoidance (RHA) and low-avoidance rats (RLA). The Wistar and RHA rats, known to exhibit

inhibitory control deficits, displayed higher frequency of licking than LE and RLA rats.

2.2. Cluster analysis allowed to classify rats according to the frequency and intensity of licking in three groups showing: high intensity and frequent licking (Compulsive Drinkers), low intensity but frequent licking (Habitual Drinkers) and low intensity and low-frequency licking (Low Drinkers).

2.3. The Wistar strain showed a higher frequency and intensity of licking, and had the largest group of Compulsive Drinkers when compared with the other strains, indicating that this rat strain is the most suitable for studying compulsive behaviour.

2.4. The acquisition of frequency of licking was similar in Compulsive and Habitual Drinker rats. However, the early and pronounced intensity of licking across the SIP sessions was the licking pattern that best distinguished the Compulsive Drinkers from the other clusters, suggesting a lack of goal-directed control over habitual processes.

2.5. The hyperactivity of the lateral orbitofrontal cortex and the basolateral amygdala observed in Compulsive Drinkers compared with Low Drinkers pointed towards an alteration of the brain circuits modulating goal-directed control in SIP.

3. Chronic dietary tryptophan (TRP) depletion demonstrated the involvement of the serotonergic system in compulsive behaviour in SIP:

3.1. After chronic dietary TRP depletion, Wistar HD rats increased compulsive licking in SIP compared with Wistar LD and Lister Hooded rats, and this increment was accompanied by reduced striatal serotonin 2A receptor binding.

3.2. The Lister Hooded strain did not exhibit acquisition of compulsive drinking in SIP, showing a similar SIP drinking than Wistar LD rats.

3.3. The chronic dietary TRP depletion did not alter the SIP behaviour of the non-vulnerable Lister Hooded strain. However, this strain showed increased spontaneous locomotor activity, and a reduction of the serotonin 1A receptor binding in the frontal cortex.

3.4. The chronic dietary TRP depletion was effective in reducing serotonin and its metabolite in different brain areas of Wistar and Lister Hooded rats. Only the depleted Lister Hooded rats showed an increase in dopamine in the nucleus accumbens and a decrease in noradrenaline in the prefrontal cortex.

4. Chronic dietary TRP depletion pointed towards the serotonergic involvement in the alteration of the gut microbiota in vulnerable populations to compulsivity in SIP:

4.1. HD rats exhibited reduced faecal bacterial diversity compared with LD rats, irrespectively of the diet.

4.2. TRP-depleted HD rats, the only group increasing compulsive licking in SIP, showed lower bacterial evenness and a highly functionally organized community compared with non-depleted HD and LD groups of rats.

4.3. Cluster analysis revealed that TRP-depleted HD had a different bacterial community structure compared with the remaining rat groups.

4.4. The chronic dietary TRP depletion effectively reduced peripheral plasma serotonin levels in High Drinker and Low Drinker rats, while plasma brain-derived neurotropic factor levels were not altered.

10

Dissemination of Scientific Results

A) From the present work

The research work developed in the present work has been published and disseminated through various forms. Hereafter, they are categorized by type of publication.

1. Articles [1]

- **Merchán, A.,** Mora, S., Gago, B., Rodriguez-Ortega, E., Fernández-Teruel, A., Puga, J.L., Sánchez-Santed, F., Moreno, M. & Flores, P. (2018) Excessive habit formation in Schedule-Induced Polydipsia: microstructural analysis of licking among rat strains and involvement of the orbitofrontal cortex. *Genes, Brain and Behavior*, e12489 (JCR I.f. 3.496; Q1 in Behavioural Neuroscience). https://doi.org/10.1111/gbb.12489

- **Merchán, A.**, Navarro, S.V, Klein, A.B., Aznar, S., Campa, L., Suñol, C., Moreno, M. & Flores, P. (2017) Tryptophan depletion affects compulsive behaviour in rats: strain dependent effects and associated neuromechanisms. *Psychopharmacology*, 234, 1223-1236 (JCR I.f. 3.222; Q2 in Neuroscience). http://doi.org/10.1007/s00213-017-4561-5

- **Merchán, A.,** Sánchez-Kuhn, A., Prados, A., Gago, B., Sánchez-Santed, F., Moreno, M. & Flores, P. (Submitted) Behavioral and biological markers for predicting compulsive drinking in schedule-induced polydipsia.

- **Merchán, A.**, Pérez-Fernández, C., López, M.J., Moreno, J., Moreno, M., Sánchez-Santed, F. & Flores, P. (Submitted) Dietary tryptophan depletion alters the faecal bacterial community structure of compulsive drinker rats in schedule-induced polydipsia.

[1] Journal Citation Reports of published articles at the end of this chapter

2. Oral Communications

- **Merchán, A.,** Sánchez-Kuhn, A., Prados, A., Sánchez-Santed, F., Moreno, M. & Flores, P. (Sept., 2018) *Behavioural markers and biomarkers for predicting compulsivity in schedule-induced polydipsia.* XXX International Conference of the Spanish Society for Comparative Psychology, Ávila, Spain.

- **Merchán, A.,** Flores, P., López, M.J., Moreno, J., Moreno, M. & Sanchez-Santed, F. (July, 2017) *Tryptophan depletion by diet alters gut microbiota of compulsive drinker rats in Schedule-Induced Polydipsia.* II International Congress of Psychobiology, Ávila, Spain. **Special Mention Award for the best oral communication.**

- **Merchán, A.,** Mora, S., Moreno, M. & Flores, P. (July, 2015) *Expression of immediate early gene c-Fos in compulsive rats selected by Schedule-Induced Polydipsia.* I International Congress of Psychobiology, Oviedo, Spain.

- **Merchán, A.,** Navarro, S., García-Martín, S., Vilches, O., Flores, P. & Moreno M. (Sept., 2014) *Tryptophan depletion diet exacerbates compulsive drinking in rats selected by Schedule-induced Polydipsia.* XXVI International Conference of the Spanish Society for Comparative Psychology, Braga, Portugal.

3. Posters

- **Merchán, A.,** Sánchez-Kuhn, A., Prados, A., Sánchez-Santed, F., Moreno, M. & Flores, P. (May, 2018) *Behavioural markers and Biomarkers for Predicting Compulsivity: Behavioural inflexibility and reinforcer devaluation predicts compulsive drinking in rats.* II Iberoamerican Congress of Neuropsychology and XIV Congress of the Andalusian Society of Neuropsychology, Almería, Spain.

- **Merchán, A.,** Moreno, M. & Flores, P. (Sept., 2017) *Role of excessive and habitual drinking in rats classified by microstructure of schedule-induced polydipsia: implication of the lateral orbitofrontal cortex.* XVII Biennial European Behavioural Pharmacology Society Meeting, Crete, Greece.

- **Merchán, A.,** Mora, S., Moreno M. & Flores, P. (July, 2016) *Increased c-Fos expression in Orbitofrontal Cortex and Basolateral Amygdala in Compulsive rats selected by Schedule-induced Polydipsia.* 10th FENS Forum of Neuroscience, Copenhague, Denmark.

- **Merchán, A.,** Navarro, S., Campa, L., Suñol, C., Klein, A., Aznar, S., Moreno M. & Flores, P. (Sept., 2015) *Strain dependent effects of tryptophan depletion diet on compulsive drinking behavior and associated neuromechanisms.* European Brain and Behaviour Society & European Behavioural Pharmacology Society Joint Meeting, Verona, Italy.

- **Merchán, A.,** Navarro, S., García-Martín, S., Vilches, O., Flores, P. & Moreno M. (Oct., 2014) *El triptófano en la conducta compulsiva: Alimentación carente de triptófano aumenta la bebida compulsiva en la Polidipsia inducida por Programa.* X Congress of the Spanish Society of Health and Clinical Psychology, Almería, Spain. **Second Prize "Serafín Lemos" for the best poster.**

- **Merchán, A.,** Navarro, S.V., García, S., Moreno, M. & Flores, P. (Sept., 2013) *Tryptophan Depletion Diet in Low versus High Drinker Rats Selected by Schedule-induced Polydipsia.* XV Biennal European Behavioural and Psycopharmacology Society Meeting, La Rochelle, France.

B) Collaborations and other research topics

During the elaboration of the present work, the collaboration with other researchers and postgraduate students has led to the following publications.

1. Articles

- Mora, S., **Merchán, A**., Vilchez, O., Aznar, S., Klein, A.B., Ultved, L., Campa, L., Suñol, C., Flores P. & Moreno M. (2018) Reduced cortical serotonin 5-HT_{2A} receptor binding and glutamate activity in high compulsive drinker rats. *Neuropsychopharmacology*,143,10-19 (I.f. 4.249; Q1 in Pharmacology). https://doi.org/10.1016/j.neuropharm.2018.09.004

- Lins, S., Doka, A., Bottequin, E., Odabasic, A., Pavlovic, S., **Merchán, A.,** Golasa, A. & Hylander, F. (2015) The Effects of Having, Feeling, and Thinking on Impulse Buying in European Adolescents. *Journal of International Consumer Marketing*, 27, 414–428. (SJR. 0.26; Q3 in Marketing). http://dx.doi.org/10.1080/08961530.2015.1027028

- Flores, P., Sánchez-Kuhn, A., **Merchán, A.,** Vilches, O., García-Martín, S. & Moreno, M. (2014) Schedule-Induced Polydipsia: Searching for the Endophenotype of Compulsive Behavior. *World Journal of Neuroscience*, 4, 253-260 (RG. 0.29). http://dx.doi.org/10.4236/wjns.2014.43029

2. Oral Communications

- Moreno, M., **Merchán, A.,** Vilches, O., García-Martín, S., Navarro, S., Sánchez-Kuhn, A. & Flores, P. (Oct., 2014). *Schedule-induced Polydipsia: Searching for the phenotype of compulsivity.* X Congreso de la Sociedad Española de Psicología Experimental & XI Congreso de la Sociedad Española de Psicofisiología y Psicología Cognitiva y Afectiva. Murcia, España.

3. Posters

- Prados-Pardo, A., Martín-González, E., Mora, S., **Merchán, A.,** Flores, P. & Moreno, M. (July, 2018) *Compulsivity, a common trait in different neuropsychiatric disorders: preclinical studies on schedule-induced polydipsia.* Workshop in Animal and Human Behavior: Using Computational Approaches to Build a Two-way Bridge, Cambridge, United Kingdom.

- Prados-Pardo, A., Martín-González, E., Mora, S., **Merchán, A.,** Flores, P. & Moreno, M. (July, 2018) *Glutamatergic drugs in compulsivity: preclinical studies on schedule-induced polydipsia.* 11th FENS Forum of Neuroscience, Berlin, Germany.

- Sánchez-Kuhn, A., **Merchán, A.**, Montaño, S., Flores, P. & Moreno M. (Sept., 2014) *Effects of Schedule-Induced polydipsia acquisition on emotional tests*. XXVI International Conference of the Spanish Society for Comparative Psychology, Braga, Portugal.

C) Scientific Divulgations

The following activities were carried out each once a year from 2014 to the present day for the purpose of the scientific divulgation of the obtained results.

- Participation in the European Researcher's night, in the framework Open Researchers, approved by the European Commission within the Marie Sklodowska-Curie Actions. Arranged by the research Results Transfer Office (OTRI) from the University of Almería.

- Participation in the Week of Science, aimed at high-school students, at the University of Almería.

JOURNAL PROFILE: GENES, BRAIN AND BEHAVIOR

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LANGUAGES
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CATEGORIES
BEHAVIORAL SCIENCES - SCIE

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PUBLICATION FREQUENCY
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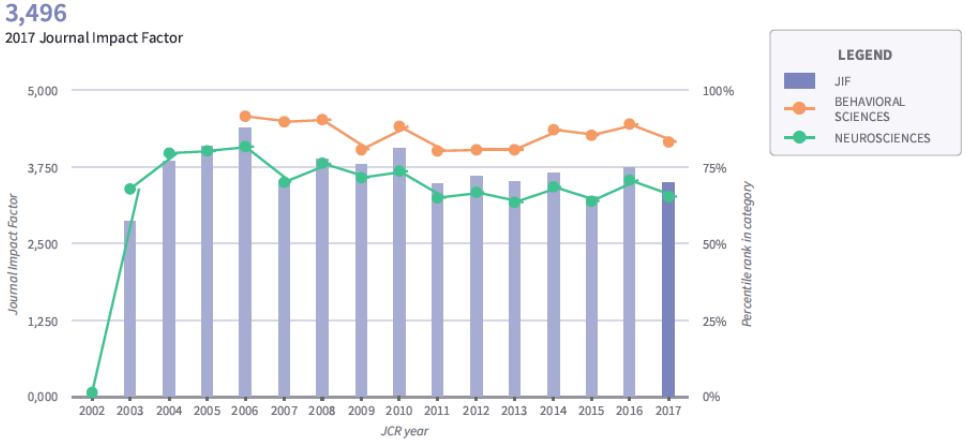

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2016	6/51	Q1	89.216	76/259	Q2	70.849
2015	8/51	Q1	85.294	93/256	Q2	63.867
2014	7/51	Q1	87.255	80/252	Q2	68.452
2013	10/49	Q1	80.612	93/252	Q2	63.294
2012	10/49	Q1	80.612	84/252	Q2	66.865
2011	10/48	Q1	80.208	86/244	Q2	64.959
2010	6/48	Q1	88.542	63/239	Q2	73.849
2009	10/49	Q1	80.612	66/231	Q2	71.645
2008	5/47	Q1	90.426	53/221	Q1	76.244
2007	5/45	Q1	90.000	64/211	Q2	69.905
2006	4/42	Q1	91.667	37/200	Q1	81.750
2005	NA	NA	NA	40/200	Q1	80.250
2004	NA	NA	NA	41/198	Q1	79.545
2003	NA	NA	NA	64/198	Q2	67.929
2002	NA	NA	NA	195/197	Q4	1.269

JOURNAL PROFILE: PSYCHOPHARMACOLOGY

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ISO: Psychopharmacology
JCR Abbrev: PSYCHOPHARMACOLOGY

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CATEGORIES
NEUROSCIENCES - SCIE

PHARMACOLOGY & PHARMACY - SCIE

PSYCHIATRY - SCIE

PUBLICATION FREQUENCY
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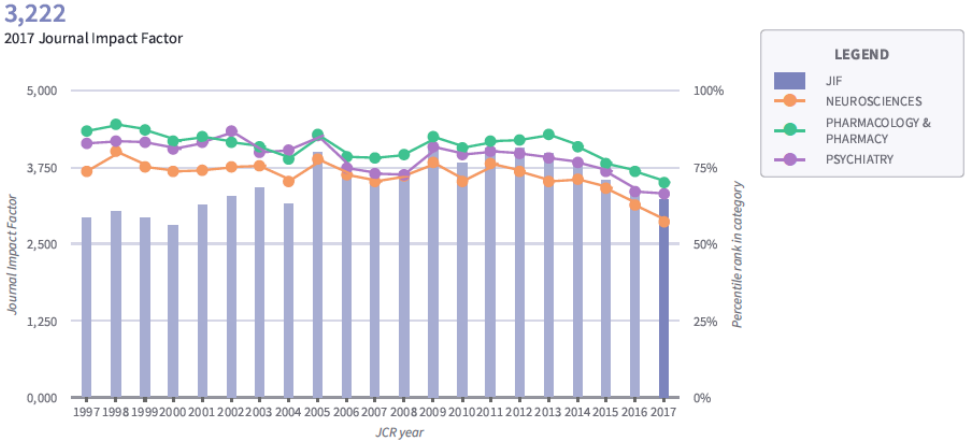
2017 Journal Impact Factor



Journal Citation Report : Impact factor

JCR Year	NEUROSCIENCES			PHARMACOLOGY & PHARMACY			PSYCHIATRY		
	Rank	Quartile	JIF Percentile	Rank	Quartile	JIF Percentile	Rank	Quartile	JIF Percentile
2017	112/261	Q2	57.280	79/261	Q2	69.923	48/142	Q2	66.549
2016	97/259	Q2	62.741	68/257	Q2	73.735	47/142	Q2	67.254
2015	82/256	Q2	68.164	61/255	Q1	76.275	38/142	Q2	73.592
2014	73/252	Q2	71.230	47/255	Q1	81.765	33/140	Q1	76.786
2013	75/252	Q2	70.437	37/256	Q1	85.742	30/136	Q1	78.309
2012	67/252	Q2	73.611	42/261	Q1	84.100	28/135	Q1	79.630
2011	58/244	Q1	76.434	43/261	Q1	83.716	26/130	Q1	80.385
2010	71/239	Q2	70.502	47/252	Q1	81.548	27/128	Q1	79.297
2009	54/231	Q1	76.840	36/237	Q1	85.021	22/117	Q1	81.624
2008	62/221	Q2	72.172	46/219	Q1	79.224	28/101	Q2	72.772
2007	63/211	Q2	70.379	45/205	Q1	78.293	26/94	Q2	72.872
2006	55/200	Q2	72.750	43/199	Q1	78.643	24/94	Q2	75.000
2005	45/200	Q1	77.750	28/193	Q1	85.751	14/94	Q1	85.638
2004	59/198	Q2	70.455	42/187	Q1	77.807	18/90	Q1	80.556
2003	49/198	Q1	75.505	34/185	Q1	81.892	18/87	Q1	79.885
2002	49/197	Q1	75.381	32/188	Q1	83.245	12/88	Q1	86.932
2001	52/198	Q2	73.990	28/186	Q1	85.215	14/81	Q1	83.333