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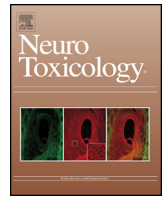
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# Mining the brain metabolome to understand behavioural disruptions induced in mouse fed *Hypochoeris radicata* (L.), a neurotoxic plant for horse



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## ABSTRACT

Mining the brain metabolome to understand behavioural disruptions induced in mouse fed *Hypochoeris radicata* (L.), a neurotoxic plant for horse.

C57BL/6J mice orally exposed to 9% *H. radicata* (HR) are metabolically competent laboratory animals which can be used as model of Australian stringhalt, a neurological horse disease induced by HR ingestion. So, the present study was conducted to assess the brain metabolome and the behavioural performances of mice fed with a 9%-HR-based diet for 21 days. By the end of the period of exposure, mice were investigated for motor activity and coordination, anxiety level, learning and memory performances, social behaviour and rewarding properties of for the plant. Thus, the animals were sacrificed and the brain metabolome was studied using <sup>1</sup>H NMR spectroscopy. HR-exposed mice displayed a motor hyperactivity in several tasks, a less resignation in the forced swimming test, and paradigm place preference for the plant. A bootstrap-based regularized canonical analysis performed on merged behavioural and metabolic datasets showed a clear relationship in HR-treated mice between an increase in cerebral scyllo-inositol, an increased motor activity, and seemingly rewarding properties of HR. These results underlie the interest of such a dual approach to characterize functional end-points of a pathophysiological model of the Australian stringhalt in equine species.

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## 1. Introduction

*Hypochoeris radicata* (HR) is a plant of the Compositae or Asteraceae family (Huntington et al., 1989; Slocombe et al., 1992) widely spread in temperate countries. It is responsible for a neurological disease in horses that pasture on invaded grasslands (Araujo et al., 2008; Araya et al., 1998). This disease, named Australian stringhalt, observed for the first time in Australia in 1848, is in increase for many years because of global climate changes. Indeed, the droughts, facilitating the development of this plant, led to some actual epidemic episodes in late summer and

early autumn in France. Clinically, this neurological disease is characterized in horses, the only target species, by an abnormal gait, an involuntary hyperflexion of both hind limbs during movement, most often, leading to deep functional consequences and possible relapses in horses even without eating the plant again (Domange et al., 2010). Currently, therapeutic treatments remain inappropriate to cure sick animals: neither the toxic dose of this plant, nor the pathogenesis and the pathophysiology of the disease are known. Therefore, a toxicological approach based on the use of global methods seems to be promising to better characterize both the set of unknown target organ disruptions and the classes of secondary metabolites that are putatively responsible for such disruptions. We previously carried out a metabolomic study on C57BL/6J mice (Domange et al., 2008) with the objective to reveal some disruptive metabolic information linked to HR ingestion without reproducing the disease itself. Even though mice did not display any visible clinical disorder, this strain was considered as a

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“metabolically competent” laboratory animal model. Thus, in this species, we showed that HR ingestion has a dose-dependent significant effect on the global metabolism of both liver and brain. We showed that this latter organ could be considered as one of the putative main target tissue in which some metabolic biomarkers were revealed. One of the main metabolic biomarker is the *scyllo*-inositol, a metabolite found in some other neurodegenerative diseases like Alzheimer’s disease (Griffith et al., 2007) and chronic alcoholism (Viola et al., 2004) whereas its stereoisomer, the *myo*-inositol is linked to various behavioural disorders (Barkai et al., 1978; Shimon et al., 1997). Thus, the *scyllo*-inositol related metabolic disturbances observed in the previous metabolomic study we performed (Domange et al., 2008) were indicative of some putative disruptive metabolic pathways involved in HR toxicity. However, the functional consequences of such disrupted metabolic events observed in mice remain to be established. So, the aim of the present study was to investigate various behavioural aspects of 9%-HR treated-mice and the link existing between metabolic and behavioural disturbances. In order to study most of the putatively altered cerebral functions, animals were tested using an appropriate set of behavioural tasks exploring the locomotor activity, the motor coordination, the spatial learning and memory abilities, the levels of anxiety and depression, the social behaviour and the rewarding properties of the plant. These tests include the open-field, the Locotronic<sup>®</sup> apparatus, the Y-maze, the Morris water maze, the elevated-plus maze, the hole-board test, the forced swimming test, the resident-intruder model, and the conditioned place preference test, respectively. Thus, a bootstrap-based canonical analysis was performed to get a correlation between changes in the brain metabolomic fingerprints achieved by <sup>1</sup>H NMR and the current behavioural phenotyping of the same animals.

## 2. Materials and methods

All experiments were conducted under European Union Directive for the use and care of laboratory animals (Directive 2010/63/EU), and were approved by the institutional ethics committee. All efforts were made to minimize animal suffering.

### 2.1. Plant materials

Flowers of HR were collected from eight different pastures colonized by this weed, between August 2005 and November 2005, in the south of France (departments of Pyrénées-Atlantiques and Haute-Garonne) and near Paris (Yvelines) and were freeze-dried by Lyophil (Salon-de-Provence, France). Flowers were hand-picked to discard undesirable plant materials, and vegetable samples were powdered in a grinder and then stored in opaque airtight vials and frozen at  $-20^{\circ}\text{C}$ . A dietary composition analysis of HR powder samples with determination of crude fibre, crude protein, and methionine concentration as well as measurement of fat and nitrogen-free contents was achieved by Lara Europe Analyses (Toulouse, France) to adjust energy and protein contents of the HR-containing diets. The origin and the composition of the pool of flowers used were kept unchanged all along the animal experiments.

### 2.2. Diets

The powder of flowers was mixed with the commercial UAR 210 rodent feed (AUGY, France) to obtain a final concentration of 9% HR incorporated in diet. To ensure a complete covering of nutritional requirements, casein and rapeseed oil were added to the diet, after measuring the nutritional values of the flowers. Finally, diets were stored in hermetically sealed bags and frozen at  $-20^{\circ}\text{C}$  until use.

### 2.3. Animals

Twenty-four 9 week-old C57BL/6J male mice weighing about 20–30 g were purchased from Harlan laboratories (Gannat, France) and were identified by permanent marks on the tail. They were randomly assigned to one of the two experimental groups defined according to the HR dose (control and 9%) and the experiment date. They were housed in plastic cages (one cage per group of three animals) and maintained at a constant temperature ( $22 \pm 2^{\circ}\text{C}$ ) and  $40 \pm 5\%$  of relative humidity on a standard 12-h light-dark cycle (light on from 8.00 p.m. to 8.00 a.m.). The animals had free access to tap water. During the first week, the animals were fed the UAR 210 rodent diet for acclimatization. After that, HR-exposed mice daily received a diet containing 9% HR (5 g/animal/day,  $n = 12$ ) for 21 days whereas the control animals were fed the UAR standard diet (5 g/animal/day,  $n = 12$ ). The body weight was measured weekly, and diet consumption was daily recorded. By the end of this period of HR exposure, both control and HR-treated mice were assessed for their behavioural performances over two weeks during which they were still fed the standard or the 9%-HR diet. During the first week of testing, the animals were evaluated for locomotor activity in the open-field, motor coordination in the Locotronic apparatus, anxiety in the elevated-plus maze and the hole-board test, resignation in the forced swimming test, short-term memory in the Y maze, spatial learning and working memory in the Morris water maze, and social behaviour in the resident-intruder test. The tests were performed according to the following order during the 3 days after 21 days of 9%-HR exposure: day 1: elevated-plus maze, Locotronic apparatus and Morris water maze, day 2: Y maze, open-field and forced swimming test, and day 3: hole-board apparatus and resident-intruder test. Then, the reinforcing properties of the plant in a conditioned place preference paradigm were studied during a second one-week-long behavioural experiment. After the behavioural testing, the animals were sacrificed by exsanguination. The brain was excised behind the optic nerve section, removed and immediately frozen in liquid nitrogen, then stored at  $-80^{\circ}\text{C}$  until the NMR spectroscopic analysis.

### 2.4. Behavioural studies

The experimental sessions were conducted every day during the dark phase of the cycle, between 9:00 and 12:00 a.m. under the red dim light. All tests were recorded by video camera and videotapes were subsequently scored by a trained observer using the Observer XT software (Noldus, Wageningen, The Netherlands).

#### 2.4.1. Open field

General motor activity was assessed on a circular open-field platform (diameter, 50 cm  $\times$  27 cm high walls, with the floor divided into 36 squares delimiting peripheral, medium and central circular areas). After a 1-min adaptation period, the mouse was placed in the centre of the maze, and was free to explore it for 5 min. The number of squares crossed with the four paws was used to measure general motor activity. Between each animal, the maze was cleaned with a 10%-ethanol solution.

#### 2.4.2. Locotronic<sup>®</sup> apparatus

The apparatus (Locotronic<sup>®</sup>, Intelli-Bio, France) was composed of a starting box (10 cm  $\times$  5 cm  $\times$  20 cm, white plastic walls, illuminated by cold white light), a corridor (75 cm  $\times$  5 cm  $\times$  20 cm) and an arrival box (10 cm  $\times$  5 cm  $\times$  20 cm, black plastic walls). The corridor was a flat ladder (bars: 3 mm diameter, spaced by 7 mm); three infrared sensors were placed above and under each bar space. The sensors were read 100 times per second to monitor the position, the number and the duration of missteps of the animal. Motor coordination and learning capacity were investigated by comparing the time spent in the apparatus and the number of leg errors

between the two trials performed 10 min apart. After each test, the apparatus was cleaned with a 10% ethanol solution.

#### 2.4.3. Elevated-plus maze

This maze consisted of a wood cross elevated to a height of 22 cm with two open (5 cm × 25 cm) and two closed arms having walls (5 cm × 20 cm × 25 cm), arranged in a manner that the arms of the same type were opposite to each other and connected by a common open central platform (5 cm × 5 cm). At the beginning of the session, the mouse was placed at the centre of the maze always faced to the same open arm. Then, the animal was let free to explore the maze for 5 min. Standard spatiotemporal measures were scored including the number of entries in the open and the closed arms, the total number of arm entries, and the cumulative time spent in the different parts of the maze (open and closed arms). An arm entry is defined if all four paws of the animal are into the arm (Rodgers and Johnson, 1995). The ratio of open arm entries and closed arm entries to the total number of arm entries was also calculated. Between each animal, the maze was cleaned with a 10% ethanol solution.

#### 2.4.4. Forced swimming test

The apparatus consisted of a Plexiglas cylinder (height: 25 cm; diameter: 10 cm) filled with water (height: 6 cm, temperature: 21–23 °C) such as a mouse cannot touch the bottom of the cylinder (Porsolt et al., 1979). The mouse was dropped into the cylinder one at a time and left there for 10 min. Then, the duration of swimming and immobility was scored. A mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water making only those movements necessary to keep its head above water.

#### 2.4.5. Hole board test

The maze consisted of a board (36 cm × 36 cm × 0.5 cm) elevated to the height of 80 cm with 16 equally spaced holes (3.5 cm internal diameter, four lines of four holes each). The mouse was placed at the centre of the apparatus and was allowed to freely explore the maze for 5 min. The number of head-dippings was scored in each of the 3 different parts of the maze (outside the maze, peripheral holes and central holes). A mouse was considered to make a head dipping if both eyes disappeared into the hole (do-Rego et al., 2006). After each test, the apparatus was cleaned with a 10% ethanol solution.

#### 2.4.6. Y maze

The maze was made of black painted wood. Each arm was 25 cm long, 14 cm high and 5 cm wide, and positioned at equal angles. The mouse was placed at the end of one arm and allowed to move freely through the maze during a 10-min session. The series of arm entries was recorded. An arm entry was considered to be complete when the four paws of the animal were completely placed in the arm. Alternation was defined as successive entries into the three arms on overlapping triplet sets. The percentage of spontaneous alternation was calculated as the ratio of actual successful overlapping alternations by the total possible triplets (defined as the total number of arm entries minus two) multiplied by 100, and was considered as representative of the immediate learning performance (Hughes, 2004). After each test, the apparatus was cleaned with a 10% ethanol solution.

#### 2.4.7. Morris water maze

The Morris water maze was made of white opaque plastic (diameter: 60 cm; walls: 27 cm in height) and filled with water (22 °C). Powder milk was evenly spread over the water surface in order to cover up the escape platform (10 cm × 10 cm). The pool was contained in a room with an extra maze visual cue. Mice were

placed next to the wall opposite the cue and were allowed to explore the maze to find the platform. Then, they stayed on the platform for at least 5 s. When they failed to reach the escape platform within 3 min, they were retrieved from the pool and placed on the platform for the same duration. Five trials were carried out with the platform and the escape latency quantified for each trial. In a sixth trial, the platform was removed and the behaviour of each mouse was assessed by measuring the time spent and the number of entries in the upper quarter of the maze. This sixth trial was carried out during a 3-min period.

#### 2.4.8. Resident-intruder test

The test was performed in the cage of a non-treated mouse that belonged neither to the control group nor to the HR-treated one. After 8 days of individual housing, a control animal or a 9% HR-treated mouse designed as the intruder was placed in the home cage of a neutral mouse named the resident. Twenty-four neutral mice were used, each being randomly allocated to a control or a HR-treated mouse. Behaviour was videotaped and scored during a 5-min session. All the following variables were measured in the resident: (1) the time of the first interaction; (2) the number of attacks displayed by the resident; (3) the exploration of the cage (duration and number of periods); (4) the exploration of the litter (duration and number of periods); (5) the number of rearing; (6) the number of grooming; (7) the period of immobility (duration and number); (8) the sniffing of the intruder (duration and number of periods); (9) the escape from the intruder (duration and number of periods).

#### 2.4.9. Conditioned place preference test

The apparatus consisted of a rectangular wood box (50 cm × 25 cm × 40 cm) with two compartments separated by a door. The compartments were differentiated by the floor texture (smooth or rough). The floor of the standard compartment was made of wood whereas the rough compartment's one was covered by sandpaper. The conditioned place preference test comprised 3 phases. In a first phase, the mice were placed for 30 min in the apparatus, and were allowed to freely explore the two compartments. This phase aimed to determine the preference of each mouse for one of the two compartments as reflected by the time spent in each of them. In a second phase, the mice were placed 10 min twice a day and every day during one week, in the rough compartment with the closed door and with a little cup containing 1 g of HR powder. This phase aimed to allow the mice to associate the rough compartment with HR. In the last phase, the door was opened and each mouse was allowed to freely explore the two compartments for 30 min. All over this test, the quantity of HR given to the HR-treated mice was the same as during the period of exposure (5 g/animal/day) divided into 2 × 1 g during the two daily sessions of the conditioned place preference test, and the last 3 g when back in the home cages. The time spent in each compartment during the first and the last phases was measured.

#### 2.5. <sup>1</sup>H NMR metabolomic fingerprinting of brain tissue extracts

The organ extraction method used here, which derives from the Folch procedure (Folch et al., 1951) and is adapted from the one described by Waters et al. (2002), allowed us to prepare lipophilic and hydrophilic extracts. Samples of brain taken from the hemiencephalon of a mouse without olfactory bulbs or cerebellum were processed as follows. After approximately 100 mg of tissue was thawed and weighed, each sample was homogenized in 2 mL of acetonitrile/water (50/50, v/v) in an ice/water bath for 30 s with a Kinematica Polytron<sup>®</sup> homogenizer and then centrifuged at 3000 × g for 10 min at 4 °C. The supernatant (aqueous extract) was removed and freeze-dried before reconstitution in 600 μL of D<sub>2</sub>O



and then frozen at  $-20^{\circ}\text{C}$  until NMR analysis. All along the extraction procedure, all samples were preserved in an ice/water bath. The reconstituted solutions were transferred to 5 mm NMR tubes.  $^1\text{H}$  NMR spectra of brain tissue extracts were acquired at 300 K on a Bruker DRX-600 Avance spectrometer at 600.13 MHz with 1D  $^1\text{H}$  “Improved Watergate” for achieving satisfactory water suppression on aqueous extracts. For each sample, 128 transients were collected into 64 K data points with a relaxation delay of 2 s. A 12-ppm spectral width was used. The data were apodized with an exponential function using a line broadening of 0.3 Hz prior to Fourier transformation. All NMR spectra were phased, and the baseline was corrected and then submitted to a bucketing procedure.

## 2.6. Statistical tests

Given that most behavioural data do not follow a Gaussian distribution, non-parametric statistical procedures were used. Data from controls were compared to those of HR-exposed mice using a Mann–Whitney test. Paired repeated measures were compared using a Wilcoxon procedure whereas the repeated measures obtained in the Morris water maze were analyzed by using a Friedman test for within-groups analysis, followed by a *post hoc* modified Wilcoxon test. For the behavioural statistical analysis, the level of significance used was  $p < 0.05$ .

Multivariate analysis of  $^1\text{H}$  NMR data acquired on brain extracts have been performed using principal component analysis (PCA) to check presence of outliers, partial least-squares-based discriminant analysis (PLS-DA) to discriminate the two populations and orthogonal signal correction based on PLS regression (OPLS) to correct NMR data before performing factorial analysis (Trygg and Wold, 2002). To establish a canonical link between the two multidimensional data sets, *i.e.* the one containing the  $^1\text{H}$  NMR fingerprints of hydrosoluble brain extracts and the behavioural data set comprising nearly 100 variables, a regularized canonical analysis was performed using mixOmics, a R package (Le Cao et al., 2009).

Because behavioural data were obtained on all animals but metabolomic data could be obtained only on the half part of the two groups of animals, a bootstrap-based procedure to repeatedly perform canonical analysis between behavioural data on one part and on metabolomic data on the other part was developed. This simple bootstrap-based procedure belongs to a more generic one used to reduce the information dimension of large datasets (Ye and Weiss, 2003). First, both data sets were corrected for multidimensional residual variance, which is orthogonal to the model (*i.e.* HR intoxication or not), using an OPLS-based procedure, which was written in R from code developed by Wise and Gallagher (<http://www.eigenvector.com/MATLAB/OSC.html>). Then, behavioural variables studied on the two consecutive one-week-long animal experiments were repeatedly merged ( $n = 500$ ) to a bootstrap NMR data set built from metabolomic variables measured on half part of the total number of animals after completion of the second one-week-long animal experiment, keeping an intra group structure for merging data. Indeed, this procedure was possible only because the brain metabolomic biomarkers were repeatedly found as *scyllo*- and *myo*-inositols in past animal experiments (Domange et al., 2008, and numerous unpublished metabolomic observations). For every bootstrap, a regularized canonical analysis was performed using the R-based mixOmics library and the resulting coordinates of variables projected on the two first principal components were kept. At the end of this bootstrap-based procedure, because only two groups of individuals were studied, it was necessary to adjust the polar coordinates for all variables to calculate a mean angular coordinate for every variable from the 500 bootstraps. Six leading variables, four corresponding to the chemical shifts of

*scyllo*-inositol and *myo*-inositol, on the one hand, and two corresponding behavioural variables (the period spent in the standard compartment at the end of the conditioned place preference test and the period spent in the deterrent compartment at the end of the same test) on the other hand, all recorded on the second one-week-long animal experiment, were used to calculate a mean angle rotation correction applied to every bootstrap. On the resulting factorial map, only variables displaying a distance from centre above 0.45 and 0.30 for NMR and behavioural variables, respectively, were projected.

Data analysis were performed using the SPSS 11.5 for Windows software (SPSS Inc., Chicago, IL, USA), Simca-P-11.0 (Umetrics, Umea, Sw), Matlab 7.6 (Mathworks, Natick, MA) and R (R Development Core Team).

## 3. Results

### 3.1. Behavioural studies

#### 3.1.1. Open field

In this test, control animals and 9% HR-treated mice crossed a similar number of squares (control: 288 (270–337), 9% HR: 296 (254–326), medians and quartiles), showing that the HR exposure did not affect the locomotor activity of the animals.

#### 3.1.2. Locotronic<sup>®</sup> apparatus

Within each of the two trials performed in this test, the locomotor activity was not significantly impaired by HR exposure as reflected by the same time spent to cover the corridor measured in the two groups. When comparing the performances between the two trials, the time spent in the apparatus was significantly decreased in each group (trial 1, control: 12.2 s (9.5–16.7), 9% HR: 12.5 s (9.8–14.3); trial 2, control 8.2 s (6.4–9.8), 9% HR: 7.4 s (6–10.6), medians and quartiles). Such results showed an increase in the activity of the animals related to the learning of the maze between the two trials and suggested the lack of motor coordination impairments in the HR-treated mice. Indeed, the number of leg errors measured in HR-treated mice was the same compared to controls and remained unchanged in both groups between the two trials (trial 1, control: 5 (3.75–6.75), 9% HR: 6.5 (3.5–8.25); trial 2, control: 6.5 (4.75–8.75), 9% HR: 6 (4–8.5), medians and quartiles).

#### 3.1.3. Elevated-plus maze

No significant variation in the variables measured in this maze was observed between the two groups, including the total number of arm entries (control: 18 (12–20), 9% HR: 18.5 (13–21.5), medians and quartiles), the open and closed arm entries (data not shown), and the time spent in both parts of the maze (open arms: control: 72 s (21–89), 9% HR: 71 s (33–98); closed arms: control: 105 s (72–151), 9% HR: 107 s (85–143), medians and quartiles).

#### 3.1.4. Forced swimming test

The latency before the 1st period of immobility was significantly increased in HR-treated mice compared to controls (+60%), which is correlated with a 24% decrease of the duration of immobility, measured in the same animals (Table 1). However, the reduction of this immobility duration was not significant. Such results showed that the HR-treated mice expressed a less depressive-related behaviour compared to controls and exhibited a lower level of resignation when faced to an inescapable environmental constraint.

#### 3.1.5. Hole board test

The number of visited holes in internal board was significantly increased in 9% HR-treated mice compared to controls (Table 2). In

**Table 1**  
Effects of HR exposure on the behaviour of mice in the forced swimming test.

	Control	9% HR
Time before the 1st period of immobility (s)	70 (56–89) <sup>a</sup>	112 (69–301) <sup>a</sup>
Mobility time (s)	282 (156–322)	360 (225–452)
Immobility time (s)	318 (278–444)	240 (148–375)

Results are expressed as median and quartiles (in brackets).

<sup>a</sup>  $p \leq 0.05$ , statistical significant difference compared to control mice (Mann-Whitney *U*-test).

**Table 2**  
Effects of HR exposure on the behaviour of mice measured in the hole board test.

Number of head-dippings	Control	9% HR
Total	40 (37–47)	54 (34–61)
In internal board	7 (6–9)	11 (7–18) <sup>a</sup>
In external board	21 (19–25)	26 (19–28)
Outside the board	14 (7–22)	11 (7–16)

Results are expressed as median and quartiles (in brackets).

<sup>a</sup>  $p \leq 0.05$ , statistical significant difference compared to control mice (Mann-Whitney *U*-test).

the same time, the number of head-dippings displayed outside the board decreased but it was not significant ( $p = 0.12$ ). Thus, the total number of visited holes and the number of visited holes in external board were similar whatever the group considered (Table 2).

### 3.1.6. Y-maze

The 9% HR-treated mice did not show any learning disabilities as reflected by their similar percentage of spontaneous alternation compared to controls (Table 3). However, HR ingestion had a significant effect on the activity of mice in the maze. Indeed, the total number of arms visited and the number of arms visited per minute were significantly increased in HR-treated mice referred to the control animals (Table 3). Over the period of testing, non-significant differences were observed between the two groups during the first 6 min of the test whereas the number of arms visited during the last 4 min of testing was significantly higher in HR-treated mice compared to controls (Table 3).

### 3.1.7. Morris water maze

The escape latency of both control and 9% HR-treated animals significantly decreased from trial 1 to trial 5 (Fig. 1A), indicating that these animals successfully learned the location of the platform. During the 6th trial performed without the platform, the 9% HR-treated mice spent more time in the upper quarter of the maze than the control mice ( $p = 0.06$ , Fig. 1B) and did fewer entries than the control mice in this part of the maze ( $p < 0.05$ , Fig. 1B).

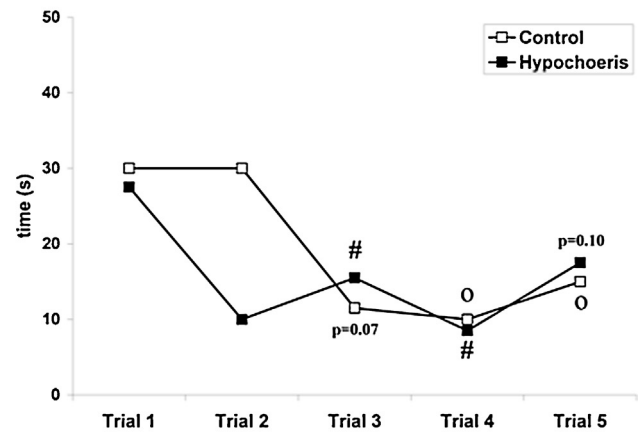
**Table 3**  
Spontaneous alternation performances of 9% HR-treated mice in the Y-maze.

	Control	9% HR
% spontaneous alternation	64.2 (56.6–69.9)	69.4 (66.7–77.5)
Total number of arm entries	44 (41–45)	47 (44–52) <sup>a</sup>
Number of visited arm(s) (per min)	4.4 (4.1–4.5)	4.7 (4.4–5.2) <sup>a</sup>
Number of visited arm(s) (during the 1st min)	7 (7–8)	8 (7–8)
Number of visited arm(s) (during the 2nd min)	6 (5–7)	7 (6–8)
Number of visited arm(s) (3–6 min)	16 (14–17)	17 (16–19)
Number of visited arm(s) (7–10 min)	13 (12–17)	17 (15–18) <sup>a</sup>

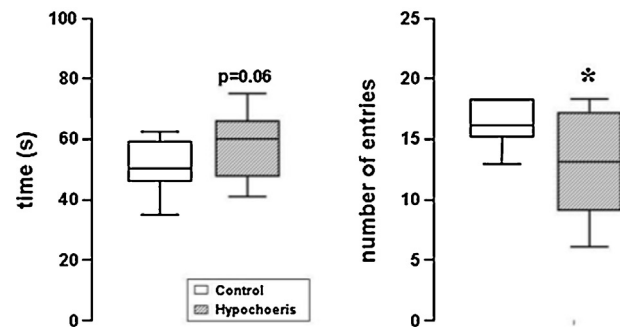
Results are expressed as median and quartiles (in brackets).

<sup>a</sup>  $p \leq 0.05$ , statistical significant difference compared to control mice (Mann-Whitney *U*-test).

## A) With the platform



## B) Without the platform



**Fig. 1.** Morris water maze: effects of HR exposure on the spatial learning performances. (A) Time spent by the control or the HR-treated animals to learn the platform over 5 consecutive trials and (B) time spent and number of entries in the quarter of the maze where the platform was located over a 3-min session without the platform. Data are expressed as median and quartiles. <sup>a</sup> $p < 0.05$ , statistical significant difference referred to trial one in control mice (Wilcoxon paired test), <sup>#</sup> $p < 0.05$ , statistical significant difference referred to trial one in HR-treated animals (Wilcoxon paired test). \* $p < 0.05$ , statistical difference between controls and 9% HR-treated mice (Mann-Whitney test).

### 3.1.8. Resident-intruder test

Faced with a control animal or a 9% HR-treated mouse as the intruder, the behaviour of the resident remained the same whatever the group considered except for variables related to the exploration of the cage and the interactions with the intruder (Table 4). Indeed, the resident mice explored their environment to a lesser extent when faced by the decrease in the number of periods ( $p < 0.05$ ) and the duration ( $p = 0.11$ ) of exploration of the cage, and by the reduction in the number of periods ( $p = 0.06$ ) and the duration ( $p = 0.13$ ) of exploration of the litter, the decreased number of exploration of the cage ( $p < 0.05$ ), and their duration ( $p = 0.11$ ), but also by the number of periods of exploration of the litter ( $p = 0.06$ ) and their duration ( $p = 0.13$ ). Some behavioural modifications regarding the interaction between the resident and the intruder have been noticed with 9% HR-treated intruder compared to controls. Indeed, the resident animals displayed a higher number of escapes and higher number of attacks, but only the first difference was significant (Table 4).

### 3.1.9. Conditioned place preference test

On the first day of testing, no significant difference in the time spent in the non-preferred compartment was observed between the two groups (Fig. 2). After one week of daily exposure twice a day to the non-preferred compartment reinforced with a cup of HR

**Table 4**  
Effects of HR exposure on social behaviour in the resident-intruder test.

	Control	9% HR
Time of the first interaction (s)	2 (1–3.75)	2 (1–3)
Number of attacks displayed by the resident	1.5 (0–5.5)	5 (1–6)
Exploring the cage		
Duration (s)	90.5 (56.75–134)	40 (4–121.5)
Number	14 (10–16)	7.5 (2.25–11.25) <sup>a</sup>
Exploring the litter		
Duration (s)	7.5 (0–61)	0 (0–2.5)
Number	2.5 (0–14.25)	0 (0–2.25) <sup>p = 0.06</sup>
Rearing (number)	5.0 (1.75–5)	4 (0–11.5)
Grooming (number)	8.5 (5–11.75)	7 (4.5–10.5)
Period of immobility		
Duration (s)	6 (0–20.5)	4.5 (0.75–7.75)
Number	4.5 (1–10.75)	3 (1.5–5)
Sniffing the intruder		
Duration (s)	140 (66.25–167.5)	128.5 (79.75–184.25)
Number	12.5 (10.25–20)	13 (8.75–16.5)
Escape the intruder		
Duration (s)	0 (0–0)	1.5 (0–28)
Number	0 (0–0)	1 (0–7.25) <sup>a</sup>

All the measuring variables concern the resident.

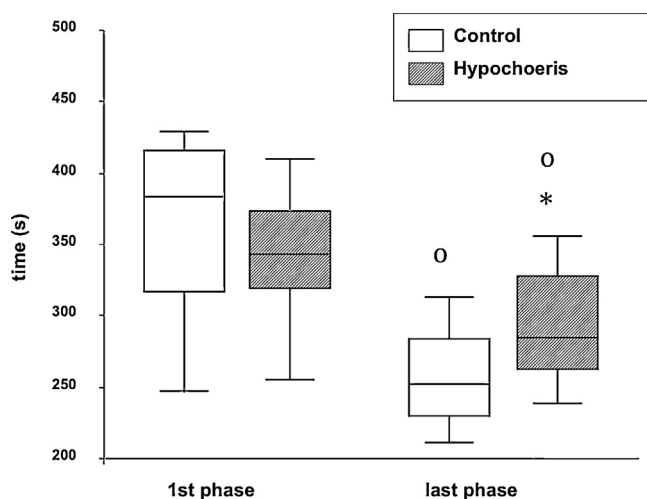
Results are expressed as median and quartiles (in brackets).

<sup>a</sup>  $p < 0.05$ , statistical significant difference compared to control mice (Mann–Whitney  $U$ -test).

powder, the HR-treated mice spent more time in this compartment compared to controls ( $p < 0.05$ ) although the animals were allowed to freely explore the two compartments of the maze. When comparing this time between the first and the last day of the test, a significant decrease was observed in both groups but it was less important in HR-treated mice than in control animals (Fig. 2).

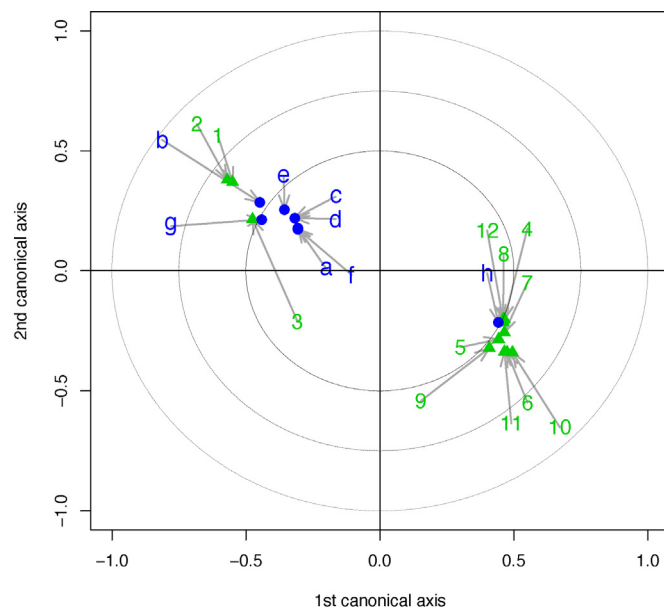
### 3.2. Metabolomic study

The two  $^1\text{H}$  NMR spectra from aqueous extracts of brain in 9% HR-treated mice and controls display a similar metabolic profile but one peak is higher in 9% HR-treated mice than in controls (not shown). It could be identified as *scyllo*-inositol, the same metabolite found as significantly increased in HR-treated mice in the previous study (Domange et al., 2008). In order to detect other metabolic differences between the two groups than a significant increase of *scyllo*-inositol in HR-treated mice, some



**Fig. 2.** Effects of HR exposure on the time spent in the non-preferred compartment in the conditioned place preference test. Data are expressed as median and quartiles of 12 mice per group. \* $p < 0.05$ , statistical difference between controls and 9% HR-treated mice ( $t$ -test). <sup>o</sup> $p < 0.05$ , statistical difference between the 1st and the 7th day of experiment ( $t$ -test for repeated measures).

multifactorial statistical analysis such as O-PLS have been performed. The O-PLS model has been applied with cross-validation to confirm the robustness of the model. The validity of such a model was attested by high values of the goodness-of-fit parameters  $R^2X$  and  $Q^2Y$  (0.648). A significant segregation between 9% HR-treated mice and controls was observed, enabling us to define some specific metabolotypes of one of the two groups. The loadings plot displayed the *scyllo*-inositol ( $^1\text{H}$  NMR corresponding variables:  $\delta = 3.34$  ppm and  $\delta = 3.35$  ppm) and the *myo*-inositol ( $^1\text{H}$  NMR corresponding variables:  $\delta = 4.06$  ppm;  $\delta = 4.07$  ppm;  $\delta = 3.63$  ppm;  $\delta = 3.53$  ppm) as the two main metabolites involved in the discrimination between the 9% HR-treated mice and the control ones (not shown). These results are in agreement with previous data (Domange et al., 2008). In order to proceed to a combined analysis of behavioural and metabolomic data, a bootstrapped canonical analysis ( $n = 500$ ) has been performed on  $^1\text{H}$  NMR variables ( $X$  matrix) and behavioural variables ( $Y$  matrix) coming from the two consecutive one-week-long behavioural animal experiments. Two orthogonal components were calculated by O-PLS to conveniently correct data keeping nearly 50% of the initial information. A good separation of the two groups of animals was obtained (not shown). The main loading variables obtained in this analysis (Fig. 3) correspond to *scyllo*-inositol chemical shifts ( $^1\text{H}$  NMR corresponding variables at  $\delta = 3.34$  and  $\delta = 3.35$  ppm, i.e. labels 2 and 1, respectively) which are positively correlated to the HR-treatment, but also to the total duration spent in the deterrent compartment at the end of the conditioned place preference test on day 7 (label g), with the three parameters of the Porsolt test, i.e. latency time before resignation (label b), time (label c) and percentage of mobility (label d), with the number (label e)



**Fig. 3.** Canonical analysis. Projection on the two first axes of the bootstrap-based regularized canonical analysis ( $n = 500$ ) performed on an OSC-corrected metabolomic ( $X$  matrix) and behaviour ( $Y$  matrix) datasets of the main loadings, i.e.  $^1\text{H}$  NMR variables (in green, distance  $> 0.45$ ) and behavioural parameters (in blue, distance  $> 0.30$ ). With a: time spent to reach the platform in the 5th trial in the Morris test; b: latency time before resignation in Porsolt test; c: time of mobility in Porsolt test; d: percentage of mobility in Porsolt test; e: number of entries in arm B in the Y maze; f: percentage of entries in arm B in the Y maze; g: the total duration spent in the deterrent compartment at the end of the conditioned place preference test; h: total duration spent in the standard compartment at the end of the conditioned place preference test or day 7; 1 and 2: *scyllo*-inositol  $^1\text{H}$  NMR corresponding variables; 5, 6, 9 and 11: *myo*-inositol  $^1\text{H}$  NMR corresponding variables. Other  $^1\text{H}$  NMR variables: 3, 4, 7, 8, 10 and 12. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and the percentage (label f) of entries in arm B in the Y-maze and with the time spent to reach the platform in the 5th trial in the Morris test (label a). On the contrary, *myo*-inositol chemical shifts ( $^1\text{H}$  NMR corresponding variables at  $\delta = 4.06$  ppm,  $\delta = 4.07$  ppm,  $\delta = 3.63$  ppm,  $\delta = 3.53$  ppm, *i.e.* labels 6, 5, 9, 11, respectively) are correlated to the absence of exposure to HR, but also to the total duration spent in the standard compartment at the end of the conditioned place preference test (label h) (Fig. 3).

#### 4. Discussion

The present toxicological study aimed to draw up a global behavioural fingerprint of mice fed a diet containing 9% HR for three weeks by testing the animals for several behavioural units including motor activity and coordination, learning and memory, anxiety, depression, social behaviour and rewarding properties of the plant. Even though this study was not able us to show in mice any behavioural disturbances comparable to those observed in intoxicated horses displaying an Australian stringhalt, originality of this study is supported by investigating the behavioural consequences of the previously described HR-related metabolic disruptions (Domange et al., 2008), and by exploring of some putative functional relationships existing between these two highly integrated levels (*i.e.* metabolism and behaviour) by using a convenient statistical procedure, a bootstrapped regularized canonical analysis. Results from the present study may suggest some aetiological hypotheses of the HR-induced pathogenesis in horse (Domange et al., 2011).

##### 4.1. Behavioural impairments observed in HR-exposed mice

In the open-field and the Locotronic tests, no significant impairment of the locomotor activity or the motor coordination was observed in the HR-exposed mice compared to controls. These results could be surprising regarding the clinical description of the disease in horses. However, it should be remembered that the purpose of the study was not to replicate in a rodent model the clinical signs of the disease, but to characterize the behavioural and metabolic clues in a subclinical step during which the organism tends to adapt itself to extrinsic perturbations to stabilize its homeostasis. Indeed, the model of HR exposure developed in mice showed a specific cerebral impairment in the *myo*-inositol/*scyllo*-inositol metabolic balance as previously described (Domange et al., 2008). Although motor activity and coordination measured in these two mazes remained both unaffected, it has to be noticed that HR-treated animals displayed a significant increase in the level of activity measured in the hole board test and the Y maze, as reflected by the increase in the number of holes visited (Table 2) and the increase in the total number of arms visited (Table 3), respectively. Such a level of activity seems to be related to a higher level of motivation of the HR-exposed animals to explore both mazes rather than to a direct motor stimulating. Compared to controls, mice exposed to HR showed some behavioural modifications which are related to a lower degree of resignation as demonstrated by the decrease in the duration of immobility observed in the forced swimming test (Table 1), the higher level of activity in the Y maze, especially during the last 3 min of the test (Table 3), and the persistence to explore the quarter of the Morris water maze where was located the platform over a 3-min session after the platform was removed (Fig. 1).

Regarding results of the two memory tests used, *i.e.* the Y maze and the Morris water maze test, no modification in learning and memory processes was detected. Indeed, the percentage of spontaneous alternation measured in the Y maze was the same in the two groups (Table 3), whereas the spatial learning performances similarly improved over the 5-trial training period

of the Morris water maze in both groups (Fig. 1). Such results suggest that HR exposure does not induce in mouse alteration of the hippocampus integrity nor memory-related brain function (D'Hooge and De Deyn, 2001).

Regarding anxiety, the 9% HR-treated mice did not display any behavioural modification in the elevated-plus maze, one of the tests most frequently used in behavioural psychopharmacology to assess the potential anxiolytic properties of drugs (Wall and Messier, 2000). However, it has to be noticed that the control animals could be divided into two subgroups based on marked differences in anxiety-related behaviour. Indeed, 6 control mice showed a low level of anxiety as reflected by a percentage of open arm entries higher than 60% whereas the 6 other ones exhibited a higher level of anxiety reflected by a lower number of open arm entries (less than 40%). Thus, the HR ingestion abolishes this strain-specific feature leading to only one group of animals presenting a low level of anxiety as reflected by a 40–50% of open arm entries (Landgraf and Wigger, 2002). This putative impact of HR exposure could be explained by the reduction of the level of anxiety in the initially most anxious animals, without having any effect in the less anxious ones. This result was in good agreement with data issued from the hole board test (Table 2), which showed a decrease in anxiety of the 9% HR-treated animals as indicated by a significant increase in the number of holes visited when they were in a reassuring context (hole board centre), and conversely, a decrease trend when they were located in a risky situation (hole board edges).

In the social interaction test, the resident mice explored their cage less frequently and displayed more interactions or flights when faced to the HR-exposed animals as intruders compared to controls. Such results are in favour of a reduction of anxiety in HR-exposed mice compared to controls with, as a consequence, an increase in the level of interactions displayed by the resident towards the intruder (File, 1980; File and Seth, 2003; Kaidanovich-Beilin et al., 2011). These results may also suggest a higher level of aggressiveness in the HR-exposed mice, leading the residents to have more interactions with intruders in order to preserve their territory or to move towards the intruder (Miczek et al., 2001). The behavioural modifications measured in the resident-intruder paradigm in HR-exposed mice could be correlated with the low level of resignation observed with the same animals in the forced swimming test (Porsolt et al., 1977). Indeed, the same kind of correlation has been reported by Bilkei-Gorzo et al. (2002) in a study investigating both anxiety and depression in mice deleted for genes encoding the neuropeptides substance P and neurokinine A.

A last behavioural feature of the 9% HR-treated mice was related to the rewarding properties of the plant as showed by the results obtained in the conditioned place preference test. By the end of the test, the HR-treated mice spent significantly more time than controls in the deterrent compartment. This test was reinforced with the presence of a cup containing the HR powder (Fig. 2). Interestingly, this result could be linked to a veterinarian's observation made on two cases of intoxicated horses in which an HR preference was observed. Indeed, even if green and good grass was present in a small-delimited square, it has been observed that these horses systematically chose HR flowers for eating first (Domange et al., 2010). Such behaviour may then partially explain the relative frequency of poisoning and suggest that a vicious circle arises due to continued preferential ingestion of HR in horses. It is also established that an interaction between rewarding behaviours and depression-like behaviours does exist. However, resulting effects regarding drug-reward exposure like morphine on the depression-like behaviour are contradictory and the meaning by which the depressive-like behaviours affect addictive behaviour still remains unknown (Tian et al., 2011). Thus, the HR-exposed

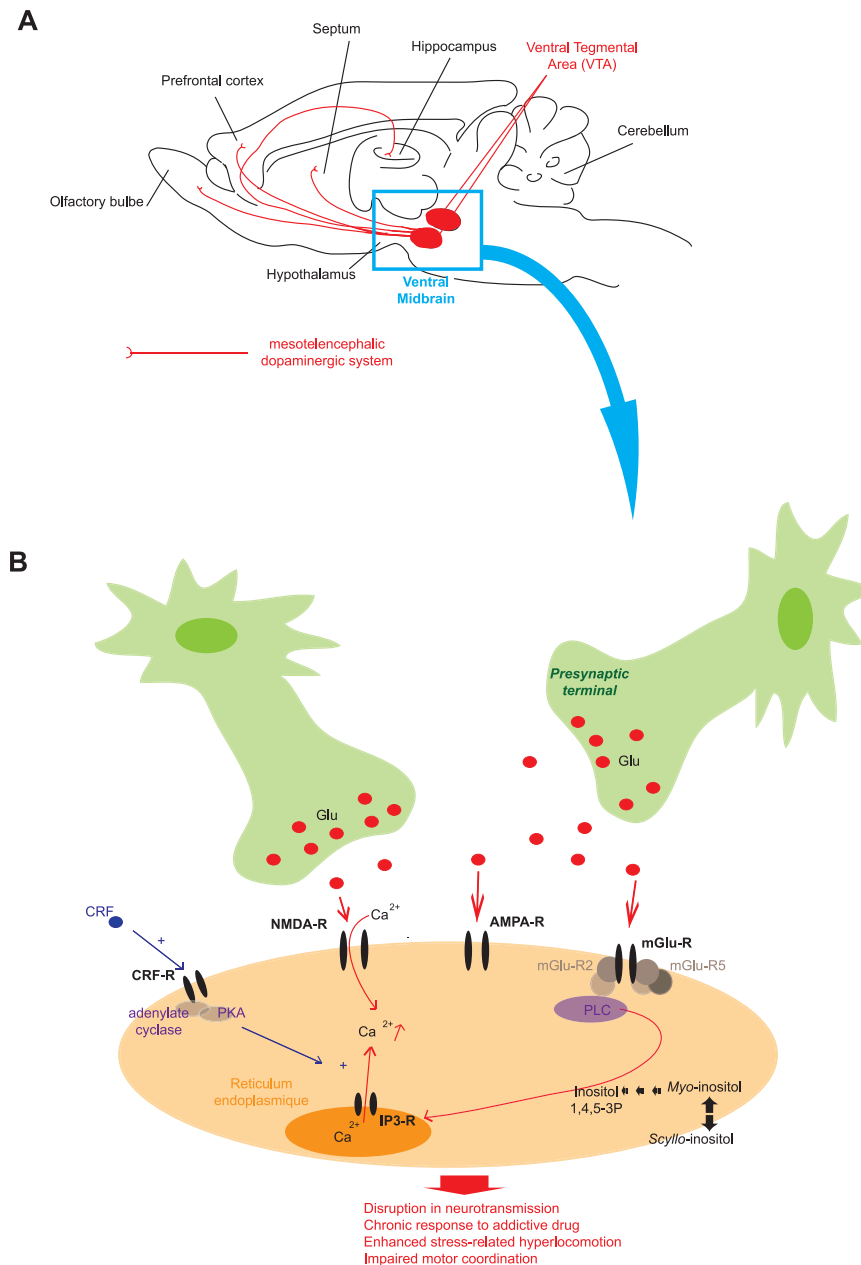


mice exhibited both a reduction of the “behavioural despair” and a conditioning place preference for the plant, suggesting the ability of this plant to modulate in a subtle way the brain metabolism involved in the regulation of different but related behavioural features like depression, anxiety and reward.

#### 4.2. Canonical analysis between behavioural and metabolic datasets

The metabolomic results obtained *via* the  $^1\text{H}$  NMR analysis of aqueous brain extracts of the same HR-exposed mice confirmed the presence of a cerebral impairment in the *myo*-inositol/*scyllo*-inositol metabolic balance as previously suggested (Domange et al., 2008). This confirms the fact that *scyllo*-inositol levels were

increased in urine, liver and brain in HR-treated animals, suggesting it was as a reliable biomarker of HR-induced disruptions. Based on the known metabolic functions of inositols, a functional association of some behavioural units with this main metabolic disruption has to be questioned. Because behavioural tests were performed in two consecutive one-week-long experiments performed on the same animals, and brain metabolomic data were obtained only on the half part of animals at completion of the second one-week-long behavioural experiment, a bootstrap-based regularized canonical analysis has been achieved to correlate the first metabolomic principal components (PC1 and PC2) with the two first behavioural PCs. Thanks to such a robust procedure, this has clearly demonstrated the existence of a specific



**Fig. 4.** Supposed metabolic pathways involved in the behavioural and metabolic HR-induced disruptions in the brain of treated mice. (A) The dopaminergic system seems to be affected. The cell bodies of neurons project to different brain areas from prefrontal cortex to caudal brain. (B) The osmolyte imbalance, in particular the altered calcium signalling system is due to an activation of phospholipase C (PLC) *via* the action of metabotropic glutamate receptors (mGlu-R). The CRF (corticotropin release factor) receptor, activated by stress-induced CRF release activates adenylylate cyclase and phosphokinase A (PKA), then potentiates the release of calcium from intracellular stores gated by inositol-triphosphates receptors (IP3-R) too. Finally, an impaired neurotransmission and an amplified stress-related hyperlocomotion could arise in response to a chronic exposure to an addictive drug.

correlation between the brain levels of *myo*- and *scyllo*-inositol on one side and behavioural variables related to depression and conditioning place preference for the plant in the other side (Fig. 3). *Myo*-inositol is the main inositol found in the mammalian and is measured in many tissues, including brain, liver and kidney (Sherman et al., 1968a,b). In the brain, the concentration of *scyllo*-inositol is very low compared to *myo*-inositol, and numerous studies have already shown that changes in *scyllo*-inositol concentrations may be relevant to several neuropathological diseases. Indeed, an increased brain level of *scyllo*-inositol was observed in various central nervous system disturbances like chronic alcoholism (Viola et al., 2004) or Alzheimer's disease (Griffith et al., 2007). Then, the results obtained in the present study are also indicative of a putative involvement of *scyllo*-inositol in mood disorders and rewarding behaviour. Indeed, the increase in brain *scyllo*-inositol concentrations observed in the HR-exposed animals is highly correlated with the increase in time spent in the deterrent compartment of the conditioned place preference test and the reduction in the duration of immobility in the forced swimming test (Fig. 3). Hence, the high cerebral level of *myo*-inositol measured in controls is projected at the opposite place of *scyllo*-inositol detected preferentially in brain of HR-exposed mice (Fig. 3). *Myo*-inositol has been shown to have antidepressant and anxiolytic properties in both humans and animals (Bersudsky et al., 1999; Einat et al., 1999, 2001; Einat and Belmaker, 2001; Kofman et al., 1998; Levine, 1997). Recently, the proteomic and metabolomic study published by Zhang et al. (2011) showed the same kind of uncoupling between two inositol isomers (*allo*- vs *myo*-inositol) in a mouse model presenting with robust high vs low anxiety phenotypes. Indeed, higher levels of *myo*-inositol in plasma (Bersudsky et al., 1999; Zhang et al., 2011) were observed in low-anxiety level mice compared to the more anxious animals. In the present study, the imbalance of the two inositols (*myo*- and *scyllo*-inositol) in the brain of HR-treated mice in favour of the isomer *scyllo*-inositol might be linked to the depressive- and rewarding-related behaviours observed in the same animals. Besides, this would be relatively specific of a short-term HR-mediated intoxication process (Domange et al., 2008). Yet, the disruptive functions of *scyllo*-inositol in the brain are still unknown.

Inositols and in particular *myo*-inositol are known to be involved in the regulation of mood through the phosphatidyl inositol second messenger system (Cohen et al., 1997; Einat et al., 2001; Einat and Belmaker, 2001; Levine, 1997) and the regulation of the intracellular calcium concentrations (Galeotti et al., 2006; Taylor, 1998). They are also involved in drug-rewarding response (Morishima et al., 2005) because of the prominent role of intracellular inositol 1,4,5-triphosphate level on  $Ca^{2+}$  signals in dopaminergic neurons and the impact of  $Ca^{2+}$  associated with action potentials and metabotropic glutamate receptor activation on neuronal activity and synaptic plasticity (Cui et al., 2007). Thus, a cascade-type link between the HR ingestion, the resulting imbalance of inositols, the consequences in terms of disrupted calcium signalling system *via* the metabotropic glutamate receptor pathway, and last the behavioural impairments, in the present study related to depression and reward could be suspected (Fig. 4).

In conclusion, the strategy used here to get a link between a behavioural phenotyping and a brain NMR-based metabolomic printing in the HR treated-mouse model appears to be very promising to provide significant functional information on the pathophysiology of a disease. Compared to controls, the HR-treated mice showed an increase in motor activity, which may be supported by a reduction in behavioural despair sustained or improved by possible rewarding properties of the ingestion of the plant. Taken together, these behavioural impairments may explain the vicious circle of plant ingestion and the clinical picture in

horses. The brain metabolic disturbances related to the imbalance in biosynthesis of inositols may functionally explain arising of some behavioural disruptions. Besides, they may account for new functional hypotheses about the pathophysiology of Australian stringhalt in horses. However, the ability of HR to induce the imbalance of *scyllo*-*myo*-inositol in HR-intoxicated horses remains to demonstrate.

### Conflict of interest statement

The authors declare that they have no conflict of interest.

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