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STUDY PROTOCOL

Effect of prenatal wheel-running exercise (before and during gestation) on cocaine psychomotor sensitization expressed in the offspring in periadolescent females and males C57BL/6J mice

BACKGROUND

Animal models of drug addiction have provided evidence for preventive effectiveness of aerobic exercise on drug of abuse vulnerability. For example, rodents housed with a running-wheel for several weeks (model of voluntary aerobic exercise) exhibited reduced rates of acquisition, maintenance, escalation or motivation of self-administration of various drugs of abuse including cocaine (see Lynch et al. 2013 and Bardo et al. 2015 for reviews). Consistent with self-administration findings, physical exercise has been reported to be protective against the psycho-stimulating effects of cocaine as well as the behavioural sensitization to those effects (Diaz et al. 2013; Geuzaine and Tirelli 2014; Lespine and Tirelli, 2015), a phenomenon which likely has an integral role in the process of drug addiction (Leyton and Vezina, 2013; Steketee and Kalivas, 2011). However, the interaction between physical exercise and drug vulnerability is far from being fully characterized. For example, little is known about the long-lasting effects of exercise on behavioural responsiveness to cocaine (and other drugs).

In our laboratory, the levels of cocaine psychomotor responsiveness were comparable in mice having undergone exercise cessation 5 days before the initiation of cocaine sensitizing treatment after a 5-week period of wheel-running (aerobic exercise), and in mice having the possibility to wheel-run continuously, both groups being less stimulated than the mice from the control condition (Lespine and Tirelli, 2015). Subsequent experiments indicated that wheel-running performed exclusively during adolescence (from the 28th to the 50th days of age) induced a durable protective effect against cocaine psychomotor sensitization in females tested later in adulthood (after a few weeks of exercise cessation). This long-term attenuating effect was less pronounced in males. Interestingly, we found a qualitatively different pattern in females and in males living with a running-wheel during adulthood from the 77th to the 99th days of age and tested later in life, their levels of behavioral responsiveness being comparable to those observed in the unexercised animals (paper in preparation). These long-term protective-like effects observed in mice exercised in youth may be related to structural and functional brain plasticity in areas involved in motivation and reward that continues to mature during adolescence.

We believe that the characterisation of the interaction between exercise and development deserves further investigations. Curiously, there is no studies having evaluated the long-lasting effects of prenatal exercise on drugs of abuse vulnerability in offspring. Yet, it is well-known that some environmental stimuli during prenatal life (before or during gestation) can subsequently affect the development and phenotypes of progeny. In rodents, it has been pointed out that prenatal maternal stress or drug exposure can increase in offspring the risk of cognitive impairments, exacerbated emotional responsiveness or drug vulnerability (e.g. Fodor et al. 2014; Hausknecht et al 2013; Maccari et al. 2014). Conversely, some reports suggest that aerobic exercise during this sensitive period induces beneficial effects on several biological and behavioural outcomes, in particular pro-cognitive effects (e.g. Aksu et al. 2012; Dayi et al. 2012; Gomez da Silva et al. 2016; Parnpiansil et al. 2003; Robinson and Bucci, 2014).

In pregnant women, regular physical exercise has not been associated with adverse consequences on foetus development or neonatal outcomes (Riemann and Hansen, 2000). In the absence of medical counter-indications, exercise during pregnancy is even recommended for its global health benefits (Prather et al. 2012) and updated guidelines have been recently published by The American College of Obstetricians and Gynecologists (2015). However, the effects of prenatal exercise on neurobehavioral outcomes received little attention. Clapp et al. (1999) reported better scores in 5-day neonates born from exercised mothers in 2 of the 6 behavioural clusters evaluated by the Brazelton scale. In a recent randomized controlled trial, Labonte-Lemoyne et al. (2016) observed that 10-day neonates of active pregnant women exhibited a lower electro-encephalographic response in a sound discrimination task as compared to control, lower amplitudes being thought to reflect an enhanced brain maturation. Given the difficulties related to human investigations, preclinical models could be useful for the understanding of the behavioural consequences of prenatal exercise in offspring.

OBJECTIVES AND HYPOTHESES

The present study principally aims at determining to which extent prenatal exercise (before and during gestation) could affect the initiation (establishment) and the expression of psychomotor sensitization induced by a representative dose of cocaine in young female and male mice. More specifically, we will assess cocaine-induced acute psychomotor-activating effects, psychomotor sensitization developing over 9 daily sessions (daily peritoneal injections of cocaine or saline) and the long-term expression of the sensitized response (30 days after the last sensitizing injection) in C57BL/6J mice born from mothers housed with or without a running wheel before and during gestation.

Based on literature and on our prior results, the mice born from exercised mothers are expected to show significantly reduced levels of cocaine responsiveness in comparison with the control mice (born from unexercised mothers). We will address this question by using a split-plot factorial design (detailed in the "EXPERIMENTAL DESIGN" section).

ANIMALS BREEDING AND HOUSING CONDITIONS

Given the number of available running-wheels, the experiment will be organized in three successive identical sub-experiments, whose collected data will be pooled for analysis. Each sub-experiment will first involve 64 females and 32 males C57BL/6J mice (experimentally-naïve) age 6 weeks. The choice of C57BL/6J strain is based on its wide use in addiction research and previous exercise-related experiments performed in our lab (Geuzaine and Tirelli, 2014; Lespine and Tirelli, 2015). Mice will be housed upon arrival in groups of 8 for one week (same-sex groups) in large transparent polycarbonate cages (38.2 x 22 cm surface x 15 cm height; TECHNIPLAST, Milano, Italy). After this acclimation period, mice will be individually housed in smaller TECHNIPLAST transparent polycarbonate cages (32.5 x 17 cm surface x 14 cm height) with pine sawdust bedding, between-animal visual, olfactory and acoustic interactions remaining possible.

For females, the two experimental housing conditions will be defined by the presence or the absence of a running wheel on the surface of the tub. They will be randomly assigned to one of these two initial housing conditions, 32 animals being housed with a running wheel (Exercise or EX) and 32 without (Sedentary or SED). The assignment into these two modalities will be based on a computer-generated randomization schedules with n=4/experimental level from each acclimation cage (n=8).

A running wheel is made of an orange polycarbonate saucer-shaped disk (diameter 15 cm, circumference 37.8 cm), which allows an open running surface, mounted on a plastic cup-shaped base (height 4.5 cm) via a bearing pin so as to being inclined from the vertical plane at an angle of 35° (ENV-044, Med Associates; St Albans, VT, USA). The base is fixed on a stable transparent acryl-glass plate. Running will be monitored and recorded continuously by means of a wireless system, each wheel being connected to a USB interface hub (DIG-804, Med Associates) which relays data to a Wheel Manager Software (SOF-860, Med Associates). To ascertain that the amount of physical exercise in the sedentary

mice is maintained to a minimum, no locked wheel (no aerobic exercise), on which a mouse can display much climbing, will be placed in their home-cage.

After 20 days in these conditions (nearly 10 weeks of age), males will be allowed to mate with females (two females into one male's cage) during a 12-to-60 hour-period depending on the presence of a vaginal plug checked every morning. Then, females will return back into their cages in the same pre-mating conditions (EX or SED) and the day will be designated as gestational day 0 (GD0). On gestational day 18 (just before birth, expected to occur on GD19-20), wheels will be removed.

The day of birth will be designated as postnatal day 0 (PND0) and pups will be left undisturbed together with their mothers during the lactation period. Note that unemployed gravid and not gravid females and males will be kept for either animal husbandry or development of future experiments in our lab. Likewise, all unemployed pups will not be sacrificed and will be used for future works.

Offspring will be weaned on 24-28 days old (depending on vaginal plug/gestation/birth timeline) and individually housed in polycarbonate cages (30 x 12 cm surface x 13 cm height) with pine sawdust bedding. Testing will begin on 38-42 days old (periadolescent; Laviola et al. 2003). Tap water and food (standard pellets, CARFIL QUALITY, Oud-Turnhout, Belgium) will be continuously available. The animal room is maintained on a 12:12 h dark-light cycle (lights on at 0700), at an ambient temperature of 20-24°C. See Figure 1. for a schematic representation of timeline and the general procedure.

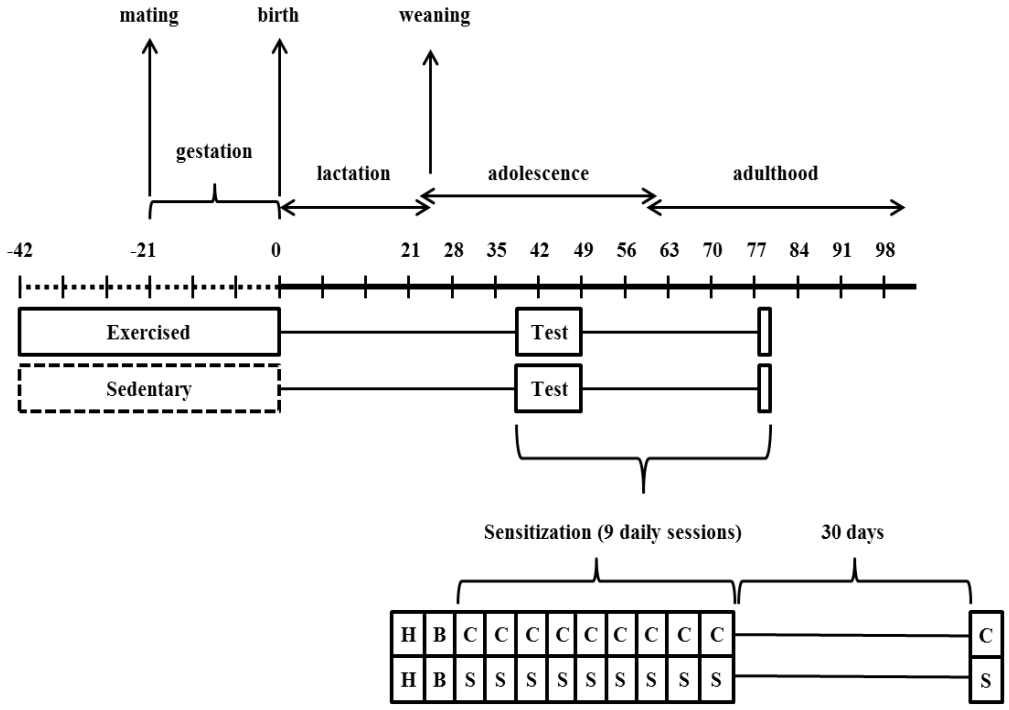

Figure 1. Timeline (in days) and general procedure. H: habituation session (without any injection), B: baseline session (under saline), C: cocaine administration, S: saline administration.

EXPERIMENTAL DESIGN

As previously mentioned, the experiment will be organized in three successive identical sub-experiments, each consisting of 32 females housed with a wheel (EX) and 32 without wheel (SED). Based on our husbandry knowledge/observations on that strain, we expect that at least 10 females per housing modality would be actually pregnant after the mating period (see Figure 2). As the number of pups in C57BL/6J average 6-7 and because we want each experimental group (within each housing condition) to be represented in each litter by one mouse (females and males receiving cocaine or saline), only litters that would comprise (at least) two females and two males will be included. Moreover, in order to employ the most homogeneous litters as possible, we will exclude litters with a number of pups ≥ 8. Based on these selection criteria, we expect to obtain 6 employable litters per housing condition in each sub-experiment (see Figure 2).

Since the four possible treatments (female/cocaine, female/saline, male/cocaine, male/saline) will be represented within each litter with one pup (n=1/group/litter), litter (block) will be incorporated as a random factor in a split-plot factorial design with the housing condition (2 levels: EX and SED) as a between-block factor and sex (2 levels: female and male) and drug (2 levels: cocaine and saline) as within-block factors (Kirk, 1995, pp. 553-562) with a total of eight experimental groups.

1) EX/Female/Cocaine group: female born from mothers exercised before and during gestation and receiving cocaine during testing.
2) EX/Female/Saline group: female born from mothers exercised before and during gestation and receiving saline during testing.
3) EX/Male/Cocaine group: male born from mothers exercised before and during gestation and receiving cocaine during testing.
4) EX/Male/Saline group: male born from mothers exercised before and during gestation and receiving saline during testing.
5) SED/Female/Cocaine group: female born from mothers unexercised before and during gestation and receiving cocaine during testing.
6) SED/Female/Saline group: female born from mothers unexercised before and during gestation and receiving saline during testing.
7) SED/Male/Cocaine group: male born from mothers unexercised before and during gestation and receiving cocaine during testing.
8) SED/Male/Saline group: male born from mothers unexercised before and during gestation and receiving saline during testing.

Previous experiments in our lab indicated that wheel-running exercise can induce a robust long-term protective effect in females on cocaine responsiveness ($\eta^2p=.34$). Cocaine reactivity in males

exercising during adolescence was also lower than their unexercised counterparts when tested a few weeks after exercise. However, these attenuating effects were smaller as compared to those showed by the females ($\eta^2p=.07$). Consequently, in order to detect at a minimally-accepted statistical power of 80% a $\eta^2p=.07$ and at an alpha level of 5% (this effect defining a one-sided housing-by-drug interaction calculated from crossed contrasts as *t*-test; see Rosenthal and Rosnow, 2009), a minimum of 15 mice/experimental group (hence 15 litters/housing condition) is required (a total of 120 offspring). A number of 18 litters/housing condition (n=6/housing condition/sub-experiment) will be used to compensate for potential attrition (loss).

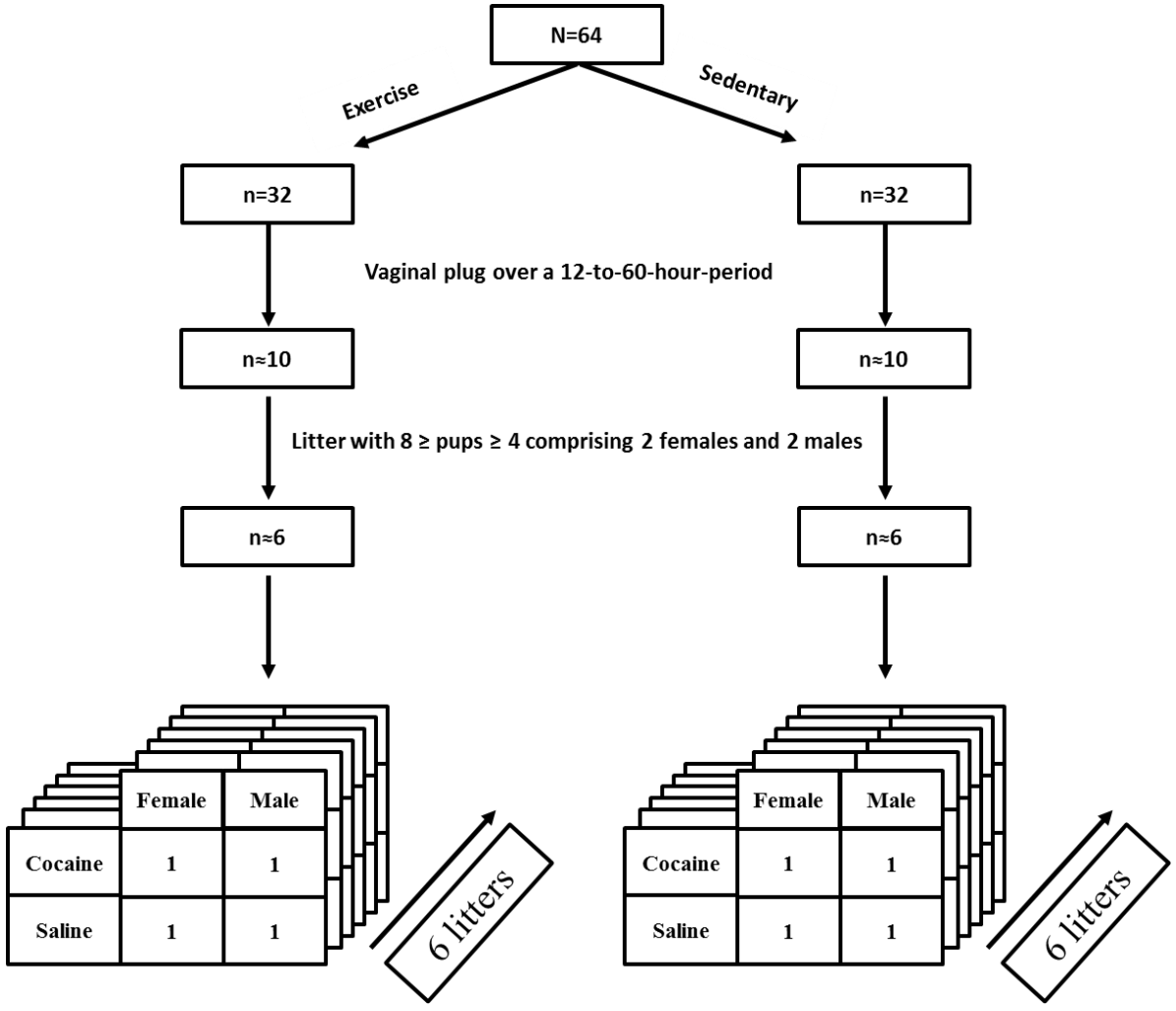

Figure 2. Split-plot factorial design with the expected progression of available number of animals defining one sub-experiment.

TESTING PROCEDURE

Psychopharmacological testing will involve the following four phases:

(1) A basal exploratory session to familiarize animals to the novelty of the test context without any injection (1st session).
(2) The assessment of the acute locomotor-stimulating effects of cocaine measured under saline (2nd daily session) and after a first injection of cocaine (3rd daily session).
(3) The assessment of the initiation of behavioural sensitization generated over 8 daily cocaine injections following the 3rd session (total of 9 sessions).
(4) The measurement of the long-term expression of sensitization 30 days after the last cocaine injection (the last day of the experimentation period), animals receiving their previous respective treatment.

Three days before the testing began, all mice will be familiarized with handling through saline injection in the animal room. Throughout testing, they will be weighted and will receive their pharmacological treatment right before being placed individually in the test chamber, the recording of ambulatory crossings lasting 30 min in all sessions.

Procedures will be conducted between 0830 and 1230; all experimental conditions being systematically represented within each test session by one mouse (2 litters with 1 litter/housing modality). Therefore, a session will include 8 mice individually tested in as many activity-chambers. After each test session, animals will return to the animal room within 10 min and the activity-chambers will clean with a disinfectant. Experimental blinding is not feasible since the experimenter inevitably has to know which pharmacological treatment to administer.

DRUG TREATMENTS

(−)-Cocaine hydrochloride (BELGOPIA, Louvain-La-Neuve, Belgium), dissolved in an isotonic saline solution (0.9% NaCl), will be injected at a dose of 8 mg/kg in a volume of 0.01 ml/g of body weight, the control treatment consisting of an equal volume of isotonic saline solution. All injections will be given via the peritoneal route (i.p.). That dose is selected on the basis of previous studies from this laboratory (Lespine and Tirelli, 2015 and unpublished experiments).

BEHAVIORAL TEST CHAMBERS

Mice will be individually tested in a battery of eight home-made activity chambers that are controlled by custom-written software for data collection. Each chamber is constituted of a removable transparent polycarbonate tub (22 x 12 cm surface x 12 cm height), embedded onto a black-paint wooden plank serving as a stable base. The lid is made of a transparent perforated acryl-glass tablet. Two photocell sources and detectors are mounted on the plank such that infrared light-beams are located on the two long sides of the tub at 2-cm heights from the floor, 8-cm apart and spaced 6.5 cm from each end of the tub.

Psychomotor activity will be measured in terms of crossings detected by the beams, one crossing count being recorded every time an ambulating mouse breaks successively the two parallel beams. The activity chambers are individually encased in sound-attenuated shells that are artificially ventilated and illuminated by a white light bulb during testing. Each shell door is provided with a one-way window allowing periodic surveillance during testing.

DATA ANALYSIS

Inferential statistics will be computed on the following data.

1) The acute reactivity scored as the absolute difference between values from the first cocaine session and the baseline session (under saline).
2) The overall reactivity to the locomotor-stimulating effects of cocaine over the initiation phase scored as the area under the curve with respect to ground (AUC Ground).
3) The sensitized locomotor activation over the initiation phase scored as the area under the curve with respect to increase (relative to the value of the first cocaine session; AUC Increase). The formulas for AUCs calculations are detailed in Pruessner et al. (2003).
4) The values of locomotor activity measured on the long-term expression session (final outcome).

These measures will be analysed with planned comparisons (or *a priori* contrasts; Rosenthal and Rosnow, 2009) based on the following focused hypotheses (tested as one-tailed *t*-test crossed contrasts):

- Interaction between housing and cocaine-related treatment in females, those born from exercised mothers being expected to display attenuated cocaine responsiveness.
- Interaction between housing and cocaine-related treatment in males, those born from exercised mothers being expected to display attenuated cocaine responsiveness.

Each contrast will incorporate the appropriate mean-square error (MSE) provided by the ANOVA analysing a split-plot factorial design in which the housing condition (2 levels) will be incorporated as a between-block factor, with the sex (2 levels) and the drug treatment (2 levels) as factorally-crossed within-block factors (Kirk, 1995). The estimates of tested effects will be provided through partial eta-squared.

ETHICAL CONSIDERATIONS

All experimental treatments and animal maintenance have been reviewed by the University of Liège Animal Care and Experimentation Committee (animal subjects review board), which gave its approval according to the Belgian implementation of the animal welfare guidelines laid down by the European Union (“Arrêté Royal relatif à la protection des animaux d’expérience” released on 23 May 2013, and

"Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes").

Moreover, the ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments) that have been developed to improve the quality of reporting in animal experiments (Kilkenny et al. 2010), were (through experimental design) and will be respected as much as possible.

DATA MANAGEMENT

Data sheets will be locked and made available on the University of Liège Open Repository and Bibliography (ORBI) on which the present protocol will be deposited.

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CONFLICTS OF INTEREST

There are no conflicts of interest.

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