The Directive 2010/63/EU on animal experimentation may skew the conclusions of pharmacological and behavioural studies

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Locomotor activity: apparatus and schedule

The sensors (20 Hz) detected any movement of mice with a frequency of 20 events per second. Data were recorded by an IBM computer with dedicated software. No movements were detected by the sensors when mice were sleeping, inactive, or performed moderate self-grooming. Scores were obtained during 1-hr intervals and expressed as counts per minute (cpm). The position of cages in the rack was such that mice of each group were equally distributed in rows and columns. The access of the authorized personnel to the animal room was not restricted and followed the routine schedule.

Elevated 0-maze (EOM) test

The floor of the apparatus was cleaned with a 10% ethanol 90% water solution after each session. Testing occurred under dim lights. The testing sessions were videorecorded and the behavioural profile was subsequently scored by a trained observer, using a computer and a dedicated software (THE OBSERVER, Noldus Information Technology, Wageningen, The Netherlands).

Progressive ratio operant procedure

We first trained food-restricted mice to nose poke in an operant chamber to obtain a highly palatable reward and then we required them to perform an increasing number of pokes to obtain the same reward. Training and testing were performed in the animals' colony room. Testing occurred under dim lights. Sessions were conducted five days a week between 09:00 h and 17:00 h in four operant chambers. Each animal was tested individually in the same chamber at the same time. Mice performed three daily sessions (starting at 09:00, 13:00, and 16:00 h). Each operant chamber consisted of a standard polycarbonate cage (Makrolon® type III cage) with sawdust bedding, in which an apparatus (PRS Italia, Roma, Italy) was positioned against the short sidewall. Subjects were placed, on day 1, on three 40-min sessions of responding in which they had to perform one nose poke to obtain one pellet (fixed ratio, FR, 1). They were subsequently placed for one day on

three 40-min sessions, in which they had to perform three nose pokes to obtain one pellet (FR3). Following the acquisition of a stable level of nose poking on the FR3 schedule, the animals were transferred to the progressive ratio schedule (PR, from the third to fifth day), which requires animals to emit an increasing number of responses in order to obtain each reward. The ratio used in this experiment was increased as follows: 3, 3, 6, 6, 10, 15, 21, 28, 36, 45, 55, 66, 78, 91, 105, 120, 136, 153, 171, 190, 210, 231, 253, 276, 300, 325, 351, 378, 406 and 435. When a mouse performed the necessary number of nose pokes, a single pellet dropped from a reservoir into the reward magazine and the magazine-light lit for 5 s while the hole-light turned off. During these 5 seconds the hole was inactive (nose pokes performed in that time interval were not counted). Each chamber was independently connected to a personal computer. A dedicated software controlled the sessions and recorded the behavioural data. We obtained three measures of reward motivation, namely, the total number of nose-pokes responses, total number of reinforcements obtained and the final ratio achieved/breakpoint.

Magnetic resonance imaging and spectroscopy

Once unresponsive to paw pinch, each mouse was transferred to a stereotaxic head frame, in prone position, under the continuous supply of anaesthetic gases through a facemask, and fixed by using tooth bar, earplugs and adhesive tape to reduce head movement. During the MR analyses, anaesthesia was maintained at 2.5-2.0 % isoflurane in oxygen (1 l/min). An integrated heating system allowed maintaining the animal body temperature at 37.0 ± 0.1 °C. Mice were left to spontaneous breathing, with no mechanical ventilation, during the whole experiment. All MRI and MRS experiments were conducted on a 4.7 T Varian/Agilent Inova animal system (Varian/Agilent Inc. Palo Alto, CA, USA), equipped with actively shielded gradient system (max 200 mT/m, 12 cm bore size). A 6-cm diameter volume coil was used for transmission in combination with an electronically decoupled receive-only surface coil (Rapid Biomedical, Rimpar, Germany). The shape of this receiver coil (1.5 cm long, 1.5 cm wide and 0.6 cm high) was designed to optimally fit the dorsal surface of the mouse head, and was positioned such as to cover forebrain areas. Gradient echo scout images were detected for placing the head of the animal inside the magnet and spin echo sagittal anatomical images (TR/TE=3000/70 ms, 13 consecutive slices of 0.8 mm thickness,

FOV=20 x 20 mm^2, matrix of 128 x 128, 2 averages) were acquired for accurate positioning of the voxel for the MRS study.

Quantitative MRS protocol, including water T2 measurements, was applied [1]. Localised shimming was performed up to water linewidths smaller or equal to < 11 Hz. Long TR and short TE parameters were selected in our pulse sequences, in order to minimize the error due to any potential carry-over influence on the relaxation times, and therefore to be able to attribute any change in signal intensities to actual changes of metabolite levels. Nevertheless, T2 measurements were performed on water signal in order to identify any change due to treatment (a set of water localised spectra was acquired from the same voxel with the same parameters but TE ranging from 23 to 300 ms, 15 values). The water signal was suppressed by using the VAPOR pre-sequence composed by seven CHESS pulses with optimized flip angles and timing in order to have a reduced sensitivity to B1 variation and thus it is highly efficient also for surface coil; 512 and 256 averages were sufficient to acquire metabolite spectra from prefrontal cortex and hippocampus with a S/N (referred to the highest signal) higher than 3 and 6, respectively. Unsuppressed water signal was acquired from the same voxel with the same parameters except for a reduced number of transients (4 instead of 256) and was used for metabolite quantification (assuming 79.9% for grey matter water content).

Spectra were analysed using LCModel [2] that calculates the best fit to the experimental spectrum as a linear combination of model spectra (spectra of metabolite solutions). Seventeen metabolites were included in the basis set: alanine (Ala), aspartate (Asp), creatine (Cr), α-aminobutyric acid (GABA), glucose (Glc), glutamate (Glu), glutamine (Gln), glycerophosphorylcholine (GPC), guanidoacetate (Gua), phosphorylcholine (PCho), myo-inositol (Ins), lactate (Lac), N-acetylaspartate (NAA), N-acetylaspartylglutamate (NAAG), phosphocreatine (PCr), scyllo-inositol, and taurine (Tau). Spectra of lipids and macromolecules were also included in the basis set. Only those metabolites that were estimated to have Cramer-Rao lower bounds (CRLB) less than 20%, which corresponded to an estimated concentration error <0.2 μmol/g, were included into the quantitative analysis. In some cases, metabolites that have resonance overlapped or very close are also given as their sum. The signals due to inositol, glutamate and glutamine underwent J-coupling

modulation with increasing TEs. However, the decreases in these signals due to J-coupling at TE=23 ms were automatically accounted for in the LCModel basis sets.

1 Canese, R. *et al.* Characterisation of in vivo ovarian cancer models by quantitative 1H magnetic resonance spectroscopy and diffusion-weighted imaging. *NMR in biomedicine* **25**, 632-642, doi:10.1002/nbm.1779 (2012).

2 Provencher, S. W. Estimation of metabolite concentrations from localized in vivo proton NMR spectra. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine* **30**, 672-679 (1993).