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# Cerebral oxidative metabolism in rats with high and low exploratory activity

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**Abstract**

To reveal brain regions most significantly related to individual differences in exploratory behaviour, oxidative metabolism was measured by cytochrome c oxidase histochemistry in 2 months old Wistar rats with persistently high (HE) or low (LE) exploratory activity in a novel environment. LE-rats had significantly higher levels of oxidative metabolism in dorsal raphe and inferior colliculi. In contrast, HE-rats had higher metabolic activity in entorhinal cortex. In conclusion, rats with different exploratory styles differ in underlying cerebral activity as measured via oxidative metabolism in regions implicated in defensive behaviours and cognitive processing of sensory stimuli.
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Studies on the neurobiology of depression have recently focused on defining endophenotypes of this disorder, and on identification of their biological substrates [20]. Among the features of depression which can be studied in animal experiments are increased anxiety and reduced motivation to engage in active behaviours. These two features appear to be reflected together in laboratory animals in terms of exploratory behaviour. Indeed, significant individual differences in rat exploratory behaviour have been reported in different test paradigms (Y-maze, 16-arm radial maze, canopy test, hole board test) [8,32]. These differences are stable in time [33] and across tests [8,32]. We have developed a technique to measure exploratory behaviour which reliably distinguishes animals belonging to clusters with combined high anxiety and low motivation to explore their environment versus low anxiety and high motivation. These rats, termed LE (low explorers) and HE (high explorers), respectively, display their characteristic behavioural profile consistently and exhibit a number of neurochemical differences in noradrenergic and dopaminergic systems [1].

Results in our laboratory and elsewhere [32] show that when the levels of voluntary exploratory behaviour are measured, the individual variability in rats is rather skewed and does not follow the normal bell shape probability distribution: there is a large cluster of animals with practically non-existent tendency to venture into unknown environment, while others tend to cluster around the relatively high exploration levels leaving only a minority of rats that occupy the middle ground. The individual differences in exploratory behaviour are evident from the first day of testing in the exploratory box but the second day is better at predicting the long-term stable individual profiles ([1] and unpublished data) and is used in this study for animal assignment into LE and HE clusters. Persistent differences in exploratory behaviour may be based on differences in the level of baseline activity of certain brain regions and circuits, which could possibly be revealed by comparative mapping of the cerebral metabolic activity [35]. The metabolic activity in the brain is dependent in large part on the availability of cellular energy, primarily in the form of ATP. ATP production from ADP is driven by a coupled process of electron transfer and oxidative phosphorylation in the enzyme complex located on the inner side of mitochondrial membrane, the major energy-synthesizing pathway utilized by the CNS [21]. Cytochrome c oxidase is a terminal enzyme of this electron transport chain, functioning as a rate-limiting enzyme in oxidative phosphorylation [43]. In this respect, the activity of cytochrome c oxidase determines the amount of ATP available in a neuron and, therefore, it can serve as a reliable metabolic marker for neuronal activity [44]. There is now ample evidence that cytochrome c oxidase activity cor-

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responds under normal conditions to the functional long-term changes in the neuronal metabolism of various nuclear groups [35]. Therefore, this method is most appropriate to relate differences in metabolic activity to stably expressed differences in a behavioural phenotype. The aim of the present study was to compare oxidative metabolism in a broad range of brain regions potentially important in anxiety, motivation, depression and drug abuse in rats with high versus low expression of exploratory behaviour.

Twenty-eight male Wistar rats of 2 months of age were used in the experiment, of which 19, weighing 288–365 g, were selected for histochemistry. Animals were tested in two separate sets (4LE-rats + 4HE-rats selected in the first, and 5LE + 6HE in the second) due to concurrent method development. Animals were obtained from Scanbur BK AB, Sollentuna, Sweden at the age of 3 weeks. They were housed four per cage in standard polypropylene cages in a light controlled room (12-h light:12-h dark cycle; lights on at 7:00 a.m.) maintained at 22 °C. Food and water were available ad libitum. Behavioural testing was carried out between 13:00 and 19:00 h. All experiments were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Ethics Committee of the University of Tartu.

The exploration box test was conducted as described previously [28]. The exploration box was made of metal and consisted of a 0.5 × 1 m open area (side walls 40 cm) with a 20 × 20 × 20 cm small compartment attached to one of the shorter sides of the open area. The open area was divided into eight squares of equal size. In the open area, four objects, three novel (a glass jar, a cardboard box, and a wooden handle) and one familiar (a food pellet) were situated in certain places (which remained the same throughout the experiment). The small compartment, which had its floor covered with wood shavings, was directly linked to the open area through an opening (size 20 × 20 cm). The apparatus was cleaned with dampened cloth after each animal. The exploration test was initiated by placing a rat into the small compartment, which was then covered with a lid for the exploration time. Open area was dimly lit (5–7 lux) for the duration of the test. The following behavioural parameters were registered: (1) latency (of entering open area with all four paws), (2) number of entries into the open area, (3) time spent exploring on the open area, (4) line crossings, (5) rearings and (6) number of unfamiliar object investigations. Manipulation of food pellet was not counted as an exploratory event. To provide an index of exploration the scores of line crossing, rearing and object investigation were summed for each animal and thus (7) the sum of exploratory events obtained. Rats were tested for their spontaneous exploratory activity in the exploration box on 2 consecutive days and decapitated 2 days after the last testing day. The animals were classified as high or low explorers based on the sum of exploratory events on the second exposure to the exploration box. Only rats that clearly differed in their exploratory activity from the animal with the statistically 'average' spontaneous exploratory activity were selected for further procedures. The mean sum of exploratory activities for LE rats of the same age group was about 1 standard deviation below average; accordingly, this parameter was 1 standard deviation above average for HE animals.

Following decapitation, brains were removed intact, frozen rapidly on dry ice, and kept in deep freezer at −80 °C until sectioning. Using a cryostat (Microm HM 560) at −20 °C, brains were coronally sectioned at 20 μm, mounted on glass slides, and desiccated at 37 °C overnight [31]. In hindsight, we should mention that overnight desiccation of frozen sections may negatively affect the enzymatic activity of the cytochrome c oxidase, reducing the sensitivity of obtained results, and thus the results in this study likely reflect only the most robust differences. Quantitative histochemical procedures followed the protocol published by Gonzalez-Lima and Cada [14] with slight modifications. In brief, the cytochrome oxidase histochemical staining procedure consisted of the following series of chemical procedures: (1) Rinsing in the 0.1M DPBS buffer (1.46 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$, 2.68 mM KCl, 136.8 mM NaCl; pH 7.4) for 5 min; (2) rinsing in 0.1 M DPBS buffer with 10%, w/v sucrose and 0.5%, v/v glutaraldehyde, pH 7.4, for 5 min; (3) four changes of 0.1 M DPBS buffer with 10%, w/v sucrose, for 5 min each; (4) metal intensification in 0.05 M Tris buffer, pH 7.4, with 275 mg/l cobalt chloride, 10%, w/v sucrose, and 0.5%, v/v dimethylsulfoxide, for 10 min; (5) incubation in DPBS containing 0.05%, w/v diaminobenzidine tetrahydrochloride, 0.01%, w/v cytochrome c, 5%, w/v sucrose, 0.02%, w/v catalase, and 0.25%, v/v dimethylsulfoxide in the dark for 1 h. The reaction was stopped by fixing the tissue in DPBS buffered formalin (for 30 min with 10%, w/v sucrose and 4%, v/v formalin). Finally, the slides were dehydrated in a series of ethanol baths (increasing from 30 to 100%, v/v ethanol), cleared with xylene, and coverslipped with Entellan (Merck, Whitehouse, NJ, USA). All procedures were performed at room temperature. During each histochemical procedure an equal number of slides with corresponding regions of interest (ROIs) from one randomly chosen LE and one HE animal were processed in the same staining solution.

Images of stained sections were digitised using Sony DXC-950P 3CCD Colour video camera on Carl Zeiss Axioplan 2 microscope with 20× magnification objective; converted to greyscale and cropped to the uniform size appropriate to the particular ROI using Graphic Converter X 5.5.2 (Lemke software GmbH, Peine, Germany). Quantitative densitometric analyses were carried out using NIH ImageJ 1.33 (National Institute of Health, USA) software applying Rodbard calibration procedure based on Kodak greyscale tablet; thus absolute greyscale values between 0 and 255 were converted to the optical density units. ROIs were identified during photographing with the aid of Paxinos and Watson's rat brain atlas [30]. The 3–6 individual measurements were made from each ROI for each animal and then averaged to obtain the single value of metabolic activity. Background optical density values were subtracted from measured results. Final optical density measures were standardized to Z scores within experiments to allow for the aggregation of results across two series of experiments. Dummy constant >5 was universally added to the results to eliminate the negative values obtained with standardization. Data were analysed using univariate ANOVA with Group (HE versus LE) as an indepen-

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