FLUORESCENCE MONITORING OF MITOCHONDRIAL COMPONENTS OF OXIDATIVE PHOSPHORILATION OF POST-SYNAPTIC ACTIVATION IN NEOCORTICAL SLICES IN THE MOUSE

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Summary

We studied mechanisms contributing to stimulus-evoked changes in NAD(P)H fluorescence as a marker of neuronal activation in barrel cortex slices of the mouse. Electrical stimuli were set at frequency (50 Hz), which produced optimal responses and did not induce consistent bleaching of cells. With these stimuli, biphasic fluorescence changes were produced, which were composed of an initial transient decrease ("initial component", 1-3%), followed by a longer lasting transient increase ("overshoot", 1-8%). These responses, which were found to reflect mitochondrial function, were also used to test possible differences between brain function in adult control mice, and in adult mice which during lactation period underwent daily brief (10 min) mother deprivation plus sham injection. Preliminary results show that neonatally stressed adult mice have an initial component with similar amplitude, followed by a consistently higher overshoot. These results may be considered as a new and interesting direction among long-term or permanent changes induced by neonatal stress on brain structures.

Key-words: mitochondrial function; neuron metabolism; barrel cortex; postsynaptic excitation; optical imaging.

Introduction

The development of type 2 diabetes and overweight in adult humans may have its roots in early events in the life (1). Considering that overweight is the leading determinant in the development of type-2 diabetes mellitus, it seems interesting to evaluate whether an experimental model in the mouse, i.e., the association of psychological and nociceptive manipulations in neonatal life, which produces long lasting or permanent overweight, increase of plasma corticosterone and adrenocorticotropin, and increase of glycaemia and insulinaemia in non genetically vulnerable mice (2), induce also alteration in the mitochondrial metabolic function of cerebral cortex. Therefore, the present study was designed to explore cellular metabolic and mitochondrial functions in the *ex vivo* mouse somatosensitive cortex

(barrel field, IV layer) during neuronal activation that was elicited by electrical stimulation, with the aim to verify whether metabolic alterations previously showed in the stressed mice model (3) could be evidenced in the brain as well, and, if so, whether brain alterations could be put in relationship to mitochondrial function

Methods

All procedures were carried out in accordance with the guidelines of the Council of European Communities and the approval of Bioethical Committee of the Italian National Institute of Health

Animal general procedures. All mice were housed in a central facility and maintained under controlled conditions of normal humidity and temperature, with standard alternating 12-h periods of light and darkness. Different series of pregnant multiparous Laboratory-born CD-1 mice (Charles River Italia, 22050 Calco, Italy) arrived at the 14^{th} day of gestation in the vivarium where all animals were kept. Animals had free access to water and food. Mucedola S.r.l. (Settimo Milanese, Italy) supplied the diet, which contained 3.95 kcal/g equivalent to assimilable 2.7 kcal/g. All experiments were performed in winter to avoid seasonal variation in receptors sensitivity.(4).

Prenatal and neonatal procedures. The experimental procedures were performed according to Loizzo *et al.* (4) Briefly, starting on the 19^{th} day of pregnancy, females were examined twice daily (at 08:00 and 16:00) for the presence of pups. In about 12 hours from the detection of the pups, litters of homogeneous size (13 ± 1 subjects) were put together, and randomly culled to six male pups so that all pups were randomly cross fostered. In addition, male pups have a homogenous weight in order to avoid interference induced by impairment of growth, which is an important determinant in the onset of type-2 diabetes mellitus in adults (5). To avoid manipulation of control mice, we preferred to adopt the protocol with complete litters receiving the same treatment to the alternative, in which half the litter would receive one treatment, and the other half a different treatment. Firstly, the litters were randomly assigned to one of the following groups (each group consisting of two litters, at least): 1) control group: the pups were left undisturbed, but pups and dams were removed at the same moment, and put in a clean cage twice a week; 2) manipulated mice: for 21 days, the pups were daily removed (10 minutes) from the home cage and grouped in a container with fresh bedding material. During the period of maternal deprivation, each pup was gently picked up with a gloved hand, weighed and injected sc in the back with sterile saline solution (1 ml/kg) with a microsyringe (26 gauge needle). After this, they returned to the home cage with the mother. Procedures were always performed by the same experimenter .

***Postweaning protocol*.** At postnatal day 21, animals were rehoused in post-weaning cages, three animals for each cage to avoid isolation-induced stress. Animals were weighed every week, and used at about 120 days of age.

Slice preparation. At 120 days of age, mice are anesthetized deeply with a mixture of ketamine and xylazine (85 and 15 mg/ml, respectively, 0.15 ml s.c.) and decapitated. Brains are removed and placed in ice-cold cutting solution. Somatosensitive cortex (6) sections (400 μm) are cut with a vibratome, and slices transferred into room temperature artificial cerebro-spinal fluid (ACSF). Cutting and recording solutions are both 300-305 mOsm/l. After being warmed to 34°C and held for 1 hr, ACSF is changed again, and the slices are held at room temperature

until used for recording. Individual slices are transferred to the recording chamber and are perfused with warmed (34°C), oxygenated ACSF at 2 ml/min.

***NAD(P)H fluorescence imaging*.** NADH has broad excitation and emission spectra with peaks at ~350 and 460 nm, respectively. Excitation (360 nm) was delivered via a fiber optic/monochromator system (DeltaRAM V^{TM}; Photon Technology International, Lawrenceville, NJ) reflected onto the slice surface via a dichroic mirror (DMLP 400 nm, Chroma Technology, Brattleboro, VT). Fluorescence emission was collected by using a cooled interline transfer CCD camera (MicroMAX System, Princeton Instruments). All experiments used a 410 nm long-pass glass filter between the dichroic mirror and camera to maximize light capture. Pilot experiments that used a 450 ± 15 nm interference filter showed that there was insignificant distortion from longer wavelength emitters. Photobleaching was not significant for NAD(P)H measurements.

Imaging is performed after focusing onto the surface of slices, using either 10× or 40× water immersion objective (numerical apertures 0.3 and 0.8, respectively; Olympus), and collected after 2 × 2 binning of the 640 × 480 line image. After binning, individual pixels corresponded to areas of 2.6 and 0.64 μm^2 for 10× and 40× objectives, respectively. For analysis the image data were filtered by using 3 × 3 pixel averaging and presented as the change in fluorescence intensity/prestimulus fluorescence intensity ($\Delta F/F_o$). Mean camera background was 421 ± 23.3 (mean ± SD) arbitrary fluorescence units (AFU)/sec. Integration times for data collection were between 100 and 150 ms. For display the selected images were converted to "tif" format and exported to Methamorph Image. Then they were put through the median filter of the program, mapped to colour, and rendered as surface plots. Top and bottom colours were set to minimum and maximum signal values for each experimental protocol. Consequently, the colour for the pre-stimulus maps "floats" to an extent, depending on these minimum and maximum values.

Electrical stimulation (brain slice). Bipolar stimulating electrodes were used for stimulation of contra lateral body surface in layer IV of the somatosensory cortex of mice. Platinum tips (50 or 25 μm in diameter) were placed in barrel cortex. Stimuli were delivered via a Master 8 controller, DC supply, and constant current isolation unit (A.M.P.I., Jerusalem, Israel). In those cases in which trains of stimuli were used, the stimulus frequency was 50 Hz. Between successive titanic stimuli 4 min intervals were maintained. For most studies the fluorescence excitation/imaging at 3 Hz was begun 4 sec before onset of the stimulus and continued for a total of 25 sec. So that the kinetics of initial NAD(P)H oxidation events (transient negative deflections) could be assessed, the acquisition rate was increased to 10-18 Hz. In experiments to monitor the time course of recovery of NAD(P)H fluorescence changes, the acquisition rate was decreased (0.25 Hz), and longer sequences (up to 90 sec) were recorded. This paper describes results performed after preliminary experiments.

Results

***Evoked NAD(P)H transients in murine cortical slices*.** Figure 1 illustrates basic properties of the biphasic fluorescence transients evoked by a single tetanus to thalamocortical pathways inputs in control mice. The 360 nm light pulses (100 ms) were used for excitation, whereas the emission (>410 nm) was collected from the barrel cortex cell region. The stimulus (25 pulses, 70 μs, 50 Hz) was applied at the arrows and was accompanied by an immediate decrease in fluorescence.

This transient, hereafter referred to as the "initial component," decreases, and is followed by a more sustained fluorescence increase, "overshoot" (7).

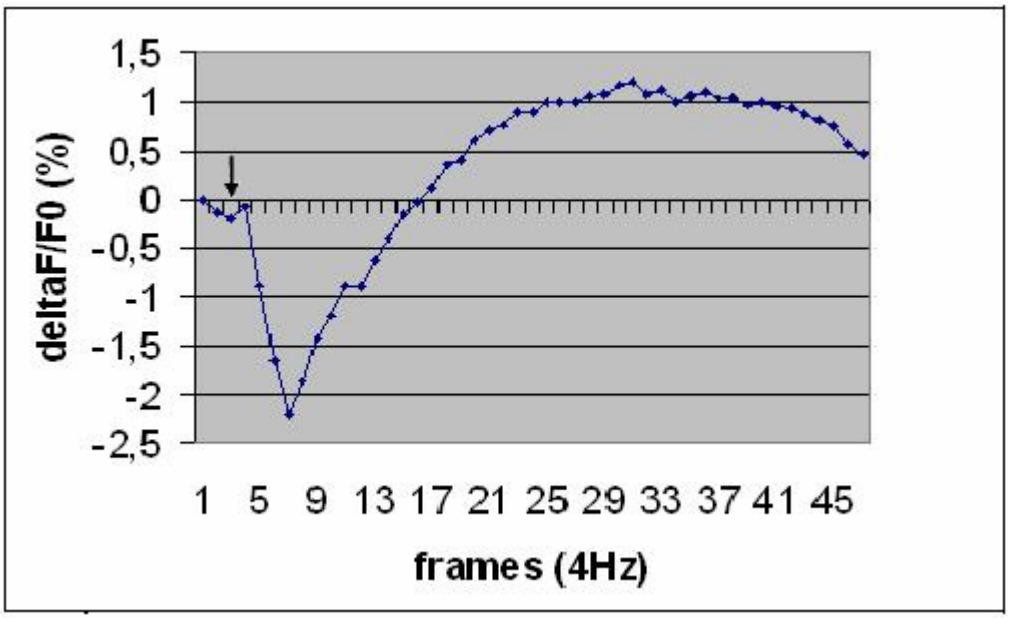

Fig 1: Example of response from control mice. Abscissa: light stimuli, frequency 4Hz. Ordinate: amplitude of response, in percent of baseline. Electrical stimulus was administered at the arrow.

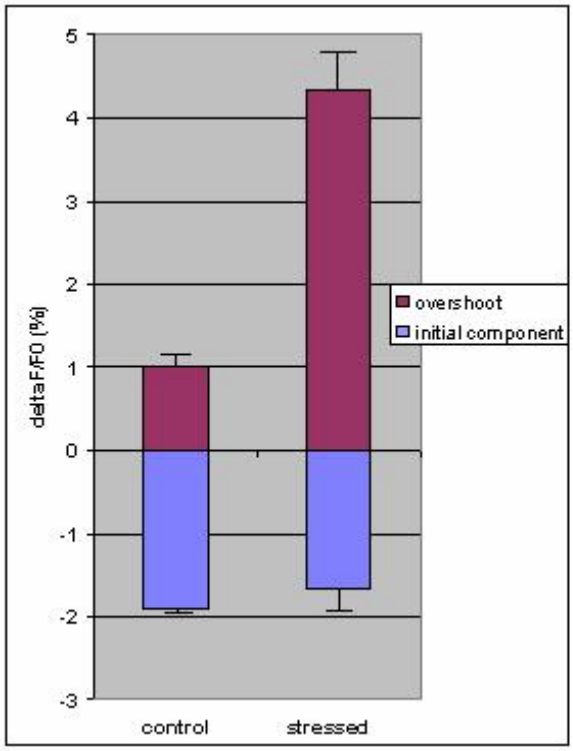

Fig 2:Response amplitude in two groups of experiments. Average of three mice, three slices per mouse, plus S.E.M. Left bar: control mice; right bar: neonatally stressed mice. Lower part of bars: first component of response (no significant difference versus neonatally stressed mice). Higher part of bars: overshoot component of response, there is a consistent difference in the amplitude in stressed mice versus controls ($p<0.01$).

Fig. 2 illustrates the differences of the response elicited by the stimulus (25 pulses, 70 μsec, 50 Hz, 1mA), at the level of the maximal response amplitude of both phases in control and stressed animals.

Discussion

These results, although preliminary in nature (these were obtained with a very small number of samples, 3 mice, 3 slices per mouse), do not evidence consistent differences between the two groups of mice in the first part of the response following the electrical stimulus. However, Fig 2 indicates a consistent difference in the second part of the response (overshoot), i.e., in the amplitude, and therefore also in the duration as well. Therefore, we can confirm that metabolic alterations previously showed in the stressed mice model (3) and evidenced as fasting hyperglycemia, probably due in part to unbalance of hypothalamus-pituitary-adrenal hormones, can be responsible for alterations evidenced in the brain of stressed mice as well, . Moreover, according to the present results, in the light of data in the literature, brain alterations can be put in relationship to mitochondrial function: in fact, Shuttleworth et al (7) showed that different glucose concentration in the bath solution may modulate overshoot in different directions. Other possibilities cannot be excluded. For example previous experiments in the literature indicate that modulation of the overshoot component is influenced by drugs which stimulate, or vice-versa inhibit, post-synaptic neuronal excitation, for example bicuculline or ouabain; also changes in the Na+ concentration of ACSF solution may influence the response (7). Neonatal repeated stressful procedures induce alterations of several receptorial families, e.g., those related to the GABA and the opioid systems (see ref (8). Therefore, much experimental work is necessary before giving correct interpretation of our data, and on the role played by receptorial systems in the “overshoot” phenomenon produced in the somatosensorial cortex in our experiments.

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