Enhanced brain performance in mice following postnatal stress

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Abstract

The double postnatal stress model (brief maternal separation plus sham injection daily applied from birth to weaning) induces metabolic alterations similar to type 2 diabetes in young-adult male mice. We verify whether 1) the stress also induces brain metabolic–functional alterations connected to diabetes and 2) different alterations are modulated selectively by two stress-damaged endogenous systems (opioid- and/or ACTH–corticosteroid-linked). Here, diabetes-like metabolic plus neurophysiologic–neurometabolic parameters are studied in adult mice following postnatal stress and drug treatment. Surprisingly, together with ‘classic’ diabetes-like alterations, the stress model induces in young-adult mice significantly enhanced brain neurometabolic–neurophysiologic performances, consisting of decreased latency to flash-visual evoked potentials ($- \sim 8\%$); increased level ($+ \sim 40\%$) and reduced latency ($- \sim 30\%$) of NAD(P)H autofluorescence postsynaptic signals following electric stimuli; enhanced passive

avoidance learning ($+ \sim 135\%$ latency); and enhanced brain-derived neurotrophic factor level ($+ \sim 70\%$). Postnatal treatment with the opioid receptor antagonist naloxone prevents some alterations, moreover the treatment with antisense (AS; AS vs proopiomelanocortin mRNA) draws all parameters to control levels, thus showing that some alterations are bound to endogenous opioid-system hyper-functioning, while others depend on ACTH–corticosterone system hyper-functioning. Our stress model induces diabetes-like metabolic alterations coupled to enhanced brain neurometabolic–neurophysiologic performances. Taken all together, these findings are compatible with an ‘enduring acute-stress’ reaction, which puts mice in favorable survival situations vs controls. However, prolonged hormonal–metabolic imbalances are expected to also produce diabetes-like complications at later ages in stressed mice.

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Introduction

Knowledge on the etiology and pathogenesis of type 2 diabetes mellitus is still a matter of debate. Investigations focused chiefly the role played by genetic, endocrine, alimentary, and environmental mechanisms (see for example Andrikopoulos (2010), Faturi *et al.* (2010), DIAGRAM Consortium (2012) and Gilbert & Liu (2012)). We underline that stressful procedures applied in pediatric ages also appear of great importance for the triggering of type 2 diabetes in adult humans. For example, clinical–epidemiologic investigation found excess obesity and type 2 diabetes in mid-adult life following exposure to adverse childhood experiences (Thomas *et al.* 2008, Bartz & Freemark 2012). Experimental laboratory studies could also induce overweight and dysmetabolic conditions in adult animals following mild

stressful procedures applied to newborn mice (Loizzo *et al.* 2006, Valenzuela *et al.* 2011). Therefore, there should be a chain of events that connect postnatal stressful experiences to type 2 diabetes in humans and type 2 diabetes models in mice, even if no inferences should be allowed between animal studies and human studies. Here, we evidence step-by-step trajectories from cause (postnatal stress) to effect (adult type-2 diabetes model) in male mice.

Diabetes-related stress mechanisms

Investigations on stress-related diseases have been focused chiefly on the central role played by the hypothalamus–pituitary–adrenal (HPA) axis, and overall by glucocorticoids in both humans and animal models (for references see Akil & Moreno (1995) and Oitzl *et al.* (2010)). However, the HPA

system upset does not explain all stress-induced alterations by itself (Kehoe & Blass 1986, Pieretti *et al.* 1991, Ploj *et al.* 1999, Coccurello *et al.* 2009, Faturi *et al.* 2010). The involvement of concurrent hormonal/neurohumoral mechanisms other than ACTH–glucocorticoids was also hypothesized to further elucidate the pathogenesis of stress-derived diseases, but clear demonstration for the identity and for the pathogenetic role of other substances (e.g. opioids) is still scanty. We hypothesized that the endocrine stress mechanism itself can explain etiology and pathogenesis of stress-related diabetes models. It is well-known that during the first 3 weeks of postnatal life in rodents, both HPA feedback control mechanisms and brain receptors for opioids and corticosteroids reach their critical development. Stressful insults applied to developing mice during this period produce long-term or permanent alterations of receptor function in the rodent brain (McDowell & Kitchen 1987, Rosenfeld *et al.* 1993, Zagon & McLaughlin 1993). Allegedly, the physiologic stress cascade is the following: in response to an acute stress the brain activates release of corticotrophin-releasing hormone (CRH) and other peptides. In turn, CRH stimulates, in the anterior pituitary, the synthesis-release of proopiomelanocortin (POMC), which is processed to ACTH, plus β -endorphin and other peptides through cleavage mechanisms. Then, ACTH stimulates the adrenal to increase cortisol (in humans) and corticosterone (in humans, rats, and mice) and other substances. Finally, following the acute stress cessation, physiologic HPA feedback mechanisms lead to decrease of the said peptides and hormones, and so on. We hypothesize that the key tool for the triggering of some stress-related diseases sits in this mechanism, i.e. in the failure of feedback mechanisms, thus leading to enduring endogenous hormone hyper-function. We demonstrated that our mild stress daily applied to male mice in the nursing period produces POMC–HPA feedback mechanism failure in the pituitary (Galietta *et al.* 2006). Consequently, enduring enhanced ACTH–corticosteroid and opioid levels in plasma and brain (Galietta *et al.* 2006) are produced, lasting up to young-adult age (Ploj *et al.* 1999, Galietta *et al.* 2006, Gustafsson *et al.* 2008). These hormonal–neurohumoral alterations are of the greatest importance, because at different ages from weaning up to adulthood they produce different behavioral, physiologic, and metabolic mechanism upsets typical of a type 2 diabetes model (Pieretti *et al.* 1991, d'Amore *et al.* 1995, Loizzo *et al.* 2002, 2003, 2006). As a counter-proof, we could prevent the enduring endocrine upset induced by stress through pharmacological tools addressed to protect those (presumed) damaged structures. Thus, we demonstrated that some type-2 diabetes-like signs produced by stress are prevented by naloxone treatment in the nursing period to block endogenous μ - δ opioid system hyper-function; whereas these and all other diabetes-like signs are prevented through administration of an antisense (AS) oligodeoxynucleotide to block synthesis–release of POMC mRNA and therefore to block both opioid and ACTH–corticosteroid system hyper-function. Also feedback

mechanism disruption and hormone enhancement induced by stress at the pituitary level, at weaning and thereafter, are then prevented, thus strongly confirming our hypothesis (Galietta *et al.* 2006, Loizzo *et al.* 2010a, 2012).

Studies on CNS complications following repeated mild stress administered during the nursing period in mice

Starting from these previous results, we decided to verify whether our mild type 2 diabetes mellitus model might allow deeper knowledge on the pathogenesis of the CNS complications of diabetes, i.e. neurological and cognitive complications, and dysfunctions of the visual system (Manschot *et al.* 2003, Gregori *et al.* 2006). Therefore, we planned to evaluate the following parameters:

- 1) Brain cortex flash-visual evoked potentials (VEPs) and rapid oscillatory potentials (OPs), because in stable type 2 diabetes and in diabetes models both color-VEP and VEP latency are consistently slower vs controls. Therefore, we planned to verify whether metabolic alterations in our diabetes model induced delayed flash-evoked responses from the visual system in our mice as well. High-frequency brain electric pattern and the gamma frequency oscillation occur in many brain regions in normal mammals and birds following sensorial and pharmacologic stimuli (Niessing *et al.* 2005, Vyssotski *et al.* 2009, Kann *et al.* 2011), and these patterns were suggested to play a role in cognitive processes and memory formation, which are at consistent risk in type 2 diabetes.
- 2) Biphasic fluorescence transients evoked by a single tetanus to visual cortical pathways inputs in mice, to see whether we could evidence cerebral metabolic dynamic changes *ex vivo* in our mice, through NAD(P)H auto-fluorescence postsynaptic signals. We expected that a chronic condition of hyperglycemia observed in our mice, which has a role in the pathophysiology of diabetes, could lead to brain damage and bioenergetics impairment (Gerbitz *et al.* 1996). Previously, Kann *et al.* (2005) using procedures analogous to ours, demonstrated severe metabolic dysfunction during neuronal activation in the hippocampus of chronic epileptic rats, through (possible) mitochondrial enzyme defects.
- 3) Step-through passive avoidance task which is frequently applied to evaluate the implications of hyperglycemia for learning and memory studies in diabetes models in animals. Cognitive deficits as possible complications in human diabetes are well known (Stranahan *et al.* 2008, McCrimmon *et al.* 2012).
- 4) Brain-derived neurotrophic factor (BDNF) levels in the cortex and hippocampus, which play a role in the pathways that control body weight and energy homeostasis. Low BDNF levels in both neurodegenerative diseases and in type 2 diabetes may in part explain the clustering of these diseases. Conversely, high BDNF expression levels in specific subfields correlate with a good

memory performance (Schaaf *et al.* 2000). Moreover, investigations by Givalois *et al.* (2004) found an interesting location of BDNF and its receptors in the median eminence, suggesting that these are presumably involved in dynamic processes such as hormone release and may be involved in the regulation of CRH homeostasis in the hypothalamus (Jeanneteau *et al.* 2012).

Materials and Methods

Ethical approval

Experiments were carried out in accordance with guidelines of the Council of European Communities 86/609/EEC. Protocols were approved by the Bioethical Committee of Istituto Superiore di Sanità (Rome, Italy), and by the Italian Ministry of Health. All efforts were made to minimize the number of animals and their suffering.

General procedures

Experimental procedures were described in previous papers (Pieretti *et al.* 1991, d'Amore *et al.* 1995, Loizzo *et al.* 2003, 2006). Series of multiparous pregnant laboratory-born outbred CD1 mice (Charles River Italia, Calco, Italy) were received at conceptual day 14 and housed one per cage. Litters of homogeneous size (13 ± 1 subjects) were put together, randomly culled to six male pups per litter, and randomly cross-fostered. To avoid manipulation of control mice, we adopted the protocol with complete litters receiving the same treatment. Experiments were performed in winter to avoid seasonal variation in receptor sensitivity (De Ceballos & De Felipe 1985).

Each litter was randomly assigned to one of the groups: 1) control (C) groups: pups were left undisturbed with their mother, except for cage cleaning twice a week, from birth up to 21 days of life (weaning); 2) stressed (W) groups: pups were daily removed from the home cage and grouped in a container with fresh bedding material. Each pup was weighed and injected under the skin of its back (s.c.) with sterile saline (1 ml/kg body weight). The double-stress model consisted therefore of brief mother deprivation plus sham injection pain. Ten minutes later, they returned to the home cage with mother. The procedure was repeated from birth up to weaning. 3) Naloxone-treated (Na) groups: pups were daily stressed for 21 days as in W groups, but they received s.c. injection of naloxone (1 mg/kg) instead of saline, with the aim of blocking the binding of endogenous opioid peptides to μ - and δ -opioid receptors. Moreover, in some experiments further positive control groups were added, i.e. 4) AS-treated (AS) groups: pups were daily stressed for 21 days as in W groups, but they were treated with AS (0.1 nmol/g body weight) acting as an antagonist for both the POMC-derived stress determinants, i.e. the ACTH–corticosterone and the endogenous opioids. Finally, 5) mother separated–not injected (Wsp) groups: pups were mother-separated as the W groups but did

not receive the pain due to sham injection. At postnatal day 21, animals were separated from mother and re-housed three per cage of each treatment group, with free access to food and water and left undisturbed. Mice were weekly weighed, and neurophysiologic–neurometabolic, metabolic, and hormonal parameters were evaluated 70–80 days after the last treatment, with the exception of the tail-flick test, which was administered at 30 days of age, and passive avoidance test, which was administered at 54 days of age (see the following paragraphs). Experimental group size included at least six mice per group and is shown in the Figures. Our protocol includes purchasing pregnant mice from a breeder. Possible interference due to prenatal travel stress was minimized by its extension to all experimental groups: our control mice did not develop any metabolic, hormonal, or behavioral alterations described for pre- and postnatal stress in the literature. At the beginning of our investigations, we practiced the injection procedure to the pups just to antagonize through drug administration the behavioral effects induced by the handling stressor alone (brief mother separation). Then, we realized that the repeated injection itself was an interesting type of stress, which was able to induce type 2 diabetes metabolic and hormonal alterations, therefore we used the double stress paradigm to study these alterations, and whether and how different alterations could be antagonized. Of the two stressors, the handling stressor alone (brief mother separation) does not induce any apparent metabolic alterations in our experimental conditions (see results of mice Wsp in this paper). Data from literature maintain that brief mother separation alone, applied during the nursing period in rodents, does not induce body metabolic alterations as we found out, but it induces behavioral alterations in adults and can induce enhanced neurophysiologic responses (Kehoe *et al.* 1995, Tang *et al.* 2008). It also induces a certain hypo-responsive hormonal answer following further stress application (less intense hypothalamic CRH and plasma corticosterone increase vs controls – see for example Plotsky & Meaney (1993)) without causing in general body metabolic alterations. Conversely, we realized that the application of mild pain alone (injection) without mother separation is hardly possible, because the procedure in any case requires pups to be withdrawn from mothers and weighed before injection in order to have a correct evaluation of the drug dose. Therefore, we moved the six pups together from their mothers also to avoid that the repeated subtraction of the six pups, one after the other, could introduce a further behavioral variable for the mother–pups interaction. We also decided to adopt a fixed-length mother separation protocol for the whole nest, for all experimental groups.

Drugs and solutions

Naloxone hydrochloride was purchased from Sigma–Aldrich Italia. Dose (1 mg/kg, weight of the base) was the lowest that prevented enhancement of pain threshold and of body weight (Pieretti *et al.* 1991, Loizzo *et al.* 2012). AS was

produced as phosphorothioate by EURO BIO Laboratories (Les Ulis Cedex, France), under the direction of one of us (S M S). The 21-base sequence of AS-POMC is 5'-TCTGGCTCTTCTCGGAGGTCA-3'; it dose dependently reduces the synthesis of some cleavage POMC-derived hormones (β -endorphin and ACTH) in *in vivo* (after s.c. administration) and *in vitro* models, whereas its mismatch compound is inactive (Spampinato *et al.* 1994, Loizzo *et al.* 2003, Galletta *et al.* 2006). Artificial cerebrospinal fluid (ACSF) contained (in mM) 126 NaCl, 3 KCl, 1.25 NaH₂PO₄, 1 MgSO₄, 26 NaHCO₃, 2 CaCl₂, and 10 glucose equilibrated with 95% O₂/5% CO₂. Cutting solution contained 3 KCl, 1.25 NaH₂PO₄, 6 MgSO₄, 26 NaHCO₃, 0.2 CaCl₂, 10 glucose, 220 sucrose, and 0.43 ketamine.

Evaluation of nociceptive threshold: tail-flick test

Between the ages of 25 and 30 days, up to 45–50 days stressed animals showed maximal response to nociceptive stimuli. At 30 days, they underwent the tail-flick test, which is believed to measure pain mediated chiefly by spinal mechanisms (Pieretti *et al.* 1991, Loizzo *et al.* 2002). The tail-flick unit (U. Basile, Comerio, Italy) consisted of an infrared source (100 W bulb), whose light was focused on a spot 1–2 cm from the tip of tail. Latency was automatically recorded until the mouse flicked its tail away from the heat source. Beam intensity was adjusted to give a tail-flick latency of 1–2 s in controls. Cut-off time was adopted (10 s) to avoid tissue damage.

Abdominal overweight (epididymal fat pads) evaluation

After sacrifice (90 days of life), epididymal fat pads were dissected distally to blood vessels, including the tissue around the testis epididymus and ductus deferens. Tissues were washed in warm saline, blotted, and both pads were weighed (total weight in grams).

Glycemic test

At the age of 90 days, mice underwent glycemic control, which was performed in the morning after 12 h fasting, with one drop of blood put on test strips inserted in One-Touch EuroFlash (Johnson & Johnson Co., Lifescan Milpitas, CA, USA). The mean of two measures was recorded.

VEPs and rapid OPs

Methods were previously described (Lopez *et al.* 2002, Guarino *et al.* 2004). At 90–100 days of age, after general anesthesia with a mixture of xylazine–ketamine (20–32 mg/kg i.p.) and locally injected lidocaine (xylocaine 2%, 0.1 ml under the scalp), four epidural stainless steel electrodes were chronically implanted, with the right posterior (active) electrode position within the area of maximal acuity of the visual cortex (Porciatti *et al.* 1999). One week later, flash-VEPs were elicited by a Grass-Instruments PS22 photic

stimulator (period 1.02 s, over 90 s, EEG sampled at 2.5 kHz). The mouse was kept in an anechoic testing cage, inside a light-, sound-, and electric-shielded room. Animals were not scotopically adapted. Values of average ambient luminance dose (lux×s) were used to estimate the number of photo-isomerizations per rod per second expressed in log units (phy) (Pugh *et al.* 1998). A stimulus intensity of 2.682 phy was chosen. Latency in millisecond and amplitude in microvolt for the three negative (upward) peaks was averaged and evaluated. OPs consist of short (20–40 ms) bursts of high-frequency rhythmic oscillations, which are evoked in the retina, lateral geniculate nucleus, and cortical areas by light stimuli in man and animals. OPs are physiologically inserted and embedded inside evoked waveforms such as VEP and can be evidenced through EEG filtering and averaging (Lopez *et al.* 2002, Guarino *et al.* 2004). Here, OPs were gathered following raw EEG digital (finite impulse response (FIR)) 64–128 Hz band-pass filtering. Latency in milliseconds of peaks appearance was averaged and evaluated.

Cortical slice preparation and NAD(P)H fluorescence imaging

The method was previously described (Loizzo *et al.* 2010b). At 90–100 days of age, mice were anesthetized with a mixture of ketamine and xylazine (85 and 15 mg/ml, respectively, 0.15 ml s.c.) and decapitated. Brains were placed in ice-cold cutting solution. Visual thalamocortical slices (400 μ m; MacLean *et al.* 2006) were cut with a Vibratome (Leica VT1000S, Leica Microsystems AG, Wetzlar, Germany) and kept in ACSF at room temperature (90 min). Individual slices were transferred to a recording chamber (RC-26GLP, Warner Instruments, Hamden, CT, USA) perfused with warmed (35 °C) oxygenated ACSF, 2 ml/min for 10 min before recording. NADH excitation (360 nm) was delivered via fiber optic/monochromator system (DeltaRAM V; Photon Technology International, Lawrenceville, NJ, USA) reflected onto slice surface via dichroic mirror (DM-LP 400 nm, Chroma Technology, Brattleboro, VT, USA). Fluorescence emission in the basal condition and following electric stimuli was collected by using a cooled interline transfer CCD-camera (MicroMAX System, Princeton Instruments, Trenton, NJ, USA). 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. Photo bleaching was not significant for NAD(P)H measurements. Data were gathered over a 2500 μ m² circle surface tangent to the stimulus electrode. For imaging treatment and evaluation see Loizzo *et al.* (2010b).

Electric stimuli

Bipolar stimulating electrodes were used. Platinum tips (50 μ m in diameter) were placed in the transition zone between layers III and IV of primary visual cortex (V1),

responses were detected in layer IV of V1. Stimuli were delivered via Master-8 controller, DC supply, and constant-current isolation unit (A.M.P.I., Jerusalem, Israel). We used for all acquisitions the same protocol: stimulus frequency 100 Hz for 1 s (interval 70 μ s) with intensity of 1 mA. Between successive tetanic stimuli, 5 min intervals were maintained. Fluorescence excitation/imaging at 4 Hz (100 ms of exposition) began 4 s before onset of the electric stimulus and continued for a total of 25 s, so that the kinetics of initial NAD(P)H oxidation events (transient negative deflections) could be assessed.

Step-through passive avoidance task

At 54 days of age, animals underwent passive avoidance test by a step-through to darkness paradigm, with one training and one retention trial (Qin *et al.* 2002). At this age, the endogenous opioid system of stressed mice did not influence anymore the behavior of animals' nociceptive threshold (unpublished results). Mice were placed in a lighted platform (8×8 cm), and the timer run until the mouse completely entered an obscure compartment (20×20×20 cm) thus determining the training entrance latency. Then, the mouse received an electric foot-shock (0.35 mA, 6 s). Memory retention was tested 24 h later without foot-shock. Entrance latency during the retention session was measured up to 300 s (cut-off time).

BDNF levels

The visual cortex and hippocampus in 90-day-old mice were dissected and stored at -80°C until analysis. BDNF levels were determined by ELISA method using the BDNF Emax Immunoassay System kit (Promega) according to manufacturer's instructions (Monteleone *et al.* 2004, 2006). The lower detection limit was 7.8 pg/ml. Intra- and inter-assay CV were 2.9 and <9% respectively.

Statistical analysis

Results are expressed as mean $\pm$ S.D. The number of animals in each treatment group is indicated in the figures. Differences were tested by ANOVA and analyzed using Holm–Sidak comparison tests. Difference among groups of data was considered significant when P was <0.05.

Results

Postnatal double stress model produces mild type 2 diabetes-like signs, all of them implicating endogenous opioid system dysfunction, with the exception of hyperglycemia

Stressed mice showed reaction latency to nociceptive stimulus delayed over controls; both naloxone and AS treatments were able to prevent the difference (Fig. 1A). ANOVA: $F(4,55) = 14.03$; $P < 0.001$. A *post-hoc* test showed values of

Figure 1 Behavioral and metabolic parameters in the five groups of mice. Nociceptive threshold at 30 days of age (A). Body weight (B), abdominal overweight (epididymal fat pads (C)) and fasting glycemia (D) at 90 days of age. Values are mean $\pm$ S.D. Twelve animals per group for tail-flick, body weight, and fat pads weight; 24 animals per group for glycemia. In (A) a *post-hoc* test shows ^a $P < 0.001$ W-mice vs controls. There is also a difference $P < 0.001$ for W-mice vs other groups. In (B) ^a $P < 0.01$ for W-mice vs controls. Na prevents completely stress-induced overweight ($P < 0.01$), whereas the preventive effect induced by AS is partial (for W-mice vs AS-mice $P < 0.06$). In (C) values of W-mice are consistently greater vs controls ($P < 0.001$). Both Na and AS treatments prevent abdominal overweight ($P < 0.01$). In (D) glycemia values of W-mice are consistently greater vs controls. AS treatment completely prevents stress-induced hyperglycemia (*post-hoc* test: ^b $P < 0.001$, W-mice vs AS-mice), whereas Na treatment has no effect (^b $P < 0.001$ of Na-mice vs controls. C is for controls; W is for stressed group; Na is for stressed plus naloxone-treated; AS is for stressed plus antisense-treated; and Wsp is for maternal separated-not injected group. Note that naloxone treatment prevents enhancement of pain threshold, body overweight, and abdominal overweight, but it is not able to prevent stress-induced hyperglycemia in adults, whereas hyperglycemia is prevented by AS. Therefore, we can hypothesize that hyperglycemia is an ACTH–corticotesterone-bound sign in our diabetes model. Conversely, naloxone treatment is more effective than AS for the prevention of stress-induced overweight, therefore increase of body weight may appear as a prevalent opioid-dependent parameter (Part of data was taken from Loizzo *et al.* (2010a)).

nociceptive threshold in W-mice were consistently higher vs all other groups ($P < 0.001$). Body weight was heavier in W-mice at 90 days of age over controls, as expected, and naloxone treatment prevented completely this effect (Fig. 1B). ANOVA: $F(4,55) = 19.80$; $P < 0.001$. A *post-hoc* test showed values of W-mice were consistently higher vs C, Na, and Wsp groups (at least $P < 0.01$), while AS treatment had a scarce preventive effect, with borderline significance. Epididymal fat pad weight, which is considered a measure of abdominal overweight, was enhanced in W-mice vs controls, and this was true as absolute weight in grams and as percent of body weight (not shown). Na and AS treatments prevented this increase (Fig. 1C). ANOVA: $F(4,55) = 20.19$; $P < 0.001$. A *post-hoc* test showed that values of W-mice were consistently higher vs all other groups (at least $P < 0.01$). Basal fasting glycemia was enhanced in W-mice vs controls. AS prevented fasting hyperglycemia, whereas naloxone treatment had no

effect at all (Fig. 1D). ANOVA: $F(4,115)=19.57$; $P<0.001$. A *post-hoc* test showed that values of W-mice and of Na-mice were consistently greater vs C, AS, and Wsp groups ($P<0.001$). Mother-deprived not-injected Wsp-mice did not show any values above controls in the four parameters.

Neurophysiologic–neurometabolic results demonstrate that the double stress applied during the nursing period produces increased efficiency of the visual–cerebral system

This statement is supported by all four experiments:

VEPs and oscillatory rapid potentials as measures of visual system efficiency

Figure 2A shows a graphic sketch of a flashing lamp and mouse head. Figure 2B shows a sample of evoked potential with the three negative peaks and the corresponding rapid OP (sinusoidal-red line). In Fig. 2C, the three negative (upward) VEP latency components, N1, N2, and N3 were recorded significantly earlier in W-mice than in controls, completing their itinerary from retina to optic cortex about 2.3–3.5 ms before C-mice did, thus showing a 7–9% faster speed of visual response cycle vs controls. Naloxone and AS treatments prevented this effect. ANOVA for N1 latency: $F(4,25)=4.14$; $P<0.02$. A *post-hoc* test showed values of W-mice were consistently lower vs other groups (at least $P<0.025$). ANOVA for N2: $F(4,25)=6.91$; $P<0.001$. ANOVA for N3: $F(4,25)=19.40$; $P<0.001$. Also for these N2 and N3 a parameters, a *post-hoc* test showed values of W-mice were consistently lower vs other groups (at least $P<0.01$). The OP latency pattern was quite similar to the VEP pattern: control mice showed OP peak latency values of 31.4 ± 0.6 , 41.9 ± 0.4 , and 51.6 ± 0.3 ms for N1, N2, and N3, respectively, and W-mice showed values of 28.7 ± 0.4 , 39.1 ± 0.3 , and 47.8 ± 0.2 ms respectively. Both Na and AS treatments resulted in raising values to control levels. Statistical analysis showed results similar to VEP, therefore these should not be reported.

NAD(P)H fluorescence imaging as a measure of mitochondrial activity

Figure 3 illustrates basic properties of biphasic fluorescence transients evoked by a single tetanus to cortical pathway inputs in mice. The 360 nm light pulses were used for excitation, whereas emissions (>410 nm) were collected from the visual cortex cell region. The electric stimulus was accompanied by a sudden decrease in fluorescence, which is correlated to mitochondrial complex-I activity (Kann *et al.* 2005, Loizzo *et al.* 2010b). This transient (initial component, ‘drop’) after decrease is followed by a more sustained fluorescence increase (‘overshoot’). Figure 3A shows the average curve obtained in the groups of mice. Brain slices gathered from W-mice showed a reaction in the initial component not different from controls. This indicates that mitochondrial complex-I activity is similar in all groups. However, the amplitude and latency of the overshoot component were consistently higher (Fig. 3C) and faster (Fig. 3D) respectively in stressed mice vs controls.

Figure 2 Flash-visual evoked potentials in the five groups of mice. Six mice per group. (A) Schematic drawing of the flashing lamp (upper part of figure) and the mouse head, with recording electrode positions (lower part, left) and ground electrode (lower part, right). (B) Sample of visual evoked potential with the three peaks N1, N2, and N3 in blue color, with rapid oscillatory potentials in red. The arrow indicates flash. Abscissa: latency in millisecond. Ordinate: amplitude in microvolt. (C) Latency of negative (upward) peaks in milliseconds, mean $\pm$ s.d. in the groups of mice. Values of W-mice are consistently faster (lower) vs controls (*post-hoc* test: $^aP<0.025$). Both Na and AS treatments prevent effectively this effect (*post-hoc* test: at least $P<0.05$). Groups are: C is for control group; W is for stressed group; Na is for stressed plus naloxone-treated; AS is for stressed plus antisense-treated; and Wsp is for maternal deprived-not injected group. We underline that the N1 peak in W-mice is recorded at a mean of 2.3 ms before the N1 peak in C mice, N2 peak 2.5 ms, and N3 peak 3.5 ms before C mice, i.e. the time lag between the flash and N1–N3 peaks is 7–9% faster in W-mice vs controls. Further experiments (not described in detail) suggest possible involvement of the endogenous cholinergic-sensitive system (perhaps retina cells): treatment with physostigmine (0.05 mg/kg i.p.) induced somehow greater speed of N1 recording in C mice vs pre-drug (mean time 30.9 ms in treated vs 32.2 ms in controls, difference not significant), but no modifications were induced in N1 latency in the other groups (see *Supplementary material, Table 1*, see section on *supplementary data* given at the end of this article). Moreover, EEG analysis performed in the domain of frequency also indicated that stressed mice showed enhanced total power of the spectrum and enhanced power of very high frequencies (90–400 Hz band) during the active wakefulness condition in W-mice vs controls (see *Supplementary material, Table 2*). These preliminary data suggest that increases in the total power of the spectrum and in the power of very high-frequency bands is produced in stressed mice over control also in steady-state conditions, and not only after visual system (flash) stimulations. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0369>.

Figure 3 Biphasic fluorescence transients recorded in the five groups of mice. (A) Six mice per group, three slices per mouse, mean of three recordings per slice. Abscissa: number of frames (4 Hz); ordinate: fluorescence level expressed as $\Delta F/F_0$. Groups are: C is for control group; W is for stressed group; Na is for stressed plus naloxone-treated; AS is for stressed plus antisense-treated; and Wsp is for maternal separated-not injected group. (B) Sample of electrode position on the visual cortex slice. Data are gathered over a $2500 \mu\text{m}^2$ circle surface tangent to the stimulus electrode and expressed as pseudo-color tones. (C) Overshoot amplitude, $n=18$ per group. Amplitude is expressed as $\Delta F/F_0$, mean $\pm$ s.d., and is measured as mean value of three consecutive sampling points in the plateau region of the curve. A *post-hoc* test showed W-mice values consistently above controls ($^aP<0.01$ vs C-mice). AS treatment completely prevented stress-induced enhancement of overshoot vs W-mice ($P<0.01$), whereas Na treatment did not induce consistent effects ($P=\text{NS}$). (D) Overshoot latency (frames). $n=18$ per group. Latency is measured as in (C), is expressed as number of frames, mean $\pm$ s.d. (the u.v. light is administered with a frequency of four frames per second). A *post-hoc* test showed $^aP<0.001$, W-mice vs controls. Both Na and AS treatments were able to prevent the effect induced by stress: *post-hoc* test showed consistent differences vs W-mice ($P<0.001$). We underline that our neuro-metabolic findings indicate that the mitochondrial complex I activity in the visual cortex is unaffected, since the discharge part of the curve (drop) is analogous in all five groups (A). Therefore, we suggest that increase of both speed and amount of synthesis of mitochondrial NADH shown by the overshoot part of the curve in W-mice is addressed to an homeostatic physiological mechanism aimed at maintaining a stable complex I activity. A similar mechanism was also suggested to take place in the motor cortex of mice at an early stage of lateral amyotrophic sclerosis transgenic model (Loizzo *et al.* 2010b).

This suggests greater and faster NADH production (e.g. Kann *et al.* (2005) and references herein). ANOVA (Fig. 3C): $F(4,85)=3.78$; $P<0.01$. ANOVA (Fig. 3D): $F(4,85)=7.48$; $P<0.001$. Na and AS treatments prevented at least in part these effects. For details see Fig. 3C and D.

Step-through passive avoidance task as a measure of behavioral performance In the first day (training trial) stressed mice remained in the starting box for a longer time than controls and naloxone-treated mice before entrance. Also during the retention session, stressed mice showed latencies significantly longer than control and naloxone groups, therefore showing better performances (Fig. 4). For the first day latency, ANOVA: $F(2,33)=13.04$; $P<0.001$. For the second day, $F(2,33)=10.63$; $P<0.001$. A *post-hoc* test showed consistent differences between W-mice and controls ($P<0.01$). Na treatment was able to prevent effects induced by stress ($P<0.01$).

BDNF levels may correlate with a good memory performance BDNF levels in the visual cortex were consistently enhanced in W-mice. ANOVA: $F(3,20)=15.09$; $P<0.001$ (Fig. 5A). Both Na and AS treatments were able to prevent this effect (*post-hoc* test: $P<0.01$). An analogous effect was recorded in the hippocampus, but differences did not reach statistical significance (Fig. 5B).

Discussion

Our data confirm and extend the previous hypothesis that a double stress paradigm (mild psychological stress plus mild pain stress) daily applied to nursing male mice produces enduring body metabolic alterations similar to mild type 2 diabetes.

Here, we show also two new series of findings: 1) following postnatal stress, body metabolic alterations are accompanied by enduring improved efficiency of the CNS and visual system in young-adult male mice and that there is a logic connection between the two phenomena (see the next paragraphs), and 2) daily postnatal treatment with the μ - δ opioid antagonist naloxone is able to prevent completely or in part a few of these alterations and treatment with the POMC mRNA synthesis-inhibitor AS completely prevents almost all of them.

Enduring improved efficiency of the CNS and the visual system

Following our stress model, metabolic 'damage' appears in young-adult mice, thus indicating an abnormal condition, i.e. a type 2 diabetes model (Loizzo *et al.* 2006). However, our stress model does not induce 'damaged' values for neurometabolic-neurophysiologic parameters but induces enhanced brain and visual system performances. These results were demonstrated through the whole experimental protocol: a) improved efficiency of flash-VEP and OP; b) increased

Figure 4 Passive avoidance test. Latency time in seconds, mean $\pm$ s.d.; 12 mice per group. (A) Day 1. (B) Day 2. A *post-hoc* test shows ^a $P < 0.01$, W-mice vs controls. Na treatment prevents the stress-induced effect ($P < 0.01$). Groups are: C is for control group; W is for stressed group; and Na is for stressed and naloxone-treated. Part of the data was published elsewhere (Loizzo *et al.* 2012).

efficiency of NAD(P)H autofluorescence postsynaptic signals following electric stimuli, in the same cortical area; c) increased behavioral performance in the passive avoidance test; and d) increased BDNF level in the cortex. In addition, our previous data in stressed mice also had shown enhanced hippocampal CA1 plasticity of population spikes (increased long-term potentiation performance) vs controls (Franconi *et al.* 2004). Therefore, a question is whether there is a relationship between increased efficiency of neurophysiologic–neurometabolic parameters and diabetes-type metabolic parameters. Here, we suggest that diabetes-type dysmetabolic findings and neurophysiologic–neurometabolic enhanced findings depend upon a single pathophysiological entity, i.e. an abnormally enduring ‘alarm stress reaction’.

In fact, within the general adaptation syndrome (Selye 1976) the alarm stress reaction is an acute reaction that involves animals reacting to threats with a general discharge of the sympathetic nervous system, priming the animal for fighting or fleeing. This includes immediate physical reactions finalized to a preparation for violent muscular action and brain accelerated response of instantaneous reflexes (Gould & Udry 1994). Indeed, following acute stress, a diabetes-like ‘damaged’ hormonal–metabolic pattern is found, i.e. increased stress hormones (ACTH and corticosterone), increasing energy mobilization, with increased plasma insulin, glucose, lipids, certain cytokines, and leptin. At the same time, acute stress in general produces stimulating effects on cellular signaling and neurophysiologic models and is followed by enhanced passive avoidance learning, although these effects may depend on the type, intensity, and duration of stressful models. Acute stress induces also increased BDNF levels in the cortex and hippocampus of rodents (Kaneto 1997, Calderon *et al.* 1999, Das *et al.* 2000, Black 2002, Bland *et al.* 2005, Schneiderman *et al.* 2005, Ahmed *et al.* 2006, Pavlova & Vanetsian 2006, Dungan *et al.* 2009, Mazurek *et al.* 2010, Shackman *et al.* 2011, Tomiyama *et al.* 2012, Uysal *et al.* 2012).

In our mice, postnatal double stress is followed by a constellation of signs quite analogous to the various effects elicited by acute stress in the above-described papers. In synthesis, liberation of nutrients (enduring enhanced plasma glucose, plus insulin, triglycerides, and total cholesterol (Loizzo *et al.* 2006) supporting enforced muscular action) corresponds to a mild type 2 diabetes, while increased energy availability in the brain (related to increased mitochondrial metabolism (Gerbitz *et al.* 1996, Kann 2012) plus enhanced brain responses at both cortical and hippocampal (Franconi *et al.* 2004) levels correspond to the brain accelerated response (see also *Supplementary data*, Table 2, see section on *supplementary data* given at the end of this article). Within this scenery, increased efficiency of the brain is fed through the increased carbohydrate and fat energy availability. Therefore, our type 2 diabetes model can be seen as an enduring physiologic answer of the organism to an acute-repeated stress applied during critical periods of development, lasting far beyond the end of stress application itself. As a speculation, under a finalistic point of view these signs could be considered initially as ‘highly favorable’ for organism survival, but not as a damage. Nevertheless, further prolonged alteration of metabolic–endocrine balance in our mice leads the body metabolism to frank disease, while the brain is expected to become damaged in a second time. We consider these damages as a consequence of overexploitation of previously described hormonal and neurohumoral systems in our mice. In fact, investigations in the literature suggest that there is a time interval between the (apparent) onset of diabetes and the onset/level of neurophysiologic damage in men (Dolu *et al.* 2003), although apart from the present data, enhanced brain efficiency during this interval had not been demonstrated yet, in humans or in experimental animals.

Moreover, our previous data also suggest that the timing to appearance of neurologic damage in type 2 diabetes models varies and appears as a variable of the neurologic structure

Figure 5 BDNF (ng/mg protein), mean $\pm$ s.d. Twelve mice per group. (A) BDNF level in the cortex: A *post-hoc* test shows ^a $P < 0.01$, W-mice vs controls. Both Na and AS treatments prevent the stress-induced effects ($P < 0.01$). (B) BDNF levels in the hippocampus. No consistent differences are found among W-mice and other groups. Groups are: C is for control group; W is for stressed group; Na is for stressed plus naloxone-treated; and AS is for stressed plus antisense-treated.

complexity. For example, we showed that a peripheral nervous system structure and/or receptor function (vas deferens intramural sensitivity to opioid agonists in stressed mice) shows damage as early as at 90 days of age (Loizzo *et al.* 2003), i.e. when brain performances do not show damage.

Stress-dependent type-2 diabetes model shows two series of dysmetabolic signs in body and brain, each of them being related to disruption of two POMC-dependent endogenous systems

The two POMC-related endocrine–neurohumoral systems (opioid and ACTH–corticosteroid) interact in their physiopharmacologic activity (Amir *et al.* 1980, Pieretti *et al.* 1994), and clear-cut separation between the effects modulated by either systems is hardly possible. However, for the first time we could demonstrate the importance of either systems for the triggering of various specific signs and symptoms of an experimental type-2 diabetes model (Loizzo *et al.* 2012). Much work should be performed to better understand the molecular bases of the previously described mechanisms involved in diabetes model pathogenesis, which are at least in part gender dependent (Loizzo *et al.* 2010c); the involvement of other hormonal–neurohumoral systems (e.g. adrenergic) should also be considered. Moreover, a recent paper (Valenzuela *et al.* 2011) through experimental models quite similar to ours showed that also the postnatal treatment of mice with the cannabinoid CB1 receptor neutral antagonist/inverse agonist rimonabant could as well prevent several metabolic signs that were produced by postnatal stress in adult animals. These data add further intriguing hypotheses to the relationships between the brain endogenous opioid and the cannabinoid systems, and also on the role played by both systems in stress-related metabolic diseases.

Conclusions

We demonstrated that the etiopathogenesis of our type 2 diabetes model is connected to postnatal stress in male mice and depends on the break-down of feedback mechanism regulation of the two different endogenous systems triggered by POMC-derived hormone unbalance, which in turn produce enduring enhancement of synthesis release of ACTH–corticosterone and opioids, and is followed by diabetes-like metabolic alterations, such as enhanced plasma glycemia, insulinemia, cholesterol and triglycerides, overweight, abdominal overweight with fat cells hypertrophy, immune alterations, and others. This hypothesis is confirmed through the pharmacological treatment applied during the critical period of development of their receptors, i.e. during the nursing period: the treatment (naloxone and AS) was able to prevent specific diabetes-like alterations (for details see Loizzo *et al.* (2012)). Of course, other receptors and mechanisms may be involved in the pathogenesis of this model, and further studies are needed to elucidate this hypothesis. We speculate that stress-derived alterations may favor long-term risk for the appearance of frank metabolic

diseases, eventually ending in diabetes complications and particularly neurologic complications: we remember that recent papers underline metabolic diseases as risk factor enhancers for Alzheimer's disease (Merlo *et al.* 2010).

Here, we suggest that studies on postnatal stressful procedures also in other mammals may contribute to the understanding of etiopathogenetic mechanisms regarding type 2 diabetes models (nongenetic and nonalimentary) and their complications. Our knowledge of timing for brain receptor development in various mammals, including humans, and their sensitivity to the various types of stressors are still scanty. We speculate that better knowledge on possible relationships between postnatal damage to brain receptors and consequent damage to related physiologic functions in infants may help in the understanding of the pathogenesis of some diseases (particularly stress-related diseases) and/or nervous system signs/symptoms (see for example Cardona *et al.* (1991), De Giorgis *et al.* (1996) and Bardin (2012)).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-12-0369>.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Author contribution statement

A L projected the research and wrote the manuscript, S M S contributed to discussion and reviewed the manuscript, G C researched data and contributed to discussion, S V contributed to discussion, A F researched data, L C researched data, A C contributed to discussion, P M researched data, P R analyzed and contributed to data evaluation, and S L projected the research, wrote the manuscript, and researched data.

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A patent for AS and its variants for the prevention and the treatment of post-traumatic stress disorder (PTSD), is held at the Ufficio Italiano Brevetti e Marchi, no. RM2007A000005.

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