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Title page

Minireview

Etiopathogenesis and Pharmacological Prevention of a Type-2 Diabetes Model in Male Mice

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***Running title page*:**

a) A stress-derived type-2 diabetes model

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ACh is acetylcholine; ACTH is corticotropin; AS is antisense oligodeoxinucleotide; CRH is corticotropin releasing hormone; HbA_{1c} is glycated hemoglobin; HSD11B1 is 11β-hydroxysteroid dehydrogenase type 1; HSD11B2 is 11β-hydroxysteroid dehydrogenase type 2; NAd is nor-adrenaline; Nal is naloxone; PND is postnatal day; POMC is proopiomelanocortin, VEPs is visual evoked potentials

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ABSTRACT

We describe a stress-derived type-2 diabetes model in male mice, and formulate new hypotheses on how the model was induced, how diabetes-like alterations were prevented through specific pharmacological treatments, and how its possible neuroendocrine pathogenesis could be hypothesized. Pregnant females arrived in our laboratory on their 14th day of conceptional age. After birth, control mice never showed any apparent behavioral-metabolic-endocrine alterations. However, application of postnatal stress (brief mother deprivation, plus sham injection, daily from birth to weaning), was followed in adult male mice by two series of diabetes-like alterations. Some alterations (e.g., body overweight, immune, neurophysiologic, neurobehavioral alterations) were selectively prevented by opioid antagonist naloxone daily administered during nursing period. Mentioned alterations plus several others (e.g., hyperglycemia, neuroendocrine alterations) were prevented by administration of specific antisense oligodeoxinucleotide, which modulated synthesis-hyperfunction of proopiomelanocortin-derived ACTH-corticosterone and endorphins in the pituitary. Surprisingly, together with metabolic alterations, enduring increment of neurophysiologic/neurobehavioral brain performances were observed, accompanied by energy compensative reactions, and brain mitochondria hyperfunction. Thus, increased glycemia/lipidemia appeared to furnish fuel necessary to cope with increased request of energy. Diabetes-like alterations were accompanied by enduring hyperfunction of opioid- and ACTH-corticosterone-endogenous structures in the brain, which were apparently due to failure of negative feed-back hormone mechanisms in the pituitary, for the control of hypothalamus-pituitary-adrenal axis. In conclusion, for the first time we can hypothesize that a diabetes-like syndrome is produced by enduring hyperfunction of two proopiomelanocortin-

dependent endogenous systems (brain opioid- and ACTH-corticosterone-systems), following failure of pituitary feed-back hormonal control, after complex stress procedures.

1.INTRODUCTION.

Despite clinical and laboratory research, no concordance still exists on etiopathogenesis of type-2 diabetes mellitus, even if genetic-epigenetic factors, pre-perinatal environment and life style play important roles (Barrès and Zierath, 2016; Bouret et al, 2015; Hanson and Gluckman, 2014). Epidemiological investigations found that adverse prenatal, as well as adverse early-life experiences in children were connected to increased lifetime risk to develop chronic metabolic alterations, such as overweight and type-2 diabetes (Barker, 1998; Birnbaum and Miller, 2015; Eriksson et al, 2014; Jiang et al, 2013; Shonkoff et al, 2012, Thomas et al, 2008).

However, neuroendocrine and neurohumoral mechanisms underlying long-term connections between early stress (including pre-natal stress) and adult metabolic conditions in humans and/or laboratory animals are still unclear (see Kumari et al, 2013).

Several investigations were carried out in laboratory animals to study stress mechanisms during perinatal periods. Early repeated psychological stress model (daily mother separation during nursing period – see section 2.1.) inspired relevant studies on psychophysiologic-behavioral consequences to adult rodents, and on their relationships to hypothalamus-pituitary-adrenal (HPA)-axis hormones, and overall to glucocorticoids (Akil and Morano, 1995; Oitzl et al, 2010). Other investigators showed that chronic stress, or long-term administration of glucocorticoids, lead to disruption of hypothalamus-pituitary-adrenal system, leading in turn to continuous high levels of glucocorticoids and to insulin resistance in adult mice (see van Donkelaar et al, 2014), and this pre-diabetic state can eventually develop into type-2 diabetes mellitus-like syndrome. Interestingly, also reactive changes in the hypothalamus came to the

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attention of researchers, as these changes were shown to trigger leptin and insulin resistance also observed in pre-diabetes (Kälin et al., 2015). In the obesity and type-2 diabetes research fields, mouse models have proven invaluable in basic science of diseases by identifying the roles of inflammation, insulin resistance, fat content of the diet and potential treatments (Islam and Loots, 2009; Heydemann, 2016).

However, pathogenetic mechanisms connecting early stress to metabolic diseases in humans and/or animal models are still matters of debate.
Here, we describe a reliable and reproducible model in male mice, as a new approach to study causes and evolution of type-2 diabetes-like syndrome in mice, in order to better investigate its etiopathogenetic mechanisms, and to develop drugs useful to prevent or reduce diabetes-like alterations and risk of complications in mice.

2. THE ANIMAL MODEL

2.1.Early stress, and its neurobehavioral-metabolic-hormonal effects in rodents.

Pioneering investigations (Denenberg and Karas, 1959; Levine, 1957; Weininger, 1954) showed that early repeated mild psychological stress alone, such as handling/mother separation, daily applied to rodents at birth or thereafter, produced upset of both behavioral and metabolic functions, accompanied by HPA-axis hormones disturbances. These investigations opened the way to a series of relevant observations addressed to study mainly psychophysiologic-behavioral consequences induced by postnatal psychological stress to adult rodents, and their relationships to HPA-axis hormones (for references see Akil and Morano, 1995; Loizzo et al, 2010a; Oitzl et al, 2010). However, the other face of the coin, i.e., the metabolic consequences of early stress, was substantially neglected during the following 3-4 decades.

2.2. Prenatal and neonatal procedures of the model.

Our investigations were addressed to study mainly the other face of the coin, i.e., the metabolic alterations following early stress in male mice. Animal care, environmental conditions and use followed rules of the Council of European Communities 86/609/EEC, and "Principles of laboratory animal care" (NIH publication No.85-23, revised 1985). Experimental procedures were approved by the Bioethical Committee of the Italian Istituto Superiore di Sanità and by the Italian Ministry of Health. All efforts were made to minimize the number of animals and their suffering. Series of multiparous pregnant outbred CD-1 mice (Charles River Italia, Calco, Italy) arrived at day 14 of conceptional age in our vivarium, where animals were kept in single plastic cages. After birth, pups of equal body weight were put together, randomly culled to six male pups nests, and randomly cross-fostered (Loizzo et al, 2006). Starting from the 2nd postnatal

day (PND), we adopted the mother deprivation stress model, but with an additional stressful condition: we coupled the usual psychological stress (10 min mother deprivation) to a mild physical pain stress caused by a subcutaneous saline injection, contextually administered daily from birth to weaning, i.e., from 2 to 21 PND (Pieretti et al, 1991). In view of the influences exerted by the endogenous opioid system on several diabetes-like alterations induced by our model (section 2.2.), experiments were performed only during the winter period, in which maximal sensitivity of opioid receptors in mice was described (De Ceballos and De Felipe,1985). After weaning, mice were caged in groups of three subjects belonging to the same treatment, and were left undisturbed in their cages, except for cage cleaning twice weekly, until experiments began (Table 1).

Reliability and repeatability of the model were tested through a reference protocol applied to series of experimental animals, in various experimental sessions, during the last 25 years, and consisting of: 1) body weight recording; 2) nociceptive test (tail-flick/hot-plate) applied at 25-30 PND; 3) fasting glycemia at euthanasia (usually at 90 PND, sometimes up to 140 PND). Other independent investigators adopted the same model as ours, including prenatal travel stress, and found results quite identical to ours in their stressed mice (e.g., Valenzuela et al, 2011). Similar models adopted by other groups produced similar results in rats (e.g., McPherson et al, 2009). We underline that changes of one or more experimental conditions may challenge reliability of results (Supplemental Data 1).

2.3. Early stress and the endogenous opioid system.

Following the stress model, we observed consistent increase of nociceptive threshold in mice, and this effect was observed from 25 PND at least up to 45

PND; moreover, stressed mice showed increase of body weight incremental curve over controls: increase was significant after 60-80 PND, was accompanied by increase of abdominal fat weight (periepididymal fat pads) and by increased volume of abdominal fat cells, and lasted at least up to 135-140 PND (d'Amore et al, 1995; Pieretti et al, 1991, Loizzo et al, 2006; Loizzo et al, 2012a). In our model, these effects were prevented in a dose-dependent way by daily injection of naloxone (from 0.1 to 1 mg kg^{-1} body weight, as weight of the base, but in most experiments the 1 mg kg^{-1} dose was adopted – Loizzo et al, 2012a) administered during the nursing period (not yet verified for fat cells volume). Therefore, we deduced that our stress model induced not only alteration of the HPA system, as it was described by several investigations following repeated perinatal stress (see Akil and Morano, 1995) but also induced consistent and prolonged upregulation of endogenous opioid system activity as well. Independent investigators from other laboratories found that, following the repeated brief mother deprivation model alone (no sham injection), enduring alteration of brain endogenous opioid peptides activity, and/or of opioid receptors, was elicited in selected brain regions in rats (Gustafsson et al, 2008; Irazusta et al, 1999; Kiosterakis et al, 2009; Ploj and Nylander, 2003; Ploj et al, 1999; Ploj et al, 2003).

2.4.Timing of opioid and corticosteroid receptors development.

We applied our postnatal stress model during the nursing period (2 to 21 PND) because during this period several physiological functions/receptors (in particular, corticosteroid and opioid receptors) pass through their critical developmental period in rodent brain, and have maximal sensitivity to stressful events; therefore, insults applied to mice and rats during this period may produce long-term or permanent alteration of receptors functions (McDowell and

Kitchen, 1987; Rosenfeld et al, 1993; Zagon and McLaughlin, 1993). We hypothesized that involvement of both receptors systems was necessary to explain etiopathogenesis of stress-related metabolic alterations, and that the acknowledged chain of hormone release elicited by our stress model, i.e.: corticotropin releasing hormone (CRH)-->proopiomelanocortin-->ACTH plus ß-endorphin (and possibly other hormones) played an important role as well. Proopiomelanocortin is an important ring of the hormonal HPA chain, since it gives rise to equimolecular amounts of ACTH and ß-endorphin through cleavage mechanisms (Vale et al,1981). Therefore, we addressed our investigations to explore physiological/regulatory mechanisms that are at the basis of homeostasis/hyperactivity of both opioid and ACTH-corticosterone endogenous systems, in order to evidence step-by-step trajectories from cause (stress) to effect (adult type-2 diabetes-like syndrome) in male mice (see Chapter 3).

3. THE NEUROENDOCRINE BASIS OF THE MODEL.

Following our stress model, mice showed several metabolic, behavioral, immunological and neuroendocrine alterations (Fig.1; see also Fig.3-6 and Supplemental Data 2), similar to those observed in human type-2 diabetes and in human prediabetes (Ferrannini, 2014). (Figure 1).

In our model we studied mechanisms which can produce these alterations.
In normal animals, following a single acute stress, plasma ACTH and corticosterone levels increase abruptly. In the absence of further stimuli, this increase triggers specific feed-back negative control mechanisms, and these hormones return to physiological steady-state levels within minutes or hours (Akil and Morano, 1995; Hackett and Steptoe, 2017) (Fig. 2A). Conversely, in case of repeated stressful stimuli, unexpected results may be produced: following daily stress through the nursing period, our stressed male mice at age 90 PND and thereafter, i.e., at least 70 days after the end of stress, still showed enduring abnormal enhancement of plasma and pituitary ACTH, and consequently showed enhancement of corticosterone plasma levels. At the same time, CRH and ACTH levels in the hypothalamus were strongly reduced in stressed mice versus controls, as expected (Fig. 2B and 2C) (Galietta et al, 2006; Loizzo et al, 2010a). Therefore, we argued that hypothalamic feed-back negative mechanisms for the control of CRH and ACTH levels were working correctly, whereas pituitary mechanisms deputed to the control of synthesis/release of ACTH and of other POMC-derived peptides in corticotropic cells failed to be modulated by enhanced corticosterone and ACTH plasma levels. Therefore, we found enduring abnormally high ACTH and corticosterone levels in the pituitary/plasma of stressed animals in the adult ages (Galietta et al, 2006;

Loizzo et al, 2010a). (Figure 2A, 2B and 2C).

This appeared as the keystone for understanding our diabetes model: repeated stress procedures apparently produced break-down of the pituitary-adrenal negative feed-back control mechanisms; this in turn resulted in a strong and enduring enhancement of plasma ACTH, and therefore of plasma corticosterone levels. These data enforced the hypothesis that both endogenous ACTH-corticosterone– and opioid-systems hyperfunction, induced by our stressful procedures (see section 2.3.), were both responsible for determining the appearance of diabetes-like alterations in adult male mice. The previous said hypothesis was further confirmed also through an inverse approach (see sections 4.1. and 4.2.).

4. UNDERSTANDING PATHOGENESIS OF SPECIFIC DIABETES-LIKE ALTERATIONS THROUGH SPECIFIC PHARMACOLOGICAL TREATMENT

4.1. Some stress-induced alterations were prevented by administration of an opioid receptor antagonist drug.

Alteration of several parameters described in adult mice, following postnatal double stressful procedures (presumably, also travel stress of pregnant mother may have induced a certain sensitization of HPA in mice fetuses–see Hiroi et al, 2016), were prevented by administering to our mice the opioid receptor-antagonist naloxone, during the nursing period, therefore these alterations can be defined as "prevalent opioid-sensitive", and include metabolic parameters (increase of body weight, and abdominal fat weight –d'Amore et al, 1996; Loizzo et al, 2010a;), increase of food caloric efficiency – Loizzo et al, 2012a), increase of some brain mitochondrial parameters efficiency (such as reduced latency of NAD(P)H fluorescence imaging evoked to cortical pathway inputs in ex-vivo brain slices– Loizzo et al, 2012b), alteration of immunological parameters (increase of some cytokines of the Th-1-type released by splenocytes, decrease of some cytokines of the Th-2-type, increase of natural killer cell activity, increase of splenocyte proliferative activity – Loizzo et al, 2002), alteration of behavioral parameters (increased efficiency of passive avoidance test – Loizzo et al, 2012a), alteration of neurophysiologic parameters (reduced latency of visual evoked responses and oscillatory responses – Loizzo et al, 2012b), and others. (See also Fig. 3, 4, 5, and Supplemental Data 2). (Figure 3).

4.2. Several stress-induced alterations were prevented by modulating the endogenous proopiomelanocortin activity.

We observed that both endogenous opioid- and ACTH-corticosterone-systems activities are linked, at the anterior pituitary level, to promote the synthesis/release of the pro-hormone proopiomelanocortin. Therefore, in one of our laboratories (Spampinato et al, 1994) antisense oligodeoxinucleotides were designed to bind to a selected target *m*RNA sequence by Watson–Crick base pairing, leading to a formation of double-stranded sequence, which resulted in blockade of *m*RNA processing or translation. Thus, we found that following administration, during the nursing period, of an antisense oligodeoxinucleotide complementary to a region of β-endorphin *m*RNA (AS-POMC), a dose-related reduction of synthesis of proopiomelanocortin-derived peptides ACTH and β-endorphin was produced in adult male mice (Galietta et al, 2006; Loizzo et al, 2003; Spampinato et al, 1994). Therefore, series of experiments were performed to identify those diabetes-like parameters induced by the stress model that were not (or only in part) prevented by naloxone treatment in stressed mice, but were efficaciously and dose-dependently prevented in adult mice by daily treatment with AS-POMC, administered during nursing period. These parameters were identified as "prevalent ACTH-corticosterone-sensitive", and include metabolic parameters (enhanced fasting glycemia – Loizzo et al, 2010a), some brain mitochondrial parameters efficiency (such as increased amplitude of NAD(P)H fluorescence imaging evoked to cortical pathway inputs in ex-vivo brain slices- Loizzo et al, 2012b), neuroendocrine parameters (reduced CRH and ACTH at hypothalamic level, enhanced ACTH at pituitary and plasmatic levels, enhanced corticosterone at plasmatic levels – Galietta et al, 2006; Loizzo et al, 2010a), and

some vascular parameters (reduced contracting response to noradrenaline in isolated *ex vivo* aorta rings, and enhanced relaxing response to acetylcholine – Loizzo et al, 2015). There was anyway a certain interference exerted by naloxone on some endocrine parameters. For example, AS-POMC treatment drew to normal values the abnormally enduring increase of ACTH levels, produced by stress in the pituitary and plasma, but naloxone treatment in part also reduced the abnormal increase (Loizzo et al, 2010a). Of course, all opioid-sensitive alterations produced by the stress model were also dose-dependently prevented by the administration of AS-POMC during the nursing period (Loizzo et al, 2010a; Loizzo et al, 2012a; Loizzo et al, 2012b). (Fig. 4 and 5; see also Supplemental Data 2). (Figure 4 and Figure 5).

Some alterations which belonged to the same patho-physiological "type" were in general prevented by the same treatment. For example, several alterations induced by stress on the immune system were prevented by naloxone (Fig 3) (Loizzo et al, 2002). Some metabolic alterations (alteration of body weight and abdominal weight - fat pads weight) also were prevented by naloxone, whereas hyperglycemia was not prevented by naloxone, but was consistently prevented by AS-POMC (Fig 4C) (Loizzo et al, 2010a). Antiociceptive effects induced by stress on prepuberal/puberal mice (up to about 45 PND), and enhanced performance of visual evoked potentials (VEPs) in adults were prevented by naloxone, and also by AS-POMC (Loizzo et al, 2012b), whereas decreased sensitivity of ex-vivo aorta isolated rings to noradrenaline contracting effects in stressed mice was prevented by AS-POMC, not by naloxone (Loizzo et al, 2015).

4.3. Some stress-induced alterations are still of undetermined origin.

Beside the previously described parameters, another series of parameters was modified in adult mice following postnatal stress, but we could not demonstrate with reasonable certainty, up to now, the mechanisms through which these alterations were produced. This is referred to some metabolic alterations (increase of plasma insulin, leptin and triglycerides – Loizzo et al, 2006), some behavioral alterations (reduced immobility time at Porsolt test – Franconi et al, 2004), some neurophysiologic alterations (enhanced long-term potentiation in the hippocampus – Franconi et al, 2004-; enhanced EEG total power, enhanced power of very fast frequency bands of EEG -90 to 400Hz- recorded during the active wakefulness – Loizzo et al, 2012b), some gene findings alterations (enhanced brain *m*RNA expression of 11β-hydroxysteroid dehydrogenase type 1, and reduced brain *m*RNA expression of 11β-hydroxysteroid dehydrogenase type 2 – Loizzo et al, 2010b) and others. Some effects listed here are depicted in Fig 6; see also Supplemental Data 2. (Figure 6).

5.THE "ENDURING ACUTE" STRESS HYPOTHESIS

Our stress model produces an increased activity of ACTH-adrenal axis and of endogenous opioid systems, and these activities are accompanied not only by diabetes-like dysmetabolic parameters, but also by increased efficiency of some neurophysiologic-neurometabolic parameters (Loizzo et al, 2012b; see also section 4.3. and Supplemental Data 2). We suggest that these findings, which according to previously described results are triggered by failure of pituitary feedback control mechanisms, depend upon a pathogenesis which involves a unique pathophysiological phenomenon, i.e., an abnormally enduring alarm stress reaction. Our stress model is accompanied by a constellation of physiological-metabolic alterations extraordinarily similar to those induced by acute stress reactions (see Loizzo et al, 2012b), as described in the original investigations (Selye, 1976), but with at least one main difference: alterations that occur after an acute stress (or following glucocorticoid administration) are observed for minutes or hours, whereas following our stressful procedures, alteration of said parameters, and of neurophysiologic performances, lasts for several months, and may be life-long (Loizzo et al, 2006; Loizzo et al, 2012a; Loizzo et al, 2012b). Importantly, increased brain performances require increased energy availability. Therefore, during the adulthood period in our mice, compensatory homeostatic mechanisms, including increased efficiency of energy production, are mobilized through enhanced fuel availability, which in our hypothesis is supplied by plasma hyperglycemia and hyperlipidemia. These events require enhanced mitochondrial activity, thus responding to increased energy demand by increased performance of brain and other organs (Loizzo et al, 2012b; Loizzo et al, 2010c). Mitochondrial energy reactions quite similar to those detected in our diabetes model, as measured by NAD(P)H

autofluorescence signal, are also observed in other diabetes models, as well as in other animal models of chronic diseases, thus representing unspecific early markers of neurodegeneration (Loizzo et al, 2010c; Moreira et al, 2003; Newsholme et al, 2012). Therefore, these reactions may prelude to cell damage and disease complications. We speculate that the previously described findings and the "enduring acute-stress" reactions put mice in a favourable survival situation versus controls. However, following long periods of enhanced efficiency of above described metabolic systems, an evolution of the model may start, i.e.: following overexploitation of energy-producing mechanisms, these structures may become exhausted, leading organism to frank pathological condition, and to progress of diabetes complications.

Our studies point out also to a link between central nervous system, especially the hypothalamus, and peripheral mechanisms associated with obesity that may lead to type-2 diabetes. Malfunction of central nervous system networks, that control energy intake and expenditure, is a major mechanism for the development of obesity that contributes to pathological changes in peripheral organs and tissues, including insulin resistance, driven by proinflammatory responses involving immune cells and adipocytes (Begg and Woods, 2013). Hypothalamic inflammation is characterized by glial reactivity, secretion of cytokines and increased levels of intracellular inflammatory signals. In rodent models of long-term diet-induced obesity, glial activation is sustained and the vasculature undergoes angiogenesis. In addition, hypothalamic POMC neurons, which inhibit food intake and stimulate energy expenditure, are reduced (Thaler et al., 2012).

Albeit we did not investigate any alteration of hypothalamic glial reactivity in our stress model, we remember that Gerber and Bale (2012) adopted, as a model

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of chronic stress, mice deficient in corticotropin-releasing factor receptor-2, and observed an involvement of hypothalamic astrocytes in the exacerbation of stress pathway dysregulation. Further studies are necessary to better explore this relationship between chronic stress and hypothalamic inflammatory reactions.

6. COMMENTS ON MODEL IMPLICATIONS

6.1. The stress model and female sex

Clinical and laboratory studies agree that women and men have different disease risk, since women of reproductive age are protected from metabolic and cardiovascular disease compared with postmenopausal women and men. In rats, several responses to stress and consequences of early maternal separation are gender-dependent: male and female rats appear to have different behavioral profiles and coping strategies in many behavioral experiments (Vetulani, 2013). It is therefore possible that history of early adversities express different hormonal balance and different metabolic equilibrium in the two sexes.
In fact, we applied our stress model also to female mice, and performed some investigations in our laboratories (Loizzo et al, 2010b). Different metabolic patterns were found between sexes (see Table 2). From these investigations we gathered some informations:

The first interesting information is given by the evolution of body weight. At weaning, following 20 days of stress (from 2 PND to 21 PND), body weight in stressed males is consistently lower versus their controls; but afterwards, stressed male mice gain rapidly their weight, and at 90 PND they become heavier than controls (Table 2). We underline that disturbed growth during important periods of early development, followed by rapid weight gain, was associated to increased risk of type-2 diabetes in humans (van Abeelen et al, 2012), and we speculate that this mechanism may be suggested as one pathogenetic determinant for diabetes-like syndrome in our mice as well.
Conversely, our stressed female mice did not show different body weight versus controls, neither at weaning nor in the following days up to 90 PND (Loizzo et

al, 2010b). Moreover, stressed females did not show consistent alterations of nociceptive sensitivity (at 30 PND), of fasting glycemia, of non-fasting corticosterone levels, and of HSD11B1 and HSD11B2 *m*RNA mean expression in the brain cortex, versus control females, at 90 PND, differently from males (see Loizzo et al, 2010b). (Table 2).

These results suggest that etiopathogenetic mechanisms of metabolic alterations, previously hypothesized in male mice, need different evaluation when females are considered. In particular, we underline that the pattern of HSD11B1 *m*RNA mean expression in the brain cortex was consistently enhanced in stressed male mice. HSD11B1 reduces 11-dehydrocorticosterone to corticosterone, that activates glucocorticoid receptors, thus suggesting that this mechanism may further increase physio-pharmacological activity exerted by the hyperactivity of the endogenous glucocorticoid corticosterone found in our stressed male mice, and presumably produces further metabolic "damage". Conversely, HSD11B2 pattern (which converts corticosterone to the inactive 11-dehydrocorticosterone) was consistently decreased after stress model in males, thus further enforcing the same physio-pharmacological mechanisms, as HSD11B1 does. Moreover, plasma level of corticosterone and ACTH, and brain HSD11B1 and HSD11B2 *m*RNA expression are not significantly modified in stressed females versus their controls (Loizzo et al, 2010b) (see Table 2). Thus, the present findings strongly confirm and extend the hypothesis that the absence of alteration of HPA hormones pattern induced by stress in females, and also the absence of alteration in the *m*RNA expression of HSD11B1 and HSD11B2 in females, could suggest some explanations for differences of metabolic and hormonal long-term alterations found in stressed

males versus stressed females in mice.

6.2. The stress model and overfeeding models

Several animal models of obesity described in the literature are somehow heterogenous, as they exploit spontaneous mutations or diet-induced obesity in rodents; however, these latter models are often used to study polygenic causes of obesity and are believed by several investigators to mimic better the state of common obesity in humans, and may be the best choice for testing prospective therapeutics. These animal models may also show some of the most frequent comorbidities of obesity, like hyperglycemia, insulin resistance or diabetes-like syndromes (Griffin et al, 2016; Habbout et al, 2013; Heydemann, 2016; Lutz and Wood, 2012; Reyes, 2012); similar metabolic and immunological alterations are observed in our model as well (Table 3; Supplemental Data 2). In synthesis, the three conditions (human type-2 diabetes, mouse high fat diet/overfeeding syndrome, and our stress model of type-2 diabetes) share a number of similar alterations, shown in Table 3, and we speculate that all three “syndromes” may also share some neuroendocrine pathogenetic mechanisms described in the present paper. (Table 3).

7. FUTURE DEVELOPMENTS, AND SELF-CRITICISM.

Stress-induced failure of pituitary negative feed-back mechanisms, for the control of proopiomelanocortin-derived ACTH-corticosterone and endogenous opioids hyper-activity, appears of crucial importance for the pathogenesis of the previously described diabetes-like syndrome. However, some important questions await to be confirmed and defined:

7.1. Studies on deep pathogenetic mechanisms

Molecular and gene mechanisms which are involved in the failure of negative feed-back mechanisms in the pituitary are still to be understood. Which is exactly the damage induced by stress? Our data (Galietta et al, 2006; Loizzo et al, 2010a) showed that our model produced failure of negative feedback hormones regulation in the pituitary, but of course, we need to know what does really means the statement "failure of negative pituitary feed-back mechanisms", and where and how, exactly, it is produced.

7.2. Studies on etiologic determinants

Etiology of the model also requires further investigation. The protocol we followed during several years gave results which were repeated in our laboratories with a high rate of reproducibility and reliability. However, in our papers in the past we may have not underlined with enough emphasis the possible influence exerted by prenatal travel stress on postnatal period (see also Loizzo et al, 2002). We reported that CD1 pregnant mice arrived at our laboratory on the 14^{th} day of conceptional age, following a travel from the factory. It is well known that a repeated, although soft vibration (and travel-producing vibration) applied to the whole body produces increase of circulating ACTH and cortisol/corticosterone in humans and laboratory animals (Cardinale

et al, 2010; Perremans et al, 2001). Stress applied during pregnancy sensitizes HPA axis, increasing stress-induced corticosterone secretion in preweanling rats, and prolonging stress-induced corticosterone secretion in the adult (Fride et al., 1986; Henry et al.,1994; Maccari et al., 1995; Peters, 1982; Takahashi et al., 1988). Therefore, it should be investigated whether pre-natal, enhanced endogenous corticosteroid exposure to fetuses environment could have rendered our pups more susceptible to pathological outcomes when further stress were administered in the post-natal period. This may have happened also in the absence of apparent changes of behavior and hormonal levels in steady-state/control conditions in newborns, and in fact, we always described alterations found in our postnatal stressed mice versus baseline data of postnatal "control" mice. Therefore, we may hypothesize that these latter animals' physiological and psychological parameters can be challenged only when further -postnatal- stress is also administered. Other investigators recently demonstrated that a "two-hit" type stress may produce effects similar to those we found: according to Vargas et al (2016) early life stress increases metabolic risk and HPA axis reactivity when it is combined with post weaning social isolation in rats (see also Hachett & Steptoe, 2017).

7.3. A bridge to developmental roots of some chronic diseases of adults?

Much work was dedicated to studies of developmental bases of adult diseases. Animal models described in the literature indicate that pre- and post-natal stress may be followed, in adult ages, by increased risk for neurobehavioral, metabolic, cardiovascular, renal diseases (see Jiang et al, 2013; Reagan, 2012; Vargas et al, 2016). Present data suggest that pathophysiologic basis of diabetes-like metabolic alterations in our model may reside in the failure of pituitary feedback control mechanisms, which is triggered by stress applied during critical period of

development of receptors, and/or other cell organs. Therefore, we speculate that even subtle manipulations of stress parameters (types, timing, duration, intensity, repeatability, combination of pre- and post-natal stress, and presumably sex of subjects) may produce increased risk for those adult body and brain chronic diseases, which are presumed to have had their origin during developmental periods, through involvement of different constellations of neuroendocrine/neurohumoral malfunctions.

7.4. Possible therapeutic applications.

In perspective, therefore possible therapies to treat type-2 diabetes should be considered, when there is a reported history of prolonged stress condition, in the early age windows reported in our studies and described in this review. To achieve this goal, antagonists of the endogenous opioid receptors like naloxone and oligodeoxinucleotide-based therapies aimed to block the expression of POMC-derived peptides could be hypothesized as prophylactic strategies.

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8. CONCLUSIONS.

We describe an early stress-derived mouse model which produces metabolic alterations similar to those found in human type-2 diabetes. In our opinion, main novelties of our investigations consist in that we could correlate etiopathogenesis of these alterations to three sceneries:

a) A type-2 diabetes model in male mice, following early complex stress model, is obtained when our complete protocol of experiments is applied. A synergistic pre- and post-natal stress mechanism can be suggested as a main cause for the triggering of previously described effects (see also Maccari et al, 2014). Of course, also other types of stress, of different intensities/times of application, may induce analogous effects (see Vargas et al, 2016).

b) Following our stress model two (partly) different series of diabetes-like alterations are produced in adult mice. One series depends mainly on hyperfunction of the endogenous opioid system, and its related alterations are prevented through the administration of naloxone during nursing period. The other series depends mainly on hyperfunction of the ACTH-corticosterone system, and its related alterations are prevented through the administration of an AS-POMC, which reduces both endogenous ACTH, and endogenous opioids synthesis/hyperfunction at brain and pituitary levels, through proopiomelanocortin synthesis modulation. Enduring enhanced functions of endogenous opioid system, together with ACTH-corticosterone system enhanced activity, are both necessary conditions for the triggering of diabetes-like alterations in our model.

c) We showed that hyperfunction of these two systems is accompanied by failure of feedback hormonal control mechanisms induced by our stress procedures at

pituitary level. This appears of great importance to induce enduring hyperfunction of the two endogenous systems here described, and therefore to induce triggering of diabetes-like syndrome.

Finally, as a finalistic speculation, our stress model produces findings which are compatible with an 'enduring acute-stress' reaction, which puts mice in favorable survival situations versus controls. However, prolonged hormonal–metabolic imbalances produced by stress are expected to also produce diabetes-like complications at later ages in stressed mice.

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SL is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Wrote or contributed to the writing of the manuscript: A. Loizzo, Spampinato, Campana, S. Loizzo.

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Footnotes:

DECLARATION OF INTEREST

Authors have nothing to declare.

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REPRINT REQUESTS

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Figure Legends

Fig.1. Main diabetes-like effects induced by stress

Data are expressed in percent versus controls, ± SEM. Data in control mice at about 90 PND were: fasting glycemia= 90 ± 4 mg dL^{-1}(Loizzo et al, 2010a); fasting plasma triglycerides = 86 ± 5 mg dL^{-1}(n=12 – Loizzo et al, 2010b); fasting plasma insulin: 24 ± 0.6 pMol L^{-1} (Loizzo et al, 2006); body weight = 42.5 ± 0.5 g (Loizzo et al, 2010b); abdominal fat weight (epididymal fat pads) = 1.2 ± 0.2 g (Loizzo et al, 2010a); non-fasting plasma corticosterone = 23 ± 4 ng mL^{-1}(Loizzo et al, 2010a); plasma leptin = 4.5 ± 0.4 ng mL^{-1}(Loizzo et al, 2006). Locomotor activity was recorded by radar devices in 6 control and 6 stressed mice during 7 consecutive days (Migliore, 2007). *is for P< 0.05; ** is for P< 0.01; ***is for P< 0.001.

Fig 2. Proposed pathogenesis of the diabetes model

A) According to the normal-physiological feed-back mechanism, following single stress hormone levels increase, but return to normal values within minutes or hours, due to the hypothalamic and pituitary feed-back regulatory mechanisms (see arrows).

B) However, during the stress period an important pathophysiologic mechanism ensue: day by day, apparently a cumulative adversity increases stress sensitivity and risk of adverse health outcomes. Therefore, repeated stress results in break-down of pituitary feed-back negative control mechanisms in developing mice, finally resulting in long-lasting, exaggerated synthesis/release of ACTH and corticosterone in adults (red arrows in the figure). Hormone levels during this period are shown in the graph C.

C) The graph shows hormone levels in stressed mice at 90 PND (black

columns), as percent of control values (white columns). In this case, please note that CRH and ACTH levels in the hypothalamus is strongly decreased versus controls (due to effective hypothalamic feed-back control mechanisms), whereas pituitary and plasmatic ACTH, and plasmatic corticosterone are strongly and enduring increased at least for 70 days after the end of stress, presumably due to failure of pituitary feed-back control hormones mechanisms). All these effects are prevented in animals stressed and treated with AS-POMC (shaded columns), while treatment with naloxone produces minor or no changes (not reported here, published in Loizzo et al, 2010a). Control values: hypothalamic CRH = 21.3 ± 1.5 pg μg^{-1} proteins; hypothalamic ACTH = 10.7 ± 0.7 pg μg^{-1} proteins; pituitary ACTH = 1816 ± 160 pg μg^{-1} proteins; plasma ACTH = 75 ± 5.2 pg mL^{-1}; plasma corticosterone = 23.7 ± 1.6 ng mL^{-1}. Plasma β-endorphin was strongly enhanced on PND 21, but did not show consistent alterations during adult ages. All data of Fig. 2C were adapted from data originally published in Galietta et al, 2006. Consistent and enduring changes of endogenous brain opioid activity were also evidenced in adult mice following postnatal stress (see sections 2.2 and 2.3 in this paper). *is for $P < 0.05$; ** is for *** is for $P< 0.001$. Please note that euthanasia was performed in non-fasting animals: in this case, the three mice in each cage were picked-up and underwent rapid euthanasia in the same moment by three different investigators, to avoid excess stress.

Fig. 3. Immunological alterations induced by stress.

The stress model induced: 1) long-term increased release of some Th-1 type cytokines (such as interleukin IL-2, interferon-γ and tumor necrosis factor-α) produced by splenocytes ex vivo, stimulated with

phytohemoagglutinin); 2) reduced release of some Th-2 type cytokines (such as IL-4 and IL-10); 3) enhanced natural killer-cell activity; 4) enhanced splenocytes proliferative activity. Data reported here are related to cytokine IL-2 (panel A), cytokine IL-4 (panel B), interferon-γ (panel D) and natural killer cells activity (panel C). Data were gathered at 110 PND in control mice (open columns), stressed mice (black columns) and mice stressed and daily treated with (-)naloxone HCl, 1 mg kg^{-1}, as weight of the base (light gray columns), during the nursing period (2 to 21 PND). A further control group was obtained through treatment of stressed mice with the (+)naloxone enantiomorph, which was devoid of antagonistic effects (not reported here), and its effects were quite similar to those obtained in mice stressed and treated with saline. The asterisk indicates consistent differences versus stressed animals (P at least < 0.05), 5 animals per group. All data of Fig. 3 were adapted from data originally published in Loizzo et al, 2002.

Fig.4. Metabolic alterations induced by stress.

The stress model induced consistent alteration of several metabolic parameters. Alteration of some parameters (e.g., body weight increment curve, panel A, and epididymal fat pads increased weight, panel B) were prevented by both naloxone and AS-POMC, whereas other alterations (e.g., increased glycemia, panel C, and increased NAD(P)H overshoot amplitude, a parameter indicative of mitochondrial activity, panel D) were not prevented by naloxone (light grey columns), but were consistently prevented by AS-POMC (shaded columns). The asterisk * indicates consistent difference versus stressed animals (P at least < 0.05). Data depicted in panels A, B and C were taken from Loizzo et al, 2010a; data in

panel D were published in Loizzo et al, 2012b.

Fig.5. Physiological and neurobehavioral alterations induced by stress. Alteration of some parameters induced by the stress model on animals (e.g., faster visual evoked potentials and delayed nociceptive responses) were prevented by both naloxone and AS-POMC, whereas other alterations (e.g., decreased contracting responses to noradrenaline in ex-vivo isolated aorta rings, and enhanced relaxing responses to acetylcholine) were not prevented by naloxone, but were prevented by AS-POMC (borderline significant in the latter case). VEP is for visual evoked potentials, in milliseconds; Nad is for noradrenaline; ACh is for acetylcholine. Data of panel A and B were published in Loizzo et al, 2012b; data of panel C and D were published in Loizzo et al, 2015.

Fig.6. Parameters with alterations of still undefined pathogenesis. We could not yet prevent the alteration induced by stress to some parameters, and their pathogenesis requires further studies. The graph shows a few data indicating alterations found in adult mice following the stress model: i.e., reduced immobility time to forced swim test (Porsolt test- we underline that our stressed mice did not show *enhanced* immobility time, as it is often found in depression models, but showed *reduced* immobility time), enhanced plasma level of insulin and leptin, enhanced 11β-hydroxysteroid dehydrogenase type 1 *m*RNA expression in the brain (HSD11B1), and reduced HSD11B2 (AU is for arbitrary units). Data of panel A were published in Franconi et al, 2004; data of panels B and C in Loizzo et al, 2006; data of panels D and E in Loizzo et al, 2010b. Asterisk indicates significant difference versus stressed groups (P at least < 0.05)

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Tables

Table 1

Timing of experimental procedures

Day -7 From birth, i.e., Day 14 of conceptional age. Pregnant mothers are transferred from the factory to our Institute

Day 1 Day of birth (PND1). Pups are culled to 6 male animals/nest, and randomly cross-fostered

Day 2 (PND2) Treatment starts for all groups receiving any drugs, while animals of the C groups are left undisturbed, except for cage cleaning twice a week

Day 21 (PND21) Treatment ends. Homogenous litters of animals are set in groups of three per cage, weighed and weaned. Then, all animals are left undisturbed

Day 30 (PND30) Animals are weighed and tested at the tail-flick/hot-plate apparatuses

Other experimental procedures are performed in ages indicated in the figures

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Table 2

Pattern of physiological and metabolic parameters in male and female mice following our stress model.

Sex & treatment	Body Weight (g)		Hot Plate (s)	Glycemia mg dL^{-1}	Corticost μg dL^{-1}	HSD11B1 AU E-04	HSD11B2 AU E-04
	21 PND	90 PND	30 PND	90 PND	90 PND	90 PND	90 PND
Control Males	15.7* ±0.1	42.5* ±0.5	39.2* ±1.8	93.0 ±4.6	2.0 ±0.22	54.4* ±5.8	4.5* ±0.54
Stressed Males	14.9# ±0.1	44.8# ±0.5	50.1# ±1.6	130.4# ±3.7	3.8# ±0.61	81.4# ±8.9	2.7# ±0.16
Control Females	14.8 ±0.1	33.3 ±0.5	35.0 ±1.1	91.9 ±3.2	2.1 ±0.6	41.7 ±4.3	3.1 ±0.48
Stressed Females	14.5 ±0.2	34.5 ±0.6	38.4 ±2.3	88.2 ±3.6	1.9 ±0.3	45.1 ±7.0	3.8 ±0.72

*Number of experimental mice for body weight is at least 60 mice per group. Hot plate was assessed at 30 PND, at least 25 mice per group. For glycemia, number is at least 20 mice per group. For plasma corticosterone al least 8 per group. Basal HSD11B1, HSD11B2, mRNA mean expression in the brain cortex, 6 mice per group. Stressful procedures were not, or minimally effective in females. All data expressed in this table were adapted from data originally published in Loizzo et al, 2010b. Values are expressed as mean ± SEM. AU is for arbitrary units. * is for consistent difference versus control females (P at least <0.05); # is for consistent difference versus controls of the same sex (P at least < 0.05).*

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Table 3.

Similarities among human type-2 diabetes, high fat diet/overfeeding model, and the present model

1) body weight gain
2) adipocytes hypertrophy
3) hyperglycemia
4) hyperinsulinemia
5) hypercorticosteronemia/hypercortisolemia
6) increased fasting leptin levels
7) inflammation with increases in inflammatory cytokines
8) sexually dimorphic response to the model
9) hypothalamus-pituitary-adrenal axis-hyperactivity/dysregulation
10) vascular alterations

From data published by Griffin et al, 2016; Habbout et al, 2013; Heydemann, 2016; Reyes, 2012, and by ourselves (single references for our groups are reported in Tables and Figures)

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Figures

Fig 1

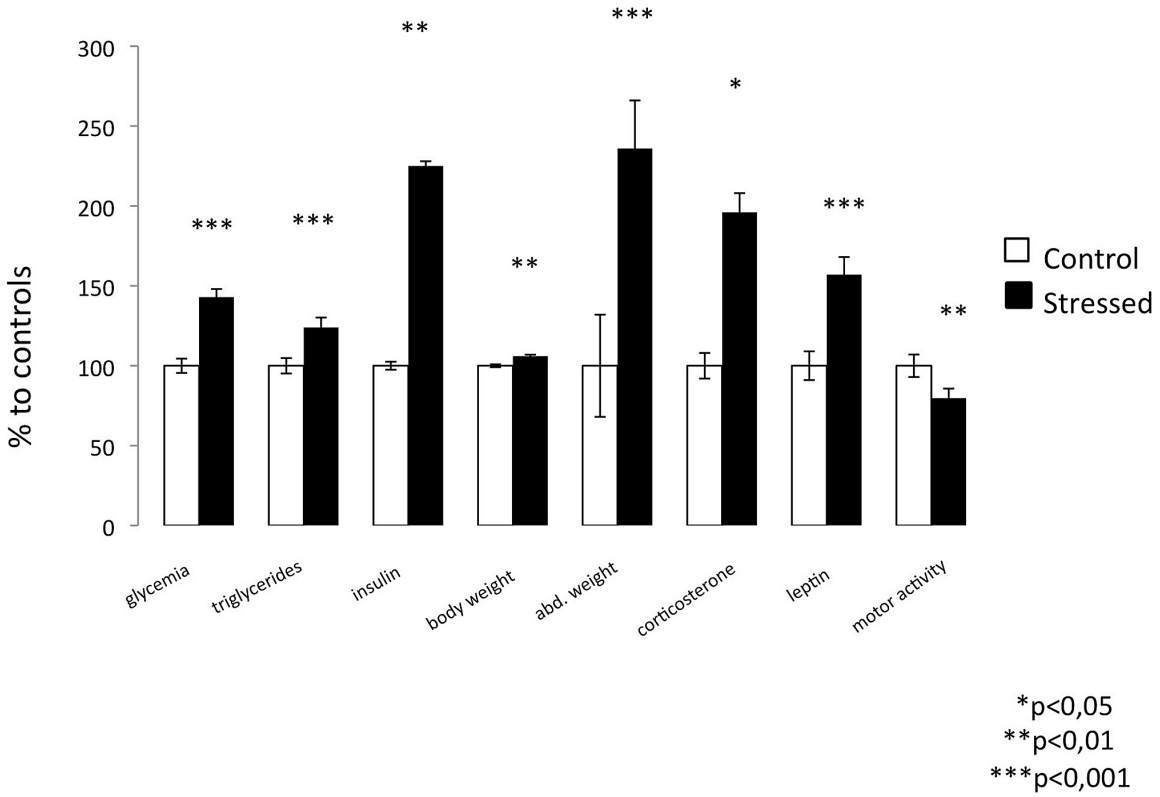

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Fig. 2

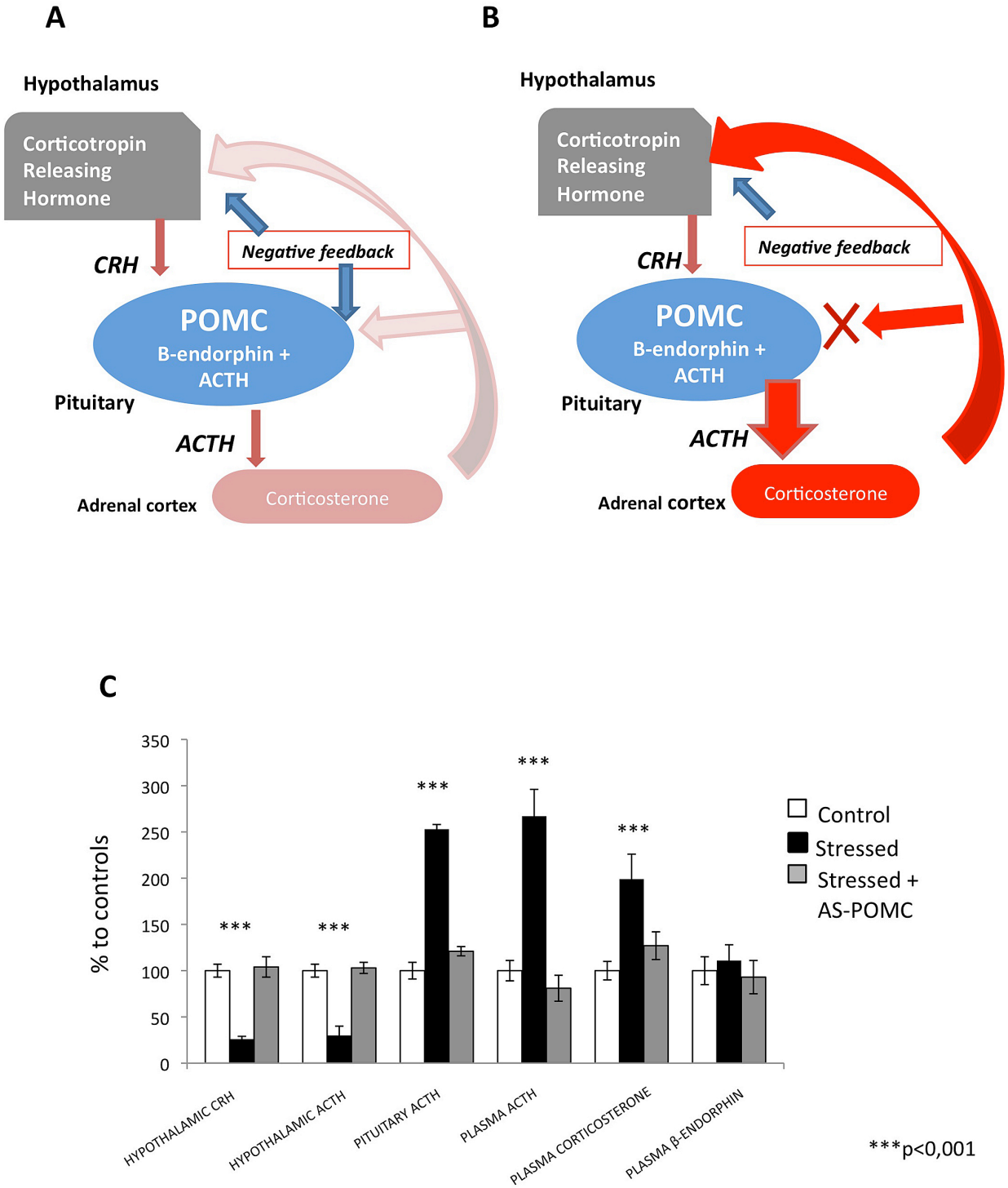

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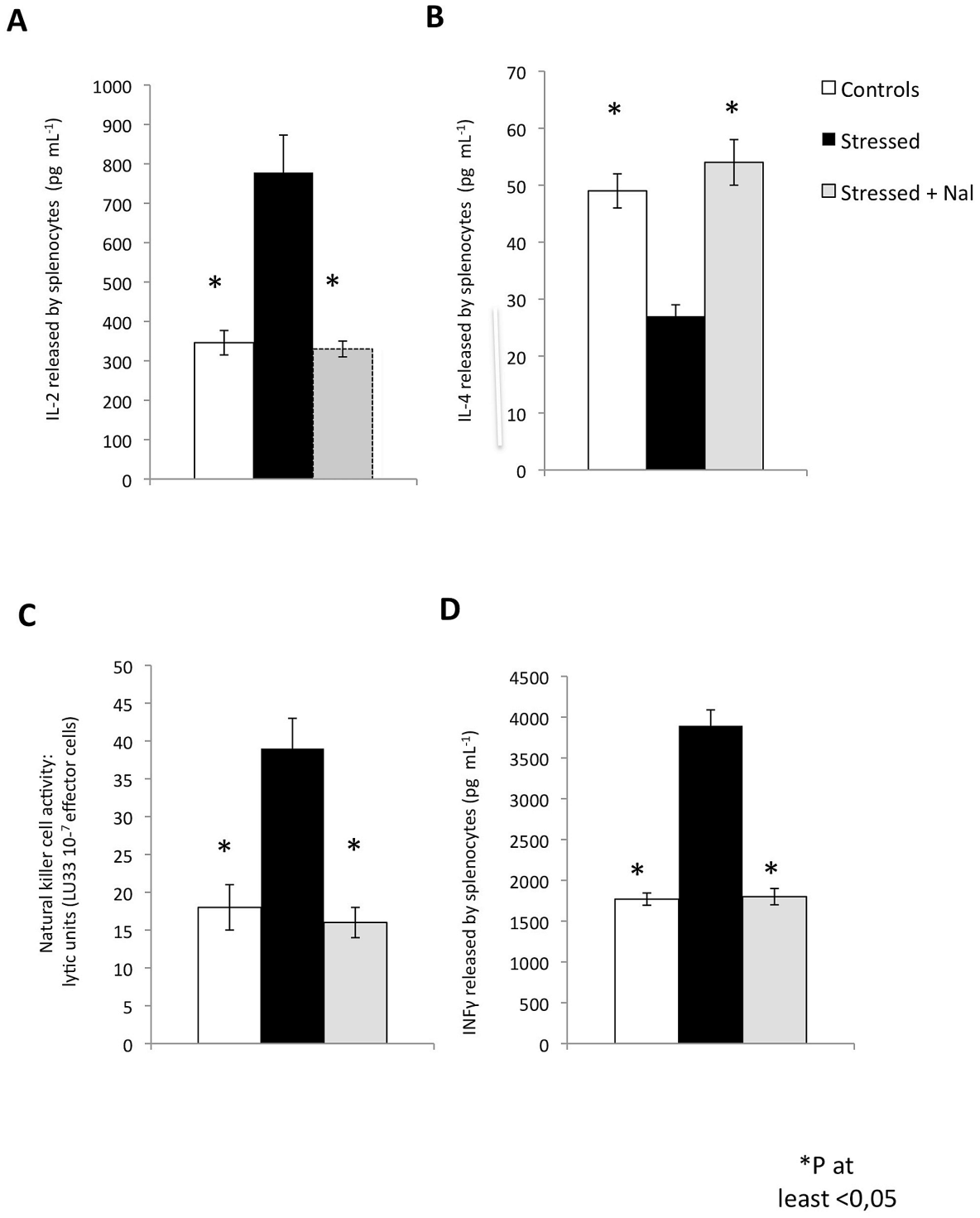
Fig.3



*P at least <0,05

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Fig. 4

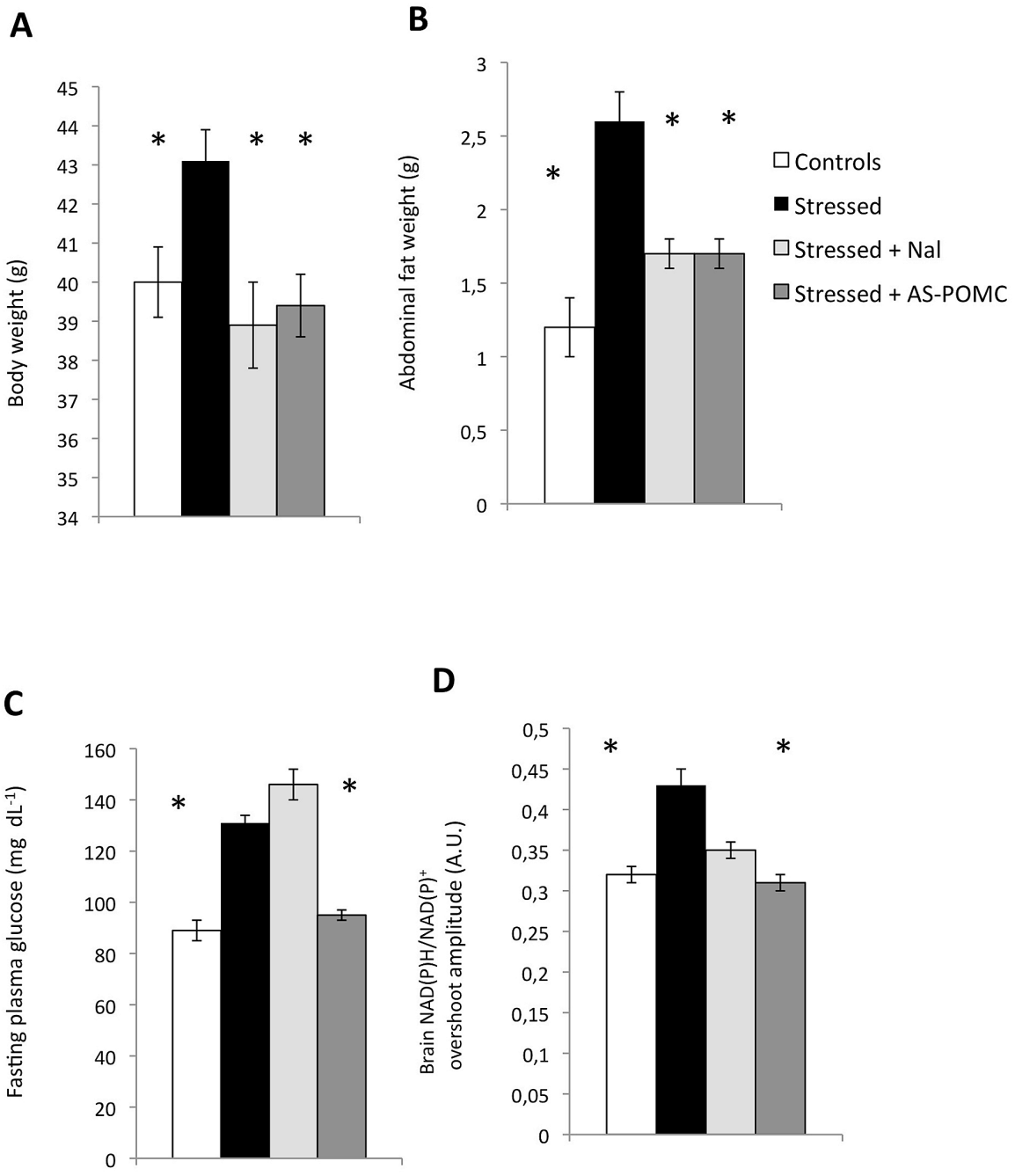

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Fig 5

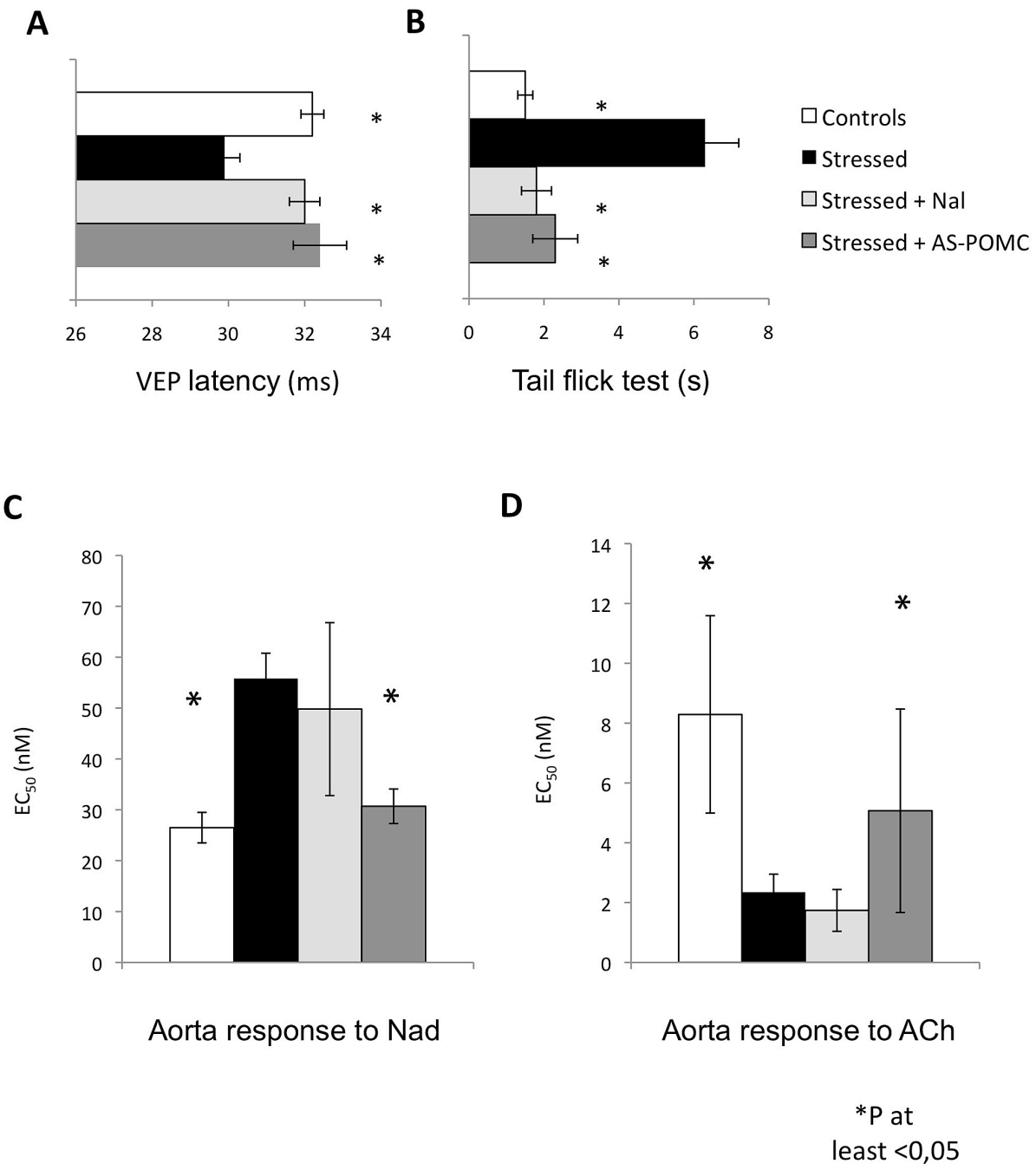

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Fig. 6