Focusing attention on biological markers of acute stressor intensity: empirical evidence and limitations

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The availability of biological markers that objectively quantify stress is a highly relevant issue. However, experimental evidence suggests that most physiological changes elicited by emotional stressors do not reflect their intensity and are not useful for this purpose. Thus, we review experimental evidence in animals and humans about the putative validity of neuroendocrine and sympathetic/parasympathetic variables to measure stress. Plasma levels of some hormones (e.g. ACTH, glucocorticoids, prolactin and catecholamines) have been found to reflect, at least under certain conditions, the intensity of emotional stressors in animals and probably in humans. However, the temporal resolution of hormone changes is insufficient to reflect the very dynamic psychological processes taking place while experiencing stressors. Cardiovascular parameters (e.g. heart rate and blood pressure) have much better temporal resolution but their validity as markers of stressor intensity either in animals or humans is problematic. Skin conductance and pupil dilation appear to be promising. Additional and more systematic studies are needed to demonstrate the actual validity of stress-induced physiological changes to quantify stress.

1. Introduction

Despite the enormous literature about stress, we have still difficulties to understand all the complexity behind situations considered as stressful and how to advance in the characterization of such complexity and their consequences in terms of health. In fact, even the definition of stress is a matter of controversies. Nevertheless, to place our present review in the appropriate context we need to adhere to a particular definition. In our view, that from Vigas (1980) has heuristic value: "stress is the response of the organism, evolved in the course of phylogeny, to agents actually or symbolically endangering its integrity. The agent inducing stress is a stressor. For an agent to become a stressor for a given organism, it has be 1) quantitatively excessive, or 2) qualitatively stressogenic". He refers to physical/systemic stressor as those actually endangering its integrity and to psychological (emotional) stressor to those symbolically endangering it.

Most researchers accept that regardless of each particular nature, stressors activate the hypothalamic-pituitary-adrenocortical (HPA) and sympathetic-adrenal-medullary (SAM) axes [Ulrich-Lai and Herman, 2009]. The key brain area in the control of the HPA axis is the paraventricular nucleus of the hypothalamus (PVN) where neurons synthesizing neuropeptides, mainly the corticotrophin-releasing hormone (CRH) and vasopressin, are located. These neuropeptides are released into the pituitary portal blood to stimulate the release of the adrenocorticotropic hormone (ACTH) in the anterior pituitary. ACTH acts on the adrenal cortex to release glucocorticoid hormones (mainly cortisol in humans and most mammals, and corticosterone in rodents). Activation of the SAM axis results in the release of adrenaline and noradrenaline from the adrenal medulla (although circulating noradrenaline can also derive from sympathetic terminals).

Systemic stimuli (e.g. infections, hemorrhage, cold, heat) directly affect homeostasis, but the challenges they represent are markedly dependent on the particular characteristic of each stimulus. Consequently, many of the physiological responses we can observe under these situations are likely to be quite specific for each particular challenge. When a challenge is so strong that cannot be solely solved with the stimulus-specific physiological responses, it becomes stressful and the non-specific stress response added to the specific one [Armario, 2015; Chrousos and Gold, 1992]. Direct evidence for these reasoning has been obtained [e.g. Goldstein and Kopin, 2007; Pacak et al., 1998; Vigas et al. 1984]. Moreover, each systemic stressor has been demonstrated to have its own brain processing pathways (signature) [Pacak et al., 2001; Sawchenko et al., 2000]. It is plausible that the non-specific response to systemic stressors (e.g. glucocorticoid and catecholamine release) could have several critical roles [Armario, 2015; Sapolsky et al., 2000]. First, to prepare the organisms for a high and immediate burst of activity (mainly through catecholamines), and contribute to increase the immediate efficacy of the particular set of specific physiological responses (both catecholamines and glucocorticoids). Second, to favor post-stress recovery

allowing return to normalcy when the situation is solved (mainly through glucocorticoids) and develop a *physiological memory* about the situation to better cope with the same or similar situations in the future.

In contrast to systemic stressors, emotional stressors do not directly represent a serious homeostatic challenge. If so, why such exposure results in the activation of the HPA and SAM axes? It is likely that the very same responses have been selected during evolution for systemic and emotional stressors because the latter ones are associated to a certain risk of being killed or wounded; for instance, when animals are searching for food or resources in a dangerous environment or in humans during the war or social conflicts. In this regard, social evaluation represents a symbolic threat and acts as a potent stressor in humans [Dickerson and Kemeny, 2004; Frisch et al., 2015]. Under emotional stressors, the activation of the stress response is anticipatory in that the organism is better prepared to cope with the situation and repair potential damage if it eventually occurs. If the emotional situation is solved without damage (e.g. escaping from a predator or an enemy), the evoked changes progressively return to resting conditions. Nevertheless, the already triggered stress response can still exert potentially beneficial effects that might be relevant in the future to cope with similar situations, for instance, favoring emotional memory, where a participation of both catecholamines and glucocorticoids is needed [Finsterwald and Alberini, 2014].

The interest for the characterization of the biological response to emotional (and physical) stressors has accompanied stress research for decades. Such a characterization could allow us not only to better understand the psychological and physiological processes underlying the response to stressors, but also to explain the connection between stress exposure and pathology. However, there has been less interest in demonstrating which biological parameters are able to detect the intensity of emotional stressors and which are not. Consequently, no particular attention has been paid to objectively demonstrate the intensity of the different types of stressors used in animal research and particularly to evaluate the intensity of the wide range of human situations presumably considered as stressful.

The biological response is likely to be a critical mediator of the pathological impact of the different stressors. Therefore, it is extremely relevant to know the magnitude and duration (area under the curve, AUC) of these responses in a population or in particular individuals. The magnitude/duration of stress-induced biological changes can be influenced by both the characteristics of stressors and individual differences. At the level of the individual, the AUC of these responses in conjunction with the particular vulnerability of the various physiological systems and brain circuits involved would play a critical role in the development of pathophysiological states or psychological alterations, including psychiatric diseases. It should be noted that we refer not only to biological variables that can measured peripherally (e.g. plasma/saliva levels of hormones or immunity-related molecules, cardiovascular parameters), but also to the neurochemical changes taking place within the brain, although the former ones are those that can be more easily measured and the focus of our attention. Importantly, we cannot deduce the biological impact on the basis of psychometric tests because the information given by tests that detect state anxiety or subjective stress in humans are very often dissociated from the biological response to stress [e.g. Fox et al., 2009; Jezova et al., 2004].

If some variables are found to be consistent markers of stressor intensity, we have some "scales" to roughly evaluate this dimension when studying one or more stressors. Most severe stressors would induce greater biological alterations and presumably greater pathological impact. In fact, in their review about the concept of stress Chrousos and Gold (1992) presented a figure assuming that the activity of the stress system is linearly related to the potency of stressors until a plateau is achieved. This is probably a common (very often implicit) assumption in the field [Schneiderman et al., 2005; Lupien et al., 2006]. It is important to characterize as many as possible biological markers of stressor intensity that are relatively unrelated physiologically (e.g. HPA axis, autonomic responses or prolactin) and measure at least two of them under the same situation. If a particular population (e.g. patients with depression or schizophrenia versus healthy subjects) respond in the same direction with all the biological variables that we know are sensitive to the intensity of stressors, we are more confident that

the two populations respond differently to stressors. When only one variable is measured, this can lead to erroneous interpretation if the population we are studying has a particular alteration in the physiological system we are using to quantify stress. That is, we cannot affirm that the patients show a generalized alteration in the responsiveness to stressors.

If we have demonstrated that the variables we are interested in are not markers of the intensity of stressors, we cannot use them to study this dimension. Importantly, this does not mean that these variables are not of interest for other reasons. First, two stressors might not differ in intensity (evaluated on the basis of two or more variables that are sensitive to this dimension), but they might differ in the quantitative response of a particular variable of interest. In this case, this particular variable might reflect certain qualitative components of the stressors. Then, we can expect that those pathologies associated to the latter variable could be more probable in the stressor eliciting the greater response, whereas those pathologies not associated to the variable would be similar after the two stressors. Second, if an individual showed a greater response in one of these variables as compared to another subject, we cannot interpret it as reflecting a greater generalized stress responsiveness, but rather as reflecting a difference in the functioning of the particular physiological system to which the variable belongs to. For instance, exacerbated blood pressure response to stress has been considered to be a major contributor to the future development of chronic hypertension, but a very relevant question is whether such a hypertensionprone individuals are more reactive to stress in general than normotensive subjects when including other stress-sensitive variables [Lovallo 2005]. In most studies this is not the case, suggesting that its high sensitivity is restricted to the cardiovascular system.

The characterization of biological markers of acute emotional stressors has to address at least three main questions: (i) whether or not they can reflect the intensity of stressors; (ii) the range of intensities in which such relationship is approximately linear; and (iii) which variables have a temporal resolution good enough to reflect the dynamic psychological processes occurring while experiencing stressors. Our

review has been focused on emotional and not physical stressors not only for the difficulty to disentangle whether the effects on physiological markers are secondary to the physical nature of the stressor or the emotional response, but also for three additional reasons. First, emotional or psychological stressors are considerably more frequent in human societies. Second, emotional stressors can precipitate major psychiatric disorders such as schizophrenia, bipolar disorder and major depression [McEwen, 2017]. Finally, a single exposure to traumatic stressors can induce post-traumatic stress disorder in humans and long-lasting effects on behavior in animals [Armario et al., 2008; Deslauriers et al., 2018]. However, the extent to which the traumatic nature of a stressor can be characterized biologically is uncertain. The possibility to use biological markers of stressor intensity is highly relevant to directly test whether intensity is the most relevant factor or, on the contrary, qualitative aspects are more critical.

2. Physiological markers of stressor intensity

Emotional stressors can markedly affect anterior pituitary hormones, including prolactin, and the autonomic nervous system (ANS). Stress-induced ANS changes result in generalized activation of the sympathetic nervous system and a more selective activation of the parasympathetic nervous system. Overall, exposure to stress leads to changes in several endocrine variables whose concentration can be measured in plasma, or, in restricted cases, in saliva (summarized in Table 1).

With no doubt, emotional stressors can differ in qualitative aspects. The debate around the specificity of the stress response has a long history in the stress field [Mason, 1974; Pacak and Palkovits, 2001]. However, it is important to note that earlier discussions about this topic included both systemic and emotional stressors, and it is now well-accepted the idea that different systemic stressors are likely to have a markedly different biological signature. Therefore, the most pertinent discussion presently is whether or not certain emotional qualities of stressors are associated to specific physiological responses. So far as stressors included within the category of emotional markedly differ in their consequences for

the organism, the set of emotions elicited by different stressors is likely to differ. In this regard, questions about the specificity of biological responses to emotional stressors are closely related to the issue of the specificity of the physiological response to different emotions, a topic that is still strongly debated [Kreibig, 2010; Mauss and Robinson, 2009]. In addition to the quality of emotional stressors, the way we can cope with them and personality factors can markedly affect the biological response. For instance, controllability (either real or perceived) and coping (passive versus active) appears to affect, at least under certain conditions, classical stress-related physiological responses. Relevant papers and discussion on this subject are available in animals [e.g. De Boer et al., 1990; Sgoifo et al., 1999] and humans [e.g. Agrigoroaie et al., 2013; Bosch et al., 2001; Breier et al., 1987; Croes et al., 1993; Olff et al., 1995; Peters et al., 1998].

Although we are aware of the potential contribution of the quality of stressors and personality traits to the biological response, these two issues are out of the scope of the present review for three main reasons. First, the precise contribution of personality traits to the response to emotional stressors is still unclear [Chida and Hamer, 2008]. Second, to the best of our knowledge, there is little information regarding how different types of emotional stressors trigger a distinct set of emotions, although some authors have paid attention to this important issue and several of its physiological correlates [Denson et al., 2009]. Third, the issue of specificity of the biological response to emotional stressors and its correspondence with the emotions they elicit is more difficult to study if we do not know the extent to which the variables are sensitive to the dimension of intensity. The latter dimension is typically considered in the study of emotions under the concept of the level of arousal [e.g. Mauss and Robinson, 2009].

Regardless of their nature, stressors can also differ in the critical dimension of intensity. How can we evaluate it? This issue can be approached by studying stressors in which the intensity can be modified while essentially maintaining their very nature. In laboratory rodents, typical designs have introduced mild but progressive changes in the normal environment of laboratory rodents, or have exposed animals

to different levels of noise or foot-shock intensities [e.g. Burow et al., 2005; Hennessy and Levine, 1978; Natelson et al., 1981]. Hennessy and Levine (1978) designed in mice an experiment that involved acute progressive changes in their regular environment (pick up and place mice in regular cages with clean sawdust, or in regular cages without sawdust, or in clearly different cages) and observed a parallel and progressive increase in plasma corticosterone. Gradual increases in plasma adrenaline and noradrenaline were observed in rats comparing the mere opening of the cages, the transfer of cages to another room, the handling the animals and immobilization (IMO) [Kvetnansky et al., 1978]. Accordingly, graded levels of foot-shock intensities resulted in progressive increases in plasma corticosterone, noradrenaline and adrenaline [Natelson et al., 1981], although corticosterone response achieved a plateau quickly and the slope was greater for adrenaline than noradrenaline. However, the two catecholamines do not discriminate between two high shock intensities [Zukowska-Grojec et al., 1988], suggesting that every biological marker eventually achieves a plateau.

The above comments highlight the limitations of plasma corticosterone as a stress marker. This is because the capability of the adrenal cortex to synthetize corticosterone saturated with intermediate levels of ACTH [Keller-Wood et al., 1983]. Therefore, plasma corticosterone is exquisitely sensitive to moderate increases in plasma ACTH but did not reflect greater increases in ACTH unless the post-stress period is evaluated. Thus, when comparing exposure to high intensity footshocks and IMO, various classical biological markers (ACTH, corticosterone and prolactin) were unable to distinguish between the two stressors when measured just after it, but clear differences emerged among all variables during the post-stress period, indicating that IMO was clearly more severe than foot-shock [Márquez et al., 2002]. Despite these well-established facts, most studies in the field of stress with laboratory animals still rely on plasma corticosterone measurement just after the stressor, what can lead to erroneous conclusions in most cases if the challenging stressor is not of low intensity. This is not a major problem in most human studies that typically used low intensity stressors.

What about other hormones? Plasma prolactin was found to be sensitive to graded levels of footshock intensities in rats [Kant et al., 1983]. Using a similar approach to that by Hennessy and Levine (1978) but introducing additional environmental changes, still of relatively low intensity, we were able to confirm in rats the sensitivity of plasma corticosterone and plasma prolactin to these changes [Armario et al., 1986a; 1986b]. Importantly, although we could intuitively assume that most of the variables that exquisitely respond to stress would be sensitive to the intensity of stressors, this was not the case. In contrast to corticosterone and prolactin, other anterior pituitary hormones (luteinizing hormone, growth hormone and thyroid stimulating hormone) did consistently respond to stressors, but their concentration was absolutely independent of the stressor intensity [Armario et al., 1986a]. This suggests an all or none rather than a gradual response in an important number of stress sensitive hormones and physiological parameters. Unfortunately, we are not aware of any other work showing, in the same experiment, variables sensitive and not sensitive to the intensity of stressors. The possibility that variables apparently sensitive to intensity are actually reflecting certain qualitative aspects of stressors rather than their intensity might be considered. However, we could then expect a dissociation between the variables when comparing qualitatively different emotional stressors. On the contrary, there are no major discrepancies among those variables to classify different types of emotional stressors in function of their intensity [e.g. Kant et al., 1983; Márquez et al., 2002].

In conclusion, the validity of plasma levels of noradrenaline, adrenaline, ACTH, corticosterone and prolactin as useful markers of stressor intensity has been repeatedly demonstrated in rats and mice. Importantly, although this restricted set of variables is potentially sensitive to the intensity of stressors, the precise dose-response relationship differs notably among the different variables [Armario et al., 2012]. Plasma corticosterone is quite sensitive to low intensity but not intermediate/high intensity stressors, plasma prolactin and ACTH are sensitive over a wide range of intensities, and plasma glucose (a surrogate of adrenaline release [Bialik et al., 1989], along with the reduction of food intake (measured over the next

24 h) are only sensitive to intermediate and high intensity stressors [Armario et al., 2012]. It is of note that stress-induced hyperglycemia is likely to be influenced by physical components of stressors when they imply enhanced glucose consumption (e.g. physical activity or cold temperature).

In humans, a series of classical studies by Frankenhauser's laboratory [Frankenhauser, 1971] demonstrated that urinary excretion of adrenaline and noradrenaline reflects both exercise intensity and graded electric shock intensities. Other data indicate that plasma adrenaline and noradrenaline reflect the degree of difficulty of two cognitive tasks [Freyschuss et al., 1990]. Regarding anterior pituitary hormones, in a study specifically designed to evaluate the sensitivity of neuroendocrine variables to the intensity of emotional stressors, we compared two academic exams of different value for medical students and observed that both prolactin and cortisol appeared to reflect intensity, paralleling state anxiety [Armario et al., 1996]. Similarly, an increase in salivary cortisol was found in medical staff after an emergency situation involving a life-threatening situation for patients, but not after a less severe condition [Sluiter et al., 2003]. When comparing in medical students the response to public speaking and simulated emergency situation, subjective but not cortisol response was stronger after the latter situation [Keitel et al., 2011], supporting a greater sensitivity of psychometric measures. Nevertheless, it should be noted that cortisol appears to be quite sensitive to manipulation of social evaluation during a speech task [Bosch et al., 2009]. In conclusion, despite the paucity of data in humans, plasma levels of catecholamines, prolactin and cortisol as well as saliva cortisol concentration (which reflects the free fraction of circulating cortisol) are likely to be sensitive to the intensity of stressors. In support of the sensitivity of cortisol, a maximum 3-fold increase in salivary cortisol can be found after the trier social stress test (TSST), which includes speech preparation, mental arithmetic and social evaluation and it is considered as a relatively strong stressor [Foley and Kirschbaum, 2010], whereas a 6-fold increase is observed in soldiers during simulation of torture [Morgan et al., 2000]. It is important to realize that in most laboratory stressful

conditions, cortisol response is clearly lower than that observed after the TSST, indicating that they are of low intensity. This can strongly affect conclusions about the putative sensitivity of biological markers of stress achieved under laboratory conditions. In a TSST study, Lennartsson and Jonsdottir (2011) observed a notable increase in ACTH, cortisol, prolactin and CV variables, with a good correlation between the three hormones and lower correlation between hormones and CV response.

Stress-induced changes in the ANS has been typically evaluated by changes in heart rate (HR), heart rate variability (HRv), mean, systolic or diastolic blood pressure (MAP, SBP, DBP, respectively), salivary □-amylase (that reflect sympathetic activation), pupil dilation (PD), skin conductance (SC) and body temperature changes (Table 2). Quite surprisingly, whereas the sensitivity of neuroendocrine variables to stressor intensity has been reasonably well-studied in rodents, to our knowledge, there are no studies in laboratory animals specifically aiming at characterizing the sensitivity of ANS-related variables to this parameter. Nevertheless, a few studies are available that typically compared two or more stressful situations. In response to the introduction in the home-cages of an electrified prod, greater HR response and hyperthermia (core temperature) were observed in rats as compared with the introduction of a non-electrified prod [Diamant et al., 1991]. Comparing various mild conditions in mice, the increases in HR and hyperthermia only discriminate between disturbances of the cages not involving handling and those procedures involving handling, but not between other additional progressive perturbations of the animals [Van Bogaert et al., 2006], suggesting so prompt saturation that any usefulness to evaluate stress intensity is questionable. In rats, differences in the HR and MAP response to air-jet versus restraint stress were observed [McDougall et al., 2005], whereas these same responses were unable to discriminate between novel environment, fear conditioning and restraint [Furlong et al., 2009]. A recent study measuring in mice the HR and HRv in response to very brief stressors that might differ in intensity (air puff, free fall and simulation of earthquake) revealed no clear differences between them [Liu et al., 2014]. This picture is further complicated by the fact that conditioned fear appears to reduce rather than to potentiate the

typical increase in HR triggered by exposure to a novel environment, whereas plasma adrenaline followed the expected higher response after conditioning [Nijsen et al., 1998]. Moreover, cardiac deceleration can be observed under certain emotional situations [Alboni and Alboni, 2017].

In humans, the influence of shock intensity or shock probability has been studied regarding some parameters related to the sympathetic system. SC has been found to increase with the intensity of pain induced by mechanical pressure and shocks [Chapman et al., 2014; Drabant et al., 2011; Ellermeier and Westphal, 1995; Kaufman, 1965] and shock probability [Kopacz and Smith 1971], and also parallels subjective stress in surgeons performing laparoscopic versus open surgery [Berguer et al., 2001]. PD also appears to be sensitive to the intensity of pressure or shock-induced pain [Ellermeier and Westphal, 1995; Chapman et al., 2014]. Therefore, both SC and PD might be potential markers of stressor intensity. In contrast, it is unclear whether cardiovascular parameters reflect stressor intensity. HR response is particularly problematic because both cardiac acceleration (tachycardia) and deceleration (bradycardia) can be observed. The complexity is illustrated by a study about the influence of noise intensity [Turpin and Siddle, 1983] showing a progressive change from deceleration to acceleration in function of the intensity of noise. Increasing the difficulty of two different mental tasks (Stroop color word test and mental arithmetic), higher HR response was observed in the hard than the easy task, with a decrease after tasks that were impossible to solve [Carroll et al., 1986], suggesting a more critical role of active engagement in solving the task than stressor intensity. Using similar tasks in which different levels of difficulty were introduced, rating of perceived stress progressively increased, whereas changes in DBP and SBP only partially reflected such a difficulty, and the sensitivity of HR was still worst [Bohlin et al., 1986; Callister et al., 1992; Kok et al., 1995; Veldhuijzen van Zanten et al., 2004]. Another study revealed that cortisol response was clearly higher after the TSST than the Stroop test, whereas no differences were observed in HR or □-amylase, supporting a lower discriminative power of the latter measures [Skoluda

et al., 2015]. Despite these overall negative results, increasing the number of people evaluating a speech (0, 1 or 4) progressively increased salivary cortisol, HR and sympathetic activity (cardiac pre-ejection period) in subjects maintained seated during the task [Bosch et al., 2009]. This latter condition is important as minor procedural differences such as the degree of activity of the subjects during the speech (seated, standing or walking) can alter the cardiovascular response [Mlynarik et al., 2007; Nater et al., 2013; Tulen et al., 1999], whereas cortisol appears to be quite insensitive to these procedural differences (Mlynarik et al., 2007; Nater et al., 2013). In a very recent study using a task similar to the TSST that included two degrees of social evaluation (low and high), HR, DBP and SBP responses were greater after the high than the low intensity evaluation [Lu et al., 2018], suggesting sensitivity of CV variables. It thus appear that the use of the former measures to quantify stressor intensity appears to be problematic on the basis of available evidence.

3. Dynamics of the response to acute stressors

The characterization of the dynamics of the physiological response to stressors is important for proper interpretation of the results for three main reasons: (i) the response, particularly the neuroendocrine one, is frequently measured at one or a few time points; (ii) the dynamics of the variable determines its temporal resolution to reflect emotional processes underlying stressor exposure; and (iii) post-stress recovery pattern might be related to the intensity/duration of stressors and individual differences in reactivity to those stressors.

When subjects confront stressors lasting for more than a few minutes (the most typical situation in laboratory and ecological stressors), the initial primary evaluation (appraisal) of the situation becomes progressively complex due to secondary mechanisms of appraisal [Lazarus and Forman, 1984]. The subjects perceived the stressor, evaluated the situation and the possible strategies to better cope with the situation so that the initial behavioral reactions as well as the initial physiological responses can be

modified. And this process continues as long as the situation persists. Only those responses with a high temporal resolution could be potentially useful to follow the psychological processes taking place during exposure to stressors lasting for more than a few seconds. It is obvious that biochemical measures, even those with a better resolution (plasma levels of adrenaline and noradrenaline) are unable to accurately reflect the dynamics of brain processes. These caveats are very relevant for the HPA hormones, and particularly for cortisol. Despite its obvious limitations, plasma cortisol and more recently saliva cortisol have become the most extensively used marker of stress.

In animals, changes directly mediated by the ANS (e.g. HR, DBP, SBP) are observed almost immediately after exposure to stressors, and the activation of the SAM system with the subsequent release of adrenaline and noradrenaline into the circulation is also fast, with peaks in plasma levels of the two hormones in about 1 minutes, declining with a half-life of 1 minutes [Kvetnansky et al., 2009]. However, the activation of the HPA axis results in a peak of plasma ACTH not before 5 minutes after initial exposure [Kovacs and Sawchenko, 1996] and a decline with an estimated halflife of about 6-7 minutes [Lopez and Negro-Villar, 1988]. After initial ACTH release, maximum plasma levels of cortisol/corticosterone are achieved beyond the 20 minutes after initial exposure, declining with a half-life of 15-30 minutes [Nemeth and Vigas, 1973]. Importantly, exposure of laboratory animals to a stressor for more than 30 minutes results in a progressive return of the initial response to pre-stress levels, despite the persistence of the situation [Armario, 2006]. This is particularly clear regarding plasma levels of adrenaline, noradrenaline, prolactin and ACTH when exposure to the stressor lasts for several hours [García et al., 2000; Hauger et al., 1988; Kvetnansky et al., 1978; Rivier and Vale, 1987; Ruisseau et al., 1978]. There are several reasons to explain these changes, including familiarization of the animals with the prolonged situation, the existence of negative feedback mechanisms (in the case of the HPA axis) or exhaustion of certain physiological systems [Rivier and Vale, 1987]. In any case, measures taken after prolonged exposure to a stressor would not reflect the initial impact and can lead to misleading interpretations of the intensity of

such a stressor. Prolonged exposure to acute stressors has also revealed a strong dissociation between plasma ACTH and corticosterone concentrations in that the progressive decrease in plasma ACTH is not paralleled by a similar decrease in plasma corticosterone [García et al., 2000; Hauger et al., 1988; Rivier and Vale, 1987]. In humans, there are also numerous reports of dissociation between the two hormones [Borstein et al., 2008]. The processes involved in such dissociation have not been studied, but there are several putative mechanisms, including the sympathetic innervation of the adrenal gland or the influence of stress-induced humoral factors such as cytokines [Borstein and Chrousos, 1999; Borstein et al., 2008].

Although we have until now focused mainly in the peak of the response to determine whether or not a particular variable is sensitive to the intensity of stressors, the presumably impact of a stressor is likely to be also related to the duration of the biological changes it elicits. That is, the AUC of the response. Therefore, the dynamics of the post-stress return to baseline is also important in that a slow post-stress recovery would maintain activation for a longer period of time and could exacerbate stress-associated pathologies. In this regard, we have demonstrated in rats that the severity of stressors affects not only the peak response, but it is positively related to the duration of the activation of the HPA axis during the post-stress period [García et al., 2000; Márquez et al., 2002; Rabasa et al., 2015]. With ANS variables, there is evidence in rodents that higher intensity stress conditions can delay the post-stress recovery of HR, HRv and body temperature [Diamant et al., 1991; McDougall et al., 2005; Sgoifo et al., 1999; van Bogaert et al., 2006], although their capability to detect intensity might be poor unless stressors greatly differ [Vianna and Carrive, 2005]. In humans, the post-stress recovery appears to be dependent on the duration of stress exposure and the particular variable measured [Anfilogoff et al., 1987; Mezzacappa et al., 200; Richter et al., 1996; Vinkers et al., 2013]. Unfortunately, how the intensity of stressors affects poststress recovery of biological markers has not been specifically addressed, despite claims about the importance of studying recovery and not only reactivity (Linden et al., 1997). Nevertheless, certain characteristics of stressors can markedly affect the post-stress dynamics. For instance, a clear safety signal emerges under

completion of certain stressors (e.g. touching the ground after parachute jumping), in contrast to certain psychosocial stressors (e.g. social evaluation), where perseverative cognition and rumination can prolong the response [Ottaviani et al., 2016; Zoccola et al., 2012].

4. How our previous experience with a stressor can change the response of biological markers to the same stressor?

If we want to compare the impact of different emotional stressors in a particular population with the specific purpose of knowing their potential pathological impact in any other human or animal population it is important to consider the possibility that the data obtained in this particular population cannot be generalized to other populations. For instance, an exam might be less stressful for university students than for other people who are not used to be examined. Similarly, a public speech might be less stressful if you focus on subjects who are used to do so [Jezova et al., 2016].

In rodents, it is likely that the response to low intensity stressors is reduced if the animals have been exposed for a long-time to laboratory routines typically involving transportation and exposure to novel environments, thus minimizing the intensity of these stressors in more naïve animals. Surprisingly, to our knowledge, there are no studies on this regard.

In animal models, repeated experience with a given stressor typically results in a reduction of plasma levels of noradrenaline, adrenaline, glucose and HPA hormones, that of prolactin being less consistent [see Martí and Armario, 1998; Armario 2015]. Although this phenomenon has been considered to follow the rules of habituation, we have presented data strongly suggesting that the reduction of the HPA response to repeated stressors does not appear to confirm a habituation process [Rabasa et al., 2015]. In brief, adaptation was stronger and faster with more severe stressors, maximally observed even with a single exposure to severe stressors, extremely long-lasting, negatively related to the interval between the

exposures and positively related to the length of daily exposure. Therefore, we proposed to consider the process of adaptation to daily repeated stress as “tolerance”. This name can apply to both physical and psychological stressors and does not require to perfectly fit to very specific rules.

Some human studies have explored the endocrine and autonomic stress responses after repeated emotional stressors. Repeated exposure to psychosocial stress with the TSST using five consecutive daily exposures or three exposures with a 4-week interval between stress sessions has shown a progressive reduction of the cortisol response [Kirschbaum et al., 1995; Schommer et al., 2003]. In addition, in the latter study salivary free cortisol, total plasma cortisol, ACTH, and HR responses showed a significant decrease across the three stress sessions, whereas no such decrease could be observed for the levels of noradrenaline and adrenaline [Schommer et al., 2003], suggesting a differential sensitivity of biological markers to adaptation to repeated stress. Moreover, individual differences in the impact of repeated experience with the TSST has also been reported [Kirschbaum et al., 1995; Schommer et al., 2003, Wust et al., 2005]. Therefore, it is problematic to use repeated exposure when evaluating the intensity of a particular stressful situation.

A phenomenon opposite to adaptation has also been described after prior stress exposure, particularly in animal models. Prior exposure to acute and chronic severe stressors in animals causes a facilitation or sensitization of the HPA response to further (different) acute stressors that last for several days or even a few weeks [Belda et al 2015]. We have demonstrated in rats that the intensity of the stressors as evaluated by the classical stress markers discussed in the present review is the critical factor to induce non-specific sensitization [Belda et al 2016]. Therefore, the development of HPA sensitization might also be useful as a marker of the intensity of a traumatic situation, with the advantage that it can be evaluated at least for several days after the trauma.

5. Conclusion and perspectives

The search for biological markers of specific pathologies is a hallmark of current research in humans. However, rigorous criteria of validation is very often lacking. This applies to biological markers of stress. Particularly relevant is the precise curve relating changes in a specific marker with the intensity of stressors. If the changes in a marker are very small because the presumably changes in the intensity of the stressful situations under study are small, experimental noise caused by uncontrolled and unknown factors would be higher than the expected changes in the marker, making it difficult to obtain reliable results. Unfortunately, this is the case in most human studies where laboratory stress conditions are of lower intensity than that expected in real life situations, not to say under traumatic events. It is difficult for ethical reason to implement laboratory stressors of greater severity, however, in order to properly evaluate the usefulness of biological response to stressors in humans we need to pay more attention to naturally occurring stressful situations of intermediate to high intensity and measure the markers at the most appropriate times. Although the relationship between biological measures of stress and psychometric scales of state anxiety and perceived stress is controversial, the use of both types of measurements are recommended.

The above review of experimental results suggests that some neuroendocrine variables have the potential to appropriately reflect the intensity of stressors when the mean of a group of subjects is used. However, due to the uncertainty about the possible contribution of the qualitative aspects of stressors and the interaction of such characteristics with personality factors and coping strategies, more than one independent marker of intensity should be used to correctly evaluate intensity. The extent to which cardiovascular and hemodynamic parameters can be sensitive is still unclear, but they could be potentially useful, particularly because they have a much better temporal resolution than neuroendocrine variables. This characteristic could be particularly relevant to reflect secondary appraisal, which can change the way the ongoing situation is perceived and determine the duration and magnitude of the physiological

response. In this regard, we should pay attention not only to the speed of changes in response to the initial exposure to the stressor, but also to the speed of return of the variable to baseline. It is beyond the initial more stereotyped response to stressors that we expect a strong contribution of individual differences (genetics, gender, personality, presence of a psychopathology). This is an extremely important topic that has been explored particularly in the study of the post-traumatic stress disorder in order to explain why there are subjects particularly vulnerable to traumatic situations whereas others are clearly resilient [Southwick and Charney, 2012].

In order to approach to the methodological complexity of implementing psychological stress at the laboratory in humans, a plausible option is to use virtual reality. In this line, a virtual reality adaptation of the TSST induced robust HPA and SAM responses in salivary cortisol and α-amylase that were comparable to an in-vivo TSST [Zimmer et al., 2019]. Virtual reality offers the opportunity to realistically simulate stressful experiences of different intensity and to introduce qualitative changes in the procedures, which could benefit future experimental studies in the laboratory aiming to explore changes in physiological markers with repeated stress.

To further complicate the picture, individual differences in the physiological response to stressors (in some cases strongly influenced by cognitive processing of the situation) can combine with a particular susceptibility of each organ/physiological system (not related to stress) to exacerbate a specific stress-associated pathology in some individuals and another different pathology in others. That is, even if two subjects manifest a similar physiological response to stressors, those with a familial history of hypertension will be prone to develop hypertension as compared to subjects without familial antecedents. The characterization of individual differences is on the basis of personalized medicine and one of the most challenging topics in stress research.

We suggest that the best strategies to appropriately characterize biological markers of stress in humans require simultaneous measurement of variables belonging to different physiological systems and the use of specific naturalistic stressors for which differences in intensity could be expected. For instance, response to exams differing in their consequences for the subjects or to expectancy about the diagnostic of pathologies differing in severity. Complementary evaluation of emotions elicited by particular stressors can help to better understand the relationship between quantitative and qualitative component of emotional stressors.

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Table 1: Main endocrine variables responsive to acute stressors

Hormone	Plasma	Saliva	Direction of changes
SAM axis			
Adrenaline	X		↑
Noradrenaline	X		↑
HPA axis			
ACTH	X		↑
Cortisol (corticosterone)	X	X	↑
Other anterior pituitary hormones			
Prolactin	X		↑
GH	X		↑ (most mammals) ↓ (rodents)
TSH	X		↑↓ (a)
LH	X		↑↓ (a)

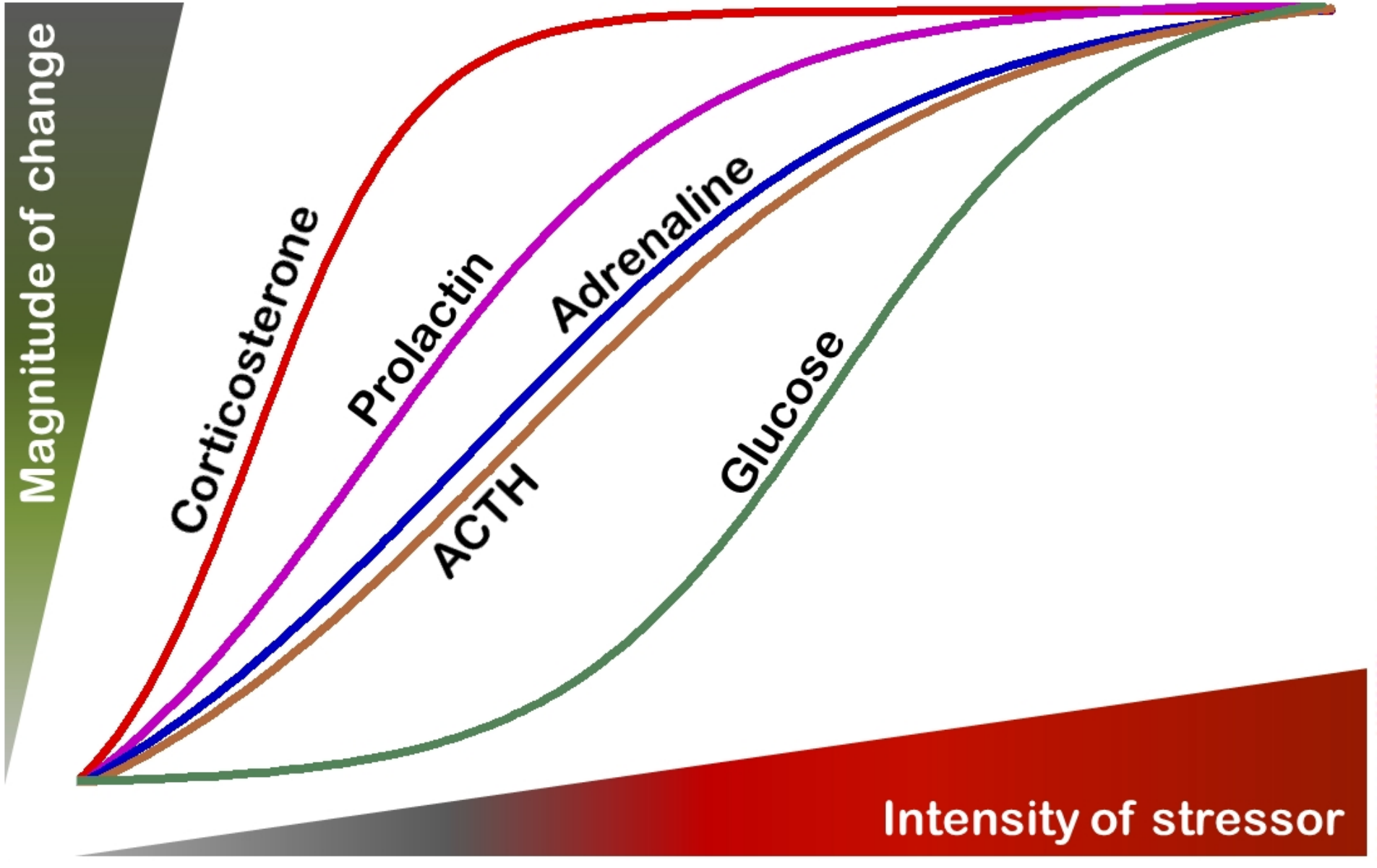
X indicates biological fluid in which they are typically measured; (a) direction of changes depends on the intensity and duration of acute stressors. ACTH: adrenocorticotropic hormone; GH: growth hormone; HPA: hypothalamic-pituitaryadrenal; LH: luteinizing hormone; SAM: sympathetic-adrenal-medullary; TSH: thyroid-stimulating hormone. Table 2: Cardiovascular and vegetative nervous system-dependent variables responsive to acute stressors

Variable	Meaning and underlying process	Direction of changes
Heart rate (HR)	Increased with sympathetic activity and decreased with parasympathetic one	↑(↓) a
Mean, diastolic and systolic arterial blood pressure (MAP, DBP, SBP)	Reflects sympathetic activity but also blood vessels resistance	↑
Heart rate variability (HRv)	Variability of inter-beats intervals Reflects parasympathetic/sympathetic balance	↓
Respiratory sinus arrhytmia (RSA)	Modulation of HR by respiratory process Reflects vagal influence on the heart	↓
Skin conductance (SC)	Electric conductance of the skin reflecting enhanced activity of sweat glands (sympathetic drive)	↑
Pupil dilation (PD)	Reflects sympathetic drive to pupil muscles	↑

a: bradycardia is observed under certain conditions

 FIGURE LEGEND

Figure 1: Relationship between the magnitude of the endocrine response to emotional stressors and the intensity of stressors (in arbitrary units). This relationship is constructed considering the peak of the response as each variable has a different time-course. Note that corticosterone response saturates with relatively low intensity and that saturation of all variables can be eventually achieved.


Magnitude of change
Corticosterone
Prolactin
Adrenaline
ACTH
Glucose
Intensity of stressor