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TITOLO DELLA TESI DI DOTTORATO:

Trait anxiety: an hidden variable in physiological and pathological processes

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PREMESSA

Nell'uomo esiste una differenza nella risposta agli stimoli stressogeni, che dipende dalla personale predisposizione all'ansia, detta specificatamente "tratto d'ansia". La differente suscettibilità all'ansia è stata studiata nei roditori creando ceppi selezionati per tratti di elevata e bassa ansia; inoltre è stato dimostrato, sia nell'uomo che nei ceppi selezionati di roditori, che differenti livelli di ansia basale influenzano la capacità dei soggetti di attuare un determinato compito, anche cognitivo. Tuttavia la suscettibilità individuale, all'interno di uno stesso ceppo di ratti naïve, è ancora poco studiata. Lo scopo di questo studio quindi è stato quello di valutare le possibili differenze interindividuali nel livello d'ansia, all'interno di una popolazione di ratti appartenenti al ceppo Wistar, e di fornire indicazioni su come uno specifico tratto d'ansia possa influenzare una successiva performance cognitiva, valutata mediante un test cognitivo ampiamente utilizzato, il Novel Object Recognition (NOR) test.

Seguendo questa linea di ricerca poi, abbiamo voluto indagare se il tratto di ansia potesse influenzare la suscettibilità del ceppo di topo C57Bl/6J all'insorgenza dell' epilessia, e se l'esposizione ad un fattore fortemente stressogeno per il topo, l'odore di un suo predatore, potesse provocare un aggravamento della malattia durante la fase cronica.

Questo lavoro di tesi mostra come esista una variabilità interindividuale all'interno di una popolazione di roditori per quanto riguarda l'ansia di tratto, cioè la componente basale di ansia insita in ogni individuo. Questo fattore può influenzare la risposta ad alcuni compiti a cui l'animale deve rispondere, come ad esempio quelli cognitivi. Il tratto d'ansia basale potrebbe anche influenzare la predisposizione all'insorgenza di una determinata malattia, oppure il decorso della malattia stessa. E' perciò molto importante considerare il tratto d'ansia basale di ciascun soggetto sperimentale in tutti gli studi che prevedano una componente comportamentale, includendo tale dato come fattore covariato nelle analisi statistiche, così da evitare errori dovuti a questa variabile nascosta.

PRELIMINARY REMARKS

Human subjects display a great variability in the predisposition to respond anxiogenically to stimuli, i.e. trait anxiety. This susceptibility has been studied in rodents through the creation of selected strains for anxiety-like behaviour, to obtain extreme anxiety traits. Moreover, anxiety has been shown to variously affect physiological processes, such as a cognitive task performance, both in humans and selected rodents strains. However, interindividual differences in basal anxiety level in naïve rats and how they may affect cognitive functioning have been poorly investigated. Therefore, the aim of this study is to provide an evidence of the huge interindividual differences in anxiety levels in a population of naïve Wistar rats and demonstrate how they can affect a widely used cognitive test, the Novel Object Recognition (NOR) test.

Following this line of research, in this study we also investigate if trait anxiety could affect pathological processes, such as the susceptibility on the onset of a neurological disease, the temporal lobe epilepsy, in a population of C57Bl/6J mice. Finally, we evaluate if the exposure to a strong stressful factor for mice, such as a predator odor, could induce an increase of the pathological process in chronic phase of the illness, for example in the number of seizures, in the same epileptic animals.

These results could show the relevance to consider trait anxiety, the propension to response in a manner more or less anxious to a specific stimulus, of each subject, in order to avoid interpretative errors during the evaluation of a specific behaviour shown by the subject.

Therefore we claim the need to consider interindividual differences in emotionality (e.g. anxiety) in general, and the need to assess anxiety level while studying rats cognitive abilities. It will be possible to include it as a covariate in the statistical analysis, in studies that schedule behavioural factors, in order to avoid interpretative errors dued to this hidden variable.

INTRODUCTION

1. Definition of anxiety and other concepts

1.1 Anxiety

The term “anxiety” stems from the Greek word ἄγχω (*angho*, “to squeeze, embrace, or throttle”). The term evolved then to the Latin “*anxietas”*, i.e. trouble of the mind, and the verb “*angere*”, meaning to choke or oppress.

Kandel (1983) proposed a definition of anxiety, which still remains appropriate; according to the Author “anxiety is a normal inborn response either to a threat – to one’s person, attitudes, or self-esteem – or the absence of people or objects that assure and signify safety”. Moreover Kaplan and Sadock stated that anxiety "is characterized by a diffuse, unpleasant, vague sense of apprehension, often accompanied by autonomic symptoms, such as headache, perspiration, palpitations, tightness in the chest, and mild stomach discomfort" (1996). Anxiety has been conceptualized in many ways, and defined as “a trait, a state, a stimulus, a response, a drive and as a motive” (Endler et al., 2001).

The emergence of anxiety as a scientific construct can be traced back in the writings of Darwin (1872), who used however the term “fear”. Darwin considered fear to be an inherent and adaptive characteristic of both animals and humans that has evolved over generations through natural selection. Furthermore, Darwin identified specific behavioural characteristics common both to human and non-human animals: “*The heart beats quickly and violently […] the skin instantly becomes pale […] due to the vasomotor centre being affected in such a manner as to cause the contraction of the small arteries of the skin […]. This exudation is all the more remarkable, as the surface is then cold, and hence the term a cold sweat; whereas, the sudoriphic glands are properly excited into action when the surface is heated. The hairs also on*

the skin stand erect; and the superficial muscles shiver. In connection with the disturbed action of the heart, the breathing is hurried. The salivary glands act imperfectly; the mouth becomes dry, and is often opened and shut. I have also noticed that under slight fear there is a strong tendency to yawn. One of the best-marked symptoms is the trembling of all the muscles of the body; and this is often first seen in the lips. From this cause, and from the dryness of the mouth, the voice becomes husky or indistinct, or may altogether fail." (Darwin, 1872).

It also assumed that individual with anxiety disorders are prone to perceive false alarm, which lead to a constant state of emotional arousal, tension and subjective distress (Beck et al., 1985). Moreover these thought are mostly automatic and derived from deeper cognitive structures, called schemas.

These theories were focused on the pathological aspects of anxiety, while new theories place more emphasis on the continuities between normal and "abnormal" anxiety, moreover the links between cognition and behaviour are highlighted (Barlow, 2002; Rachman, 2004).

1.2 Prevalence and costs of anxiety disorders

Anxiety occurs so frequently in association with psychological distress, and other psychiatric illnesses. It has been noticed that research on anxiety and its disorders has accelerated since the 1980s. This growth is due to the fact that anxiety has been recognized as one of the most prominent and pervasive emotions. Moreover, the introduction of a separate category for anxiety disorder and a clearer definition of these disorders, with the introduction of the Diagnostic and Statistical Manual for Mental Disorders (DSM), also played an important role in increasing the interest paid to that aspect (Rachman, 2004). In regard to the prevalence of anxiety disorders it has been reported a one year prevalence of 10.6% and a lifetime prevalence of 16.6%. Among the various subtypes of anxiety disorders, generalized anxiety disorder (GAD) is most frequent (lifetime prevalence 6.2%) followed by phobias: 4.9% lifetime prevalence for specific phobias,

3.1% for agoraphobia and 2.5% for social phobia (Somers et al., 2006). Posttraumatic stress disorder (PTSD) and obsessive compulsive disorder (OCD) appear with lifetime prevalence of 2.1% and 1.3% respectively, whereas panic disorder is the least frequent subtype (lifetime prevalence 1.2%; Somers et al., 2006). It has been also reported GAD to be the second most frequently seen psychiatric disorder in primary care, following depression (Lepine, 2002).

With the exception of GAD and PTSD, anxiety disorders typically have an onset in adolescence or early adult life, mostly before age of 16, and a considerable degree of persistence over the patient's lifetime (Wittchen et al., 2002).

Anxiety disorders are associated with significant costs to society, which include both direct and indirect costs. The first category include psychiatric and psychological counselling, hospitalization, emergency room care and drug prescription, whereas indirect costs refers to reduced productivity, absenteeism from work and suicide (Lepine, 2002). It has been calculated the annual economic burden of anxiety disorders in United States in 1990 to be $42.3 billion, $1542 per sufferer (Greenberg et al., 1999).

The economic burden of anxiety disorders in Europe has been poorly investigated (Sobocki and Wittchen, 2005; Löthgren, 2004). Il has been provided an estimate of the total cost of GAD in France, ranging from € 2882 to € 4778 per patient per year, with and without comorbidities, respectively. In a spanish study on panic disorder, it has been indicated that the total cost was € 1568 1 year prior to diagnosis and treatment and € 996 the year after diagnosis and treatment (Salvador-Carulla et al., 1995).

1.3 Trait Anxiety vs State Anxiety

A critical role in the development of anxiety and related disorders is certainly played by stressful events and threatening situation; however it is clearly visible (even in everyday occasions) that people differ in their susceptibility to the impact of stressful events and in their vulnerability in

experiencing anxiety. The existence of individual differences in anxiety proneness is supported by both clinical and experimental evidences and has been studied regarding both its biological and cognitive determinants (e.g. Bishop, 2007; Rutter, 2009; Gregory et al., 2008).

To begin with, there is evidence of experiential, biological and cognitive determinants of anxiety vulnerability. Indeed, it has been postulated the presence of individual differences in anxiety proneness in his theory, which was based on a two-dimensional model of personality: emotional instability (neuroticism) and introversion/ extroversion (Eysenck, 1957). Introverts are thought to be more prone to conditioning and therefore to acquire conditioned anxieties and fears (Eysenck and Rachman, 1965).

It has been possible to distinguish two separate concepts within the construct of anxiety: one referring to the emotional state of an individual at a given time and in a given situation, the other referring to a variable of personality that can differentiate between different individuals (Cattel and Sheier, 1958). It was, however, Spielberger (1966) the one who formulate a conceptual framework of state-trait anxiety and proposed the definitions, still in use, of state anxiety (A-state) and trait anxiety (A-trait).

A-trait identifies a predisposition to respond anxiogenically, given the individual's normal level of anxiety, and was defined as "a motive or an acquired behavioural disposition that predispose an individual to perceive a wide range of objectively non dangerous circumstances as threatening, and to respond to these with A-state reactions disproportionate in intensity to the magnitude of the objective danger".

A-state refers to a transitory emotion "subjective, consciously perceived feelings of apprehension and tension, accompanied by or associated with activation or arousal of the autonomic nervous system".

It has been also hypothesized that individual differences in A-trait were not displayed directly in behaviour, but they determined the cognitive appraisal of a specific stimulus as threatening, which leads to an increased A-state (Purdue and Spielberger, 1966).

In order to enable the assessment of both these components the "State Trait Anxiety Inventory" (STAI) was developed, the inventory included 40 items and contained a trait scale (i.e. how the individual generally feels) and a state scale (i.e. how the individual feels at the moment) (Spielberger et al., 1970). The inventory was later revised and renamed as STAI form Y (Spielberger, 1983).

Another method to assess anxiety was developed by Endler and colleagues (1991). The "Endler Multidimensional Anxiety Scale" (EMAS) assesses both trait and state anxiety together with the perception of stressful situation. The Author proposed with this test a multidimensional model of anxiety, which posit that a threatening situation is able to induce an increase in the A-state only if it is congruent with the facet of A-trait being investigated, thus interactions are not expected when the stressful situation is not congruent with the facet of A-Trait under investigation. It is possible to distinguish four dimensions of A-trait: Social Evaluation (SE), Physical Danger (PD), Ambiguous (AM) and Daily Routines (DR). Moreover EMAS measures two facets of A-state, thus Endler distinguished between the emotional component, referred to activation of autonomic nervous system (AE: Autonomic Emotion), and the cognitive component (CW: Cognitive Worry; Endler, 1991).

A-trait has been later compared with the concept of anxiety sensivity: anxiety sensivity refers to "a specific propensity to respond fearfully to the sensations of anxiety" (Reiss, 1997).

The relation between these two concepts is still debated: it has been differentiate trait anxiety as future anxiety that is based on anxiety from the past, whereas, anxiety sensitivity assesses beliefs about the consequences of anxiety (Reiss, 1997). However, it has been proposed that anxiety sensivity is one of the three factors that contribute to trait anxiety; the other two factors are illness/injury sensivity and fear of negative evaluation (Taylor, 1995). Finally, it has been concluded that this concept can be considerate a "bridge between the so-called temperamental features of anxiety proneness (i.e. experiences and biological determinants) and the more recently introduced cognitive aspects" (Rachman, 2004).

1.4 "Normal" Anxiety vs Pathological Anxiety

Fear and anxiety can be regarded as normal and common experiences, providing adaptive reactions to potentially threatening stimuli and an appropriate motivation for action in general. Identifying objects or situations that may threaten an organism's survival activates cognitive, affective, physiological, and behavioural processes, which serve to ensure the organism's safety and to restore its homeostasis (LeDoux, 1996).

The purpose of these responses is to maintain an appropriate degree of emotionality (i.e. avoid pervasive anxiety) under non-threatening circumstances; to respond to potential threats with a (transient) "fear" proportional to the danger encountered; to permit adaptive behavioural responses, such as escape or avoidance; and to rapidly restore a "normal" emotional status once the threat has passed (Heim and Nemeroff, 1999 and Holmes, 2001).

However, a malfunctioning of mechanisms, which control these responses, due to genetic, developmental and/or environmental factors, can provoke a perturbation of equilibrium. According to the previously cited Authors this perturbation may provoke an abrupt or gradual shift to a new "stasis" ("set-point") and, in pathological cases, a clinically-defined anxiety disorder.

Indeed, pathological anxiety involves the *over*-activation of these resources, that is, anxiety can become so severe or enduring that it significantly impairs normal functions (Barlow, 2002). Although often it may be difficult to clearly distinguish normal from pathological anxiety, some criteria have been proposed analysing the different components of anxiety (Starcevic, 2006) (Table 1).

Table 1 Criteria to distinguish between normal and pathological anxiety (from Starcevic, 2006).

Panic disorder without agoraphobia
Panic disorder with agoraphobia
Agoraphobia without history of panic disorder
Generalized anxiety disorder
Social anxiety disorder (social phobia)
Specific phobia
Obsessive-compulsive disorder
Acute stress disorder
Post-traumatic stress disorder
Anxiety disorder due to a general medical condition
Substance-induced anxiety disorder
Anxiety disorder not otherwise specified

Beside, the issue about differences between "normal" and pathological anxiety is related to the debate between a dimensional or categorical nature of anxiety.

The dimensional hypothesis states that anxiety can be conceptualized as lying on a continuum; one end represent a low amount of anxiety, the opposite end a severe level of anxiety (Endler and Kocovski, 2001). According to this point of view the difference between adaptive and pathological anxiety is simply one of degree (McLean and Woody, 2001).

According to the categorical hypothesis anxiety disorders are viewed as qualitatively different from a normal level of anxiety (Endler and Kolcovski, 2001). The categorical view focuses on the different and specific features the stimuli and the responses involved in the different types of disorders. For example anxious response can be cued by a stimulus or spontaneously experienced; moreover, anxiety can be managed by avoiding the stimulus or by using ritual or safety behaviours as protection from arm (McLean and Woody, 2001).

Despite the fact that the debate remains still open, the categorical approach is certainly the most used for diagnostic purpose. In fact, categories are very helpful in communication and have a simplifying quality (i.e. the patient either has or not the disorder) (Endler and Kocovski, 2001).

The current edition of the Diagnostic and Statistic Manual of Mental Disorder, i.e. DSM-IV (American Psychiatric Association, 1994), that is the most used diagnostic manual, contains

twelve categories of anxiety disorders, plus the separation anxiety disorder in the case of children (see Table 2).

Table 2 Categorization of anxiety related disorders according to DSM-IV (APA, 1994).

Panic disorder without agoraphobia
Panic disorder with agoraphobia
Agoraphobia without history of panic disorder
Generalized anxiety disorder
Social anxiety disorder (social phobia)
Specific phobia
Obsessive-compulsive disorder
Acute stress disorder
Post-traumatic stress disorder
Anxiety disorder due to a general medical condition
Substance-induced anxiety disorder
Anxiety disorder not otherwise specified

It has to be mentioned that in both case, i.e. considering anxiety as a dimensional or categorical factor, determining the cut-off for a pathological level of anxiety is quite an arbitrary operation; however, anxiety symptoms are generally considered to be clinically relevant when they interfere with everyday functioning (McLean and Woody, 2001).

1.5 Anxiety and fear

The concept of fear and anxiety are strongly related and often difficult to separate and even from a clinical perspective this confusion remains.

It has been noted that even in DSM IV (1994) there is a lack of a real attempt to define fear and distinguish it from anxiety (McNaughton, 2010). However, a definition paragraph for anxiety is present in the third edition DSM III (American Psychiatric Association, 1987): “apprehension, tension, or uneasiness that stems from the anticipation of danger, which may be internal or

external. Some definitions of anxiety distinguish it from fear by limiting it to anticipation of a danger whose source is largely unknown, whereas fear is the response to a consciously recognized and usually external threat or danger. The manifestations of anxiety and fear are the same and include motor tension, autonomic hyperactivity, apprehensive expectation, and vigilance and scanning." (DSM-III-R, 1987).

It has been analyzed the different defensive responses to threats and proposed a categorical separation of fear from anxiety. It was based on immediacy (or certainty) versus potentiality (or uncertainty) of threat. Thus, fear is linked to a set of behaviour elicited by a predator and that are sensitive to panicolytic but not to anxiolytic drugs. On the contrary, anxiety is linked to behaviours elicited by the potential presence of a predator and are sensitive to anxiolytic drugs (Blanchard and Blanchard, 1990).

Similarly, it has been proposed a definition of anxiety as "the apprehensive anticipation of future danger or misfortune accompanied by feeling of dysphoria or somatic symptoms of tension, when there is no true threat" whether fear is "a feeling of anxiety associated with real external (or internal) threat" (Vermetten et al., 2002).

Another distinction may depend to the fact that fear is considered by many theorists as a basic emotion, therefore, it develops on emotional program the basis of an innate emotional program when confronted with an identified threat. On the contrary, anxiety is often considerate as a secondary emotion, that is an emotion in response to a primary emotional reaction, i.e. fear or anger (Barlow, 2002; Greenberg, 2002).

Furthermore, anxiety involves modulation of pre-existing fear (or frustration) (McNaughton and Corr, 2004). It has been also recognized that there is a considerable functional overlap between the generation of fear and anxiety, but there are also functional, behavioural and pharmacological distinction.

On one hand the function of fear is to move animal away from danger and the related behaviour consists in a fight/flight/freezing response. Finally fear is insensitive to anxiolytic drugs. On the

other hand anxiety has the function of moving animal toward danger, it involves inhibition of prepotent behaviours, increased risk assessment and defensive quiescence. Obviously anxiety is sensitive to anxiolytic drugs (McNaughton and Corr, 2004).

1.6 Neurobiology of anxiety

The entire organism is involved in the response to (and modulation of) stress, fear and anxious states. Moreover, these states impact upon virtually all major systems: motor, sensory, endocrine, immune, cardiovascular and, of course, neural (Millan, 2003).

It has been formulated one of the most famous theories (James, 1884; Lange, 1887), that suggested that one experiences an emotion in response to physiological changes in the body. According to the this theory, as an individual perceives a stimulus, the sensory systems send information to the brain, which reacts inducing physical (neurovegetative) manifestations (i.e. changes in heart rate, muscle tone etcetera). Thus, this theory stated that the physiological changes coincide with the emotion itself.

The Cannon-Bard theory also states that, when a person faces an event that somehow affects him or her, the nervous impulse travels straight to the thalamus where the message divides. One part goes to the cortex to originate subjective experiences like fear, rage, sadness, joy, etc. The other part goes to the hypothalamus to determine the peripheral neurovegetative changes (symptoms). According to this theory physiological reactions and emotional experience occur simultaneously.

Broca (1878) originally coined the term “limbic” for a series of phylogenetically-conserved structures, but the central role of this subcortical network of brain structures was hypothesized by Papez in 1937 (Fig.1).

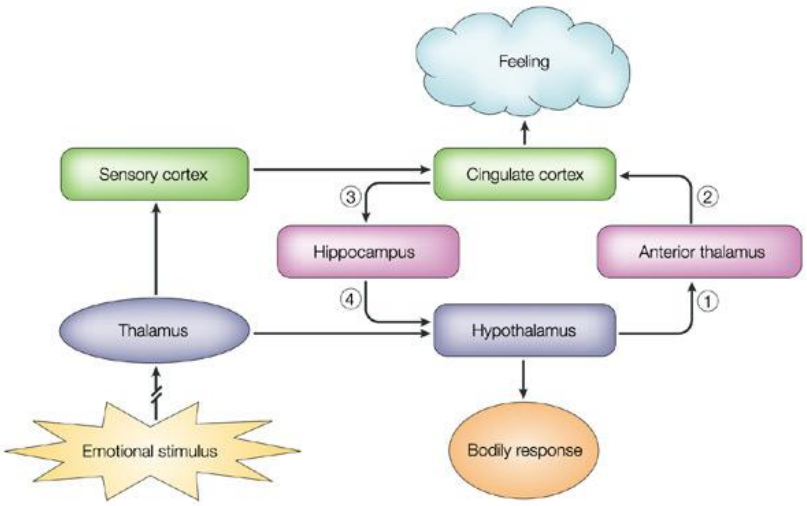

Fig.1 Papez's emotional circuit (from Dalgleish, 2004).

The Author argued that sensory messages concerning emotional stimuli that arrive at the thalamus are then directed to both the cortex (stream of thinking) and the hypothalamus (stream of feeling). Papez also proposed a series of connections from the hypothalamus to the anterior thalamus (1) and on to the cingulate cortex (2). Emotional experiences or feelings occur when the cingulate cortex integrates these signals from the hypothalamus with information from the sensory cortex. Output from the cingulate cortex to the hippocampus (3) and then to the hypothalamus (4) allows top–down cortical control of emotional responses.

In 1949, McLean integrated Papez's original circuit (hypothalamus, anterior thalamus, cingulate gyrus, and hippocampus) with other anatomically and functionally related areas (amygdala, septum, and orbitofrontal cortex).

By now several cortical regions have shown to be relevant in the various components of anxious states. The principal structures involved in anxiety, as indicated by McNaughton and Corr (2004), are presented, together with the main neuroendocrine responses.

2. Key brain structures

2.1 Periaqueductal gray

The Periaqueductal Gray (PAG) is part of the limbic midbrain area.

The earliest finding regarding the role of PAG in fear and anxiety reported that stimulation of PAG provoked deep analgesia in rats and reports of intense fear and panic, associated with autonomic changes, in humans (Reynolds, 1969; Nashold et al., 1969).

Further studies showed that electrical stimulation of rostral dorsolateral PAG in rats and cats produced threat display associated with vocalization and strong flight response, whereas stimulation of the caudal ventrolateral PAG produced immobility (Behbehani, 1995).

Thus, a columnar organization of this structure it has been proposed. Indeed, anatomical and functional evidences suggested the presence of four longitudinal columns, namely, dorsolateral, dorsomedial, lateral and ventrolateral, with distinct features, connections and functions (Bandler et al., 1991).

It has been proposed a PAG-amygdala network, involved in anxiety and activated by threatening stimuli. According to this model signals of danger, e.g. the sight of a predator, activate amygdala regions which project to the ventral PAG. Activation of this region produces freezing and analgesia.

However, as the animal encounters danger, e.g. is caught by a predator, a network in the amygdala that projects to the lateral PAG is activated and flight responses are produced, along with vocalization and autonomic responses.

Thus, there seems to be an inhibitory interaction between ventral and lateral PAG networks. Lateral PAG stimulation produces flight and defensive responses, whereas ventral PAG stimulation produces immobility and freezing (Fanselow, 1991).

Carobrez and colleagues also demonstrated that dorsal PAG participates in the mediation of anxiety/fear-like behavior elicited in the Elevated Plus Maze (EPM), showing correlation among behavioural data observed in the EPM, fear-like defensive behaviour and dorsal PAG activity. Moreover, it has been shown that dorsal PAG-NMDA-receptor blockade increased EPM open arms exploration, reducing anxiety-like behaviour (Carobrez et al., 2001**;** Kincheski and Carobrez, 2009**).**

According to McNaughton and Corr PAG is responsible of the lowest levels of control of anxiety (2004).

2.2 Hypothalamus

Hypothalamus plays a crucial role in regulating stress response. Indeed, many of the neuroendocrine and autonomic changes resulting from stress, fear and anxiety may be understood from the projections that the hypothalamic nuclei receive from many limbic and brain stem structures.

Among the first experiments showing an involvement of hypothalamus in emotional responses are the series of experiments conducted in the 1920s by Hess, who implanted electrodes into the hypothalamic region of cats. Electrical stimulation led to an 'affective defence reaction' that was associated with increased heart rate, alertness and a propensity to attack. Hess could induce animals to act angry, fearful, curious or lethargic as a function of which brain regions were stimulated (as reported by Dalgleish, 2004).

It has been reported that the blockade of gamma-aminobutyric acid (GABA) function in the posterior hypothalamus of rats elicits a pattern of physiological and behavioural arousal, consisting of increases in heart rate, respiration and blood pressure as well as intense locomotor stimulation and a selective enhancement of avoidance behaviour. Moreover, endogenous GABA

acts in the posterior hypothalamus to regulate the level of experimental anxiety in rats (Shekhar et al., 1990).

The medial hypothalamus, has been shown to be implicated in the regulation of different behaviours and physiological functions, such as food ingestion and metabolism, reproduction, and defense. This regions contains different cell groups, which are strongly interconnected and seems to be especially involved in the integration of innate responses to environmental threats (Canteras, 2002).

Medial hypothalamus has been shown to be involved in controlling defensive behaviour and its dysfunction has been related to panic disorder (Blanchard et al., 2001; McNaughton and Corr, 2004).

Moreover, it has been postulated that this structure would control the simplest behavioural reactions, when facing a situation in which immediate danger is present (McNaughton and Corr, 2004).

2.3 Amygdala

A key role in the regulation and experience of emotional states in general is certainly played by the amygdala, involved in the recognition of signals of danger and in the control of autonomic and behavioural reaction responses to external threat. There are several human and animal evidences supporting the central role of amygdala in anxiety and fear (Etkin et al., 2009).

The amygdala is a complex of different nuclei situated in the anterior portion of the temporal lobe and has a close anatomical and functional relationship with the hippocampal formation; these two structures form the two major subcortical telencephalic limbic areas. Amygdala possesses an extensive pattern of reciprocal connections with cortical, limbic, monoaminergic and other structures implicated in the emotional, cognitive, autonomic and endocrine response to stress (Gray and Magnuson, 1992; LeDoux, 2000; Carrasco and Van de Kar, 2003) (Fig.2).

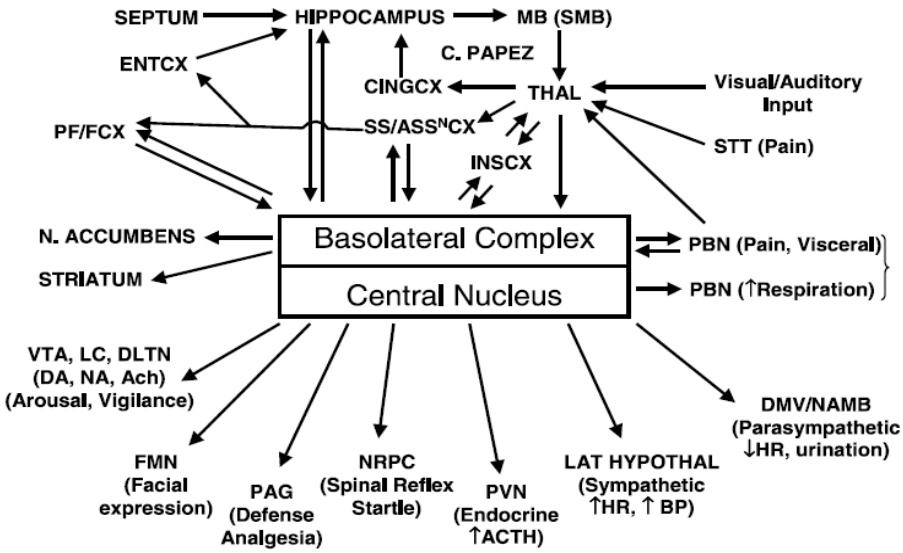

Fig.2 Schematic representation of the organization of structures involved in the integration and induction of anxious states. Abbreviations (clockwise) indicate PF/F: prefrontal/frontal; ENT: entorhinal; CX: cortex; MB (SMB): mammillary bodies (supramammillary bodies); CING: cingulate; ASSN: association; THAL: thalamus; INS: insular; SS: somatosensory; STT: spinothalamic tract; PBN: parabrachial nucleus; DMV/NAMB: dorsal motor nucleus of the vagus/nucleus ambiguous; HR: heart rate; AP: arterial pressure; LAT HYPOTH: lateral hypothalamus; PVN: paraventricular nucleus; ACTH: adrenocorticotropic hormone; NRPC: *nucleus reticularis penduncocellularis*; PAG: periaqueductal gray; FMN: facial motor nucleus; VTA: ventrotegmental area; LC: locus coeruleus; DLTN: dorsolateral segmental nucleus; DA: dopamine; NA: noradrenaline; ACh: acetylcholine and NACC: nucleus accumbens. (From Millan, 2003)

Output pathways are primarily derived from the central nucleus, which is part of the centromedial amygdala (CMA) and projects to the brain stem, hypothalamic and basal forebrain targets, and from the contiguous bed nucleus of the stria terminals, whereas the basolateral amygdaloidal complex (BLA) is principally responsible for the receipt and filtering of cortical and subcortical (mostly thalamic) sensory input (Gray and Magnuson, 1992; Walker et al., 2003). It has been reported that in rodents, BLA encodes the threat value of a stimulus, while the central nucleus is essential for the basic species-specific defensive responses associated with fear (Davis and Whalen 2001).

In humans amygdala has been shown to be activated by emotional stimuli with negative valence; moreover, lesions of the amygdala are associated with inability to label fearful facial expressions and to encode fear-based memories (Phan et al., 2002; Wager et al., 2003). Moreover, invasive stimulation of human amygdala with microelectrodes produces subjective reports of fear and anxiety (Lanteaume, 2007).

Synaptic plasticity in the amygdala has been shown to be implicated in the induction, processing and extinction of conditioned fear, in the generation of anticipatory anxiety and in the coordination of the global response to threats. Such phenomena are highly relevant to behaviour in experimental models in rodents, as well as to GAD and phobias in man (Davis, 1992; LeDoux, 2000; McGaugh et al., 2002).

Amygdala also responds to anxiety provoking environmental cues with a neutral valence, to emotional stimuli processed outside of awareness, in this case the activation is greater in the most anxious subjects, and to emotional stimuli processed under limited attentional resources (Herry et al., 2007; Etkin et al., 2004; Bishop et al., 2004).

Therefore, amygdala plays a central role in both subjective and attentional-vigilance aspects of the processing of threatening stimuli; thus, abnormalities in this system may be associated with hyperarousal and hypervigilance, which manifest in anxiety disorders (Etkin et al., 2009).

Several authors also proposed a distinction between the neural substrates of anxiety and fear, the core of this distinction has been reported to be amygdala (Fig.3).

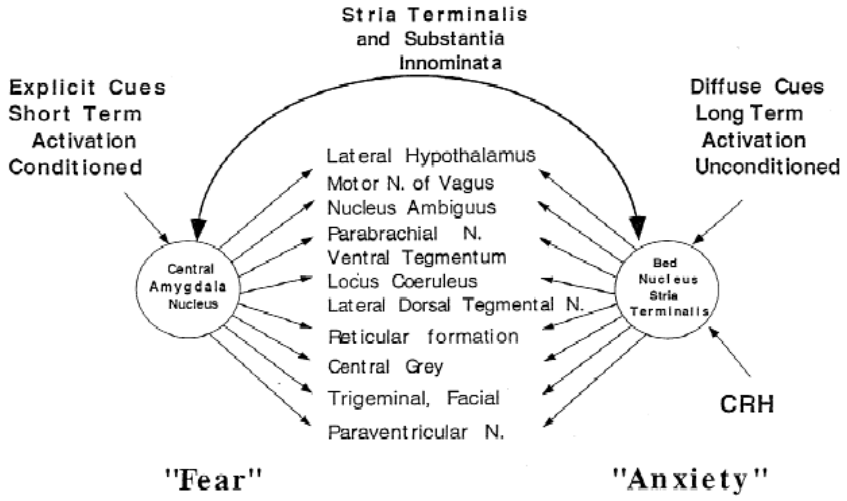

Fig.3 Hypothetical scheme suggesting a different involvement of central amygdala nucleus and bed nucleus of stria terminalis in fear versus anxiety, respectively. (from Lang et al., 2000).

Both central nucleus of the amygdala and bed nucleus of stria terminals have highly similar hypothalamic and brainstem targets known to be involved in specific signs and symptoms of fear and anxiety.

However, the stress peptide CRH seems to act on receptors in the bed nucleus of the stria terminalis. Furthermore, this nucleus seems to be involved in the anxiogenic effects of bright lights presented for a long period, but not when the same light has been previously paired with a shock. Just the opposite is the case for the central nucleus of the amygdala, which is critical for fear conditioning using explicit cues such as light or tone paired with aversive stimulation (i.e., conditioned fear) (Lang et al., 2000).

2.4 Hippocampal formation

The hippocampus plays a key role in memory function and on analysing the context in which fear is experienced. Indeed, an important aspect of fear response is the incorporation of a person's prior experience (memory) into the cognitive appraisal of stimuli. The hippocampus mediates declarative memory function (e.g., recall of facts and lists) and plays a crucial role in the integration of memory elements with those present in the context at the time of retrieval and in assigning significance for events within space and time.

This structure is also involved in mediating emotional responses to stressor, as proved by lesions of hippocampus in animal studies, which disrupt the formation of emotional memories of the context where a stressor took place (Vermetten et al., 2002).

Several authors reported that hippocampal volume is reduced in patients with long-standing depression (e.g. Campbell et al., 2004; Videbech and Ravnkilde, 2004) and severe, unremitting post-traumatic stress disorder (PTSD; e.g. Bremner et al., 1995, Gurvits et al., 1996; Lindauer et al., 2004).

However, it has been reported that trait anxiety is positively related to hippocampal volume in both depressed patients and normal controls (Rusch et al., 2001). Thus, it has been proposed that an enlarged hippocampal volume in anxious individuals could reflect an increased use. However, found a positive correlation between anxiety-like behaviour and hippocampus volume in rats with an extreme hyper-anxious phenotypes (i.e. HAB, High Anxiety Behaviour rats), but a negative correlation in normal rats with an high anxiety behaviour (Kalish et al., 2005).

Thus, despite being clearly involved in anxiety, the results suggests that the relationship between hippocampal volume and trait anxiety is quite complicated.

It has been claimed a crucial role for the hippocampal formation, which initially constituted the core of Gray's neuropsychology of anxiety (1982). It has been attributed to this formation the cognitive aspects of conventional anxiety and GAD (Gray and McNaughton, 2000).

2.5 Prefrontal cortex

The final component of every stress and anxiety response involves preparation for a response to the potential threat, which requires an integration between brain areas involved in assessing and interpreting the potentially threatening stimulus and brain areas involved in the response (Bremner et al., 2009) and this role seems to be fulfilled by the prefrontal cortex (PFC).

For instance, the medial prefrontal cortex (mPFC) has been shown to be involved in "learning the emotional and motivational value of stimuli" (Rolls, 1996). It has been also suggested that neurons in the PFC can detect changes or reversals in the reward value of learned stimuli and change their responses accordingly.

mPFC areas also modulate emotional responsiveness through the inhibition of amygdala function in response to fearful cues. Moreover area 25, which is part of mPFC, also has direct projections to brain stem and is involved in the regulation of peripheral responses to stress, such as heart rate, blood pressure and cortisol response (Vermetten et al., 2002).

The medial region of the rat prefrontal cortex also contains both mineralocorticoid and glucocorticoid receptors, in addiction lesions of the cingulate gyrus region are associated with significantly increased plasma levels of adrenocorticotropic hormone (ACTH) and cortisol (CORT) (Diorio et al. ,1993). It has been claimed that these data are consistent with the idea that the prefrontal cortex mediates an inhibitory effect of glucocorticoids on stress-induced HPA activity.

The cingulate cortex is part of the PFC and has been related in particular to agoraphobia, since it appears to be involved in spatial analysis (as reported by McNaughton and Corr, 2004).The dorsal trend of the prefrontal cortex seems to be involved in complex forms of anxiety, such as social anxiety. Indeed, changes in the activation of this cortex have been found with maternal separation induced anxiety both in Rhesus monkeys and human infants (Rilling et al., 2001), and in patients with social anxiety disorders (Nutt et al., 1998).

3. Neuroendocrine responses

3.1 Serotonin

Serotoninergic neurones, originating in raphe nuclei, provide a massive input to corticolimbic structures involved in the control of anxious states. The dorsal raphe nucleus (DRN) primarily innervates the frontal cortex, dorsal hippocampus and amygdala, while the median raphe nucleus (MRN) principally projects to hippocampal formation, septum, nucleus accumbens and hypothalamus (Gray, 1987; Millan, 2003).

These networks seem to fulfil differential roles in the control of anxious states. For example, serotoninergic pathways emanating from the DRN have been shown to be specifically involved in the control of behaviour in the Vogel conflict test (Pratt, 1992). The response in this test is also accompanied by an increase in 5-HT release in the hippocampus (Matsuo et al., 1996).

Furthermore, it has been reported that a complex and non-uniform role for 5-HT in the control of anxiety disorders has also been forwarded as a function of: "(1) whether the anxious state is provoked by conditioned or unconditioned fear and (2) contrasting actions of 5-HT in specific cerebral regions, notably the amygdala as compared to the PAG" (Millan, 2003). The Author also pointed out that in assessing the role of 5-HT in the modulation of anxious states, it has to be considered that serotoninergic pathways play a profound influence upon other behaviours, such as motor function, impulsivity and cognition.

Serotonergic pathways innervating structures such as the frontal cortex, amygdala, hypothalamus and hippocampus are activated by anxiogenics stimuli, including psychosocial stress, conditioned fear and conflict procedures (Pratt, 1992; Rueter et al., 1997, Ishida et al., 2002).

Finally, serotonergic mechanisms participate in the influence of a broad range of therapeutically-employed drugs upon emotionality in general, and upon anxious states in particular. Moreover, the 5-HT releaser, methylenedioxymethylamphetamine (ecstasy), has been shown to modify

anxiety states in a dose and test-dependent manner both in experimental studies and in human subjects.

3.2 Dopamine

Dopaminergic pathways have been mainly studied within the perspective of their role in the pathogenesis of depression, drug abuse, schizophrenia and Parkinson's disease; indeed, all this disorders involve a marked dysregulation of dopaminergic transmission.

Mesocortical and mesolimbic dopaminergic pathways originate in the ventral tegmental area and play an important role in the control of mood. Indeed, dopaminergic activity may be critical for an appropriate response to stress and fear, as well as cognitive functions (Nioullon, 2002, Pezze et al., 2003).

Moreover, anxious symptoms are frequently co-morbid with (and may exacerbate) drug abuse, affective and psychotic disorders, while anxiety, particularly social anxiety, is a prominent, precocious and persistent symptom of Parkinson's disease (Millan, 2003).

It has been also reported that subjects with high levels of trait anxiety and very susceptible to panic attacks have revealed an enhancement in the activity of central dopaminergic pathways (Finlay and Zigmond, 1997; Mizuki et al., 1997).

Several experimental studies demonstrate that conditioned fear, anxiety and other stressors elicit an activation of dopaminergic pathways to the amygdala and adjacent bed nucleus of the stria terminals, to the nucleus accumbens and to the frontal cortex and this activation seems to be reversible with BDZ-treatment (Suzuki et al., 2002; Pezze et al., 2001; Finlay and Zigmond, 1997).

It has been also reported that a psychostimulant and DA releaser, dextroamphetamine, enhances the response of the amygdala to aversive stimuli in human subjects (Harari et al., 2002).

Other results, deriving from rats studies, suggested that dopaminergic action in the amygdala reflects the simultaneous promotion of excitatory input from the somato-sensory cortex, and suppression of inhibitory input from the prefrontal cortex (Rosenkranz and Grace, 2001, 2002).

Dopaminergic mesolimbic projections are critically involved in mechanisms of motivation and reward, and a substantial body of evidence suggests that their engagement contributes to the preference for (non-aversive) novel stimuli (Millan, 2003). Indeed, It should also be recalled that dopaminergic pathways fulfil an important role in the formation, retention and extinction of fear-related associations and memory (Morrow et al., 1999; Nieoullon, 2002).

Moreover, in paradigms involving the exploration of unfamiliar environments and other measures of neophobia, the reinforcing effects of dopaminergic agents may interact with their influence upon anxious states per se and can outweigh the negative impact of stress (Marinelli and Piazza, 2002).

3.3 Noradrenaline

Ascending noradrenergic projections innervate many structures variously involved in anxiety, e.g. hippocampus, amygdala, PAG, cortex, hypothalamus, and a main proportion of this input derives from the locus coeruleus (LC) (Millan, 2003).

The marked and substained activation of noradrenergic inputs derives from anxiogenics stimuli and stressful and is accompanied by emotional, cognitive and autonomic manifestations, particularly studied is NA influence in panic attacks induction. (Ishida et al., 2002; Shekhar et al., 2002)

3.4 GABA

GABAergic neurons constitute the major mode of inhibitory transmission in the Central Nervous System and several corticolimbic structures involved in the modulation of anxious states contain

these neurons (e.g. hippocampus, amygdala, lateral septum and PAG) (Cherubini and Conti, 2001, Mody, 2001).

Moreover, GABAergic pathways also have an inhibitory influence upon the release of many other neurotransmitters known to mediate anxiogenics actions. Indeed, they play a suppressive influence upon corticolimbic noradrenergic and serotoninergic projections, of which hyperactivity is implicated in the induction of anxious states (Millan, 2003).

Conditioned fear has been shown to be accompanied by an activation of GABAergic interneurons in both noradrenergic and serotoninergic cell clusters (i.e. Locus Coeruleus and Raphe Nuclei) (Ishida et al., 2002).

Further, GABAergic neurones are inhibitory to the stress-induced release of dopamine (DA), glutamate, corticotrophin-releasing factor (CRF) and several other anxiogenics mediators.

Thus, the control of anxious states by GABAergic mechanisms implies: "(1) modulation of monoaminergic transmission; (2) interactions with monoaminergic receptors postsynaptic to monoaminergic projections; and (3) actions independent of monoaminergic pathways" (Millan, 2003)

4. Stress

The term stress derives from physical sciences and generally refers to external forces or pressures on an object or person. It was firstly used to refer to physiological and biochemical reactions evoked by noxious stimuli. These reactions arouse and prepare a subject to a defensive behaviour, that is a "flight or fight" response (Cannon, 1929).

It has been provided an operational definition of stress as "any challenge to homeostasis that requires an adaptional response (Newport and Nemeroff, 2002). Often stress is a consequence of a change in the external environment that perturbs the internal mileu."

Stress involves therefore a stressor, i.e. a stimulus, and a stress response.

Stressors include different types of stimuli such as trauma, injury or major life events. It has been developed a list of potentially stressful events, examining the medical records of over 5,000 medical patients as a way to determine whether stressful events might cause illnesses. The result was the Social Readjustment Rating Scale (SRRS) (Holmes and Rahe, 1967).

Stress is generally acknowledged to play a critical role in the pathogenesis of many psychiatric disorders (Newport and Nemeroff, 2002). There is, however, a profound difference in the vulnerability to stressors among individuals; some people exhibit a lower threshold of tolerance for stress, which also seems to predispose them to stress-induced illness or precipitation of a psychiatric syndrome. This vulnerability is explained by the diathesis/stress model, which was originally used to describe an underlying pathogenic mechanism that remains latent and harmless until activated by sufficient stress (Ingram et al., 1998).

Diathesis/stress models recognize that both genetic inheritance and environmental acquired factors contribute to the vulnerability to stress and played a role in influence of stress in psychiatric and psychological disorders such as Generalized Anxiety Disorder, Post Traumatic Stress Disorder, and Depression (Gregory et al., 2008; Gillespie et al., 2009; Afifi et al., 2010; Rutter, 2009).

4.1 Eustress vs Distress

The word *stress* and its definition take origin by the physiologist Hans Selye, who discovered that different physical stimuli can activate the so called hyphotalamic-pituitary-adrenal axis (HPA axis). Generally, stress can be defined as a condition in which a perturbation of homeostasis (the equilibrium) of the organism exists, as a consequence of any kind of stimulus or factor, internal or external.

The functional response that the organism can use in order to response to adverse environmental stimuli (called "*stressors*"), that can be a risk for life, is defined "coping strategy". In particular,

in nature animals can react to an external stressful stimulus with a *proactive strategy*, in which animals show an active control on the environmental stimulus, exibit a predisposition to the exploration and response to the stimulus without fear; or animals can react with a *reactive strategy*, showing a passive behaviour on the same stimulus.

Generally, stress is a condition that occurs life of each person; when the subject become, for any kind of reason, incapable to react to the stressors, so stress can become an aetiological factor in the development of a pathological state.

As regards the characteristics of stress, Selye identify two different type of stress: eustress, i.e. positive stress, and distress, i.e. negative stress.

Since it is not the nature of the stress to induce a pathological state, but the incapability of subjects to adapt to, frequency and duration of the stressful stimulus are the key players for the development of pathological condition. On the basis of these two parameters, it is possible to distinguish an *acute stress* and a *chronic stress*: the first is the state in which subject has a sudden decrease in the predictability and/or control of relevant external factors; the second is the state in which subject has in front of unexpected external factors that are uncontrollable for long time.

Thus the adaptive responses are sufficient to reinstate homeostasis and terminates after this re-balance (Engelmann et al., 2004). Distress in the contrary requires a level of performance beyond the potential of the individual (Shelly, 2003).

To conclude, a stressor may cause eustress or distress, depending on its quality and intensity, and on how the stressor is perceived and interpreted by the individual (Engelmann et al., 2004).

4.2 Neurobiology of stress

Both, beneficial and deleterious effects of stress are thought to involve the action of corticosterone/cortisol secreted from the adrenal glands into the blood.

One of the main systems activated during the stress response in order to cope with stressors is the hypothalamic-pituitary-adrenal (HPA) axis (Fig.4). In fact, the release of glucocorticoids by the adrenal glands serves both to alert the organism to environmental or physiologic changes and to defend homeostasis.

The key role in these responses played by the HPA axis was first suggested by Selye (1956), who also proposed a model for bodily responses to short-term or long-term stressors: the General adaptation syndromes, which include three stages.

The first stage is alarm and occurs as the threat or stressor is identified or realized and the body prepares to deal with it. During this phase activation of the HPA axis, the nervous system (SNS) and the adrenal glands take place and the main stress hormones cortisol, adrenaline, and noradrenaline, are released to prepare the "fight-or-flight" response. The second stage is resistance (or adaptation), which manifest if the stressor persists and involves coping strategies and the attempt of the body to adapt to the strains or demands of the environment. Stress hormones levels may return to normality but if the stressful condition persists the body remains in a state of arousal.

The final stage is exhaustion: all of the body's resources are depleted and the body is unable to maintain normal function. The initial autonomic nervous system symptoms may reappear (sweating, raised heart rate etc.). If stage three is extended, long term damage may result as the capacity of glands, especially the adrenal gland, and the immune system is exhausted and their function may be impaired, resulting in decompensation (Selye, 1956).

4.3 Hypothalamic-Pituitary-Adrenal (HPA) Axis

Perception of a stressful situations causes initially the activation of the neurons of the parvocellular portion of the hypothalamic paraventricular nucleus (PVN). Indeed hypothalamus receives inputs from many different brain pathways. The PVN synthesises and releases corticotrophin releasing hormone (CRH) and vasopressin (AVP). Both neuropeptides are released from neurosecretory nerve terminals at the median eminence and are transported to the anterior pituitary through the portal blood vessel system of the hypophyseal stalk.

In the anterior pituitary CRH and AVP stimulate the production and secretion of adrenocorticotropic hormone (ACTH) from corticotrope cells. Finally ACTH is transported by the blood to the adrenal cortex of the adrenal gland, where it stimulates the release of glucocorticoids (corticosterone in rats, cortisol in humans) into the blood.

The HPA axis is regulated by a negative feedback system at multiple levels via glucocorticoid receptors within the hypothalamus and the anterior pituitary, which detect the circulating level of glucocorticoids this feedback serves to protect the mechanism against spillover. If level goes above the norm production of the initial substances from PVN is down-inhibited (Vermetten et al., 2002; Kalat, 1995).

HPA dysregulation occurs mainly in terms of an exaggerated CRH and glucocorticoids secretion, which have been described in several psychiatric disorders, such as melancholic depression, anxiety and emotional disturbances (Ehlert et al., 2001). CRH hypersecretion is assumed to result by the disinhibition of the negative feedback control, which may be a consequence of longstanding hypersecretion of glucocorticoids.

Glucocorticoids play a central role in the regulation of a wide range of bodily function, such as inflammatory and cardiovascular responses, cognitive functions, e.g. information processing, learning and memory, metabolic and immune functions (Vreugdenhil and de Kloet, 1998; Sapolsky et al., 2000). They have also been shown to have a significant impact on vigilance and

cognitive performance and this influence seems to follow the Yerkes-Dodson Curve (see Paragraph 5.1) as studies have shown that circulating levels of glucocorticoids vs. memory performance follows an upside down U pattern, much like the Yerkes-Dodson curve.

It has been identified coping and defense mechanism as the most important cognitive filters responsible for the intra and interindividual differences in HPA axis responses (Ursin et al., 1998).

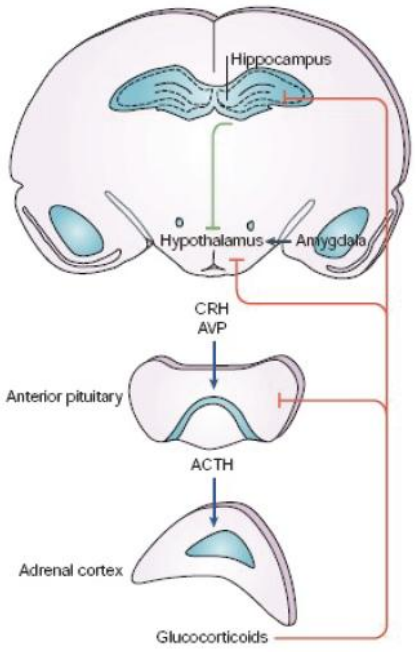

Fig.4 Schematic representation of the hypothalamus-pituitary-adrenal (HPA) axis (from Sandi, 2004).

4.4 Stress and psychopathology

From both a biological and physiological perspective stress is therefore strongly related to the concept of homeostasis, an imbalance between the demands of the threatening situation and the ability and possibility to cope with it.

Thus stress can be beneficial in term of mobilization of resources, but detrimental if the perceived demands exceed the resources (Newton and Nemeroff, 2002).

Despite the fact that all stress responses are intended to preserve homeostasis, they may result adaptive or maladaptive, in the second case they fail in achieving homeostasis and a pathophysiological, or psychological, cascade of negative events may ensue (Newton and Nemeroff, 2002).

It has been proposed a psychological diathesis model focusing on relatively stable individual differences (e.g., personality traits or cognitive styles) that increase one's vulnerability to stress and to the development of psychological disorders. These vulnerabilities are described as stable (without intervention), endogenous (i.e., resides within the person), latent (i.e., not easily observable), and likely to interact with stress (Ingram & Price, 2001). The notion that cognitive processes or appraisal are central in determining whether a situation is potentially threatening was already expressed by Lazarus (1966). Thus these processes determine if the situation constitutes a harm/loss, a challenge, or is benign and allow to select the appropriate coping strategies.

Diathesis/stress model has been applied to a broad range of disorders, including major depression, schizophrenia, chronic fatigue syndrome, PTDS and other anxiety disorders (Newton and Nemeroff, 2002).

5. The relationship between anxiety and stress

Conceptually stress and anxiety are tightly linked. Indeed stress and (state) anxiety often co-occur and anxiogenics situations lead to an activation of HPA-axis.

The link between these two aspects also derives from the fact that anxiety and fear can be a part of the stress response and constitute a component of a potential stressor (Dietrich, 2008).

With regard to the effect of stress on anxiety the multidimensional interaction model of stress, anxiety and coping proposed by Endler (1989, 1990) provide a useful conceptualization of the relationship between these three aspects from cognitive point of view (Fig.5).

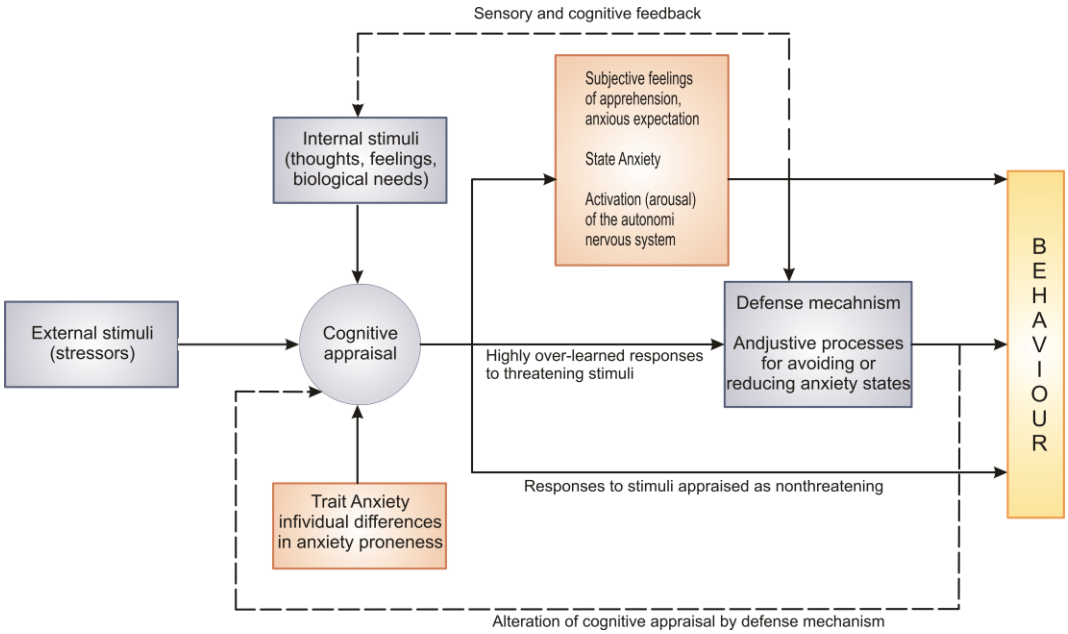

Fig.5 Multidimensional interaction model of stress, anxiety and coping (adapted from Endler, 2000).

As previously said, coping strategies are central in stress response and depend on cognitive appraisal. A coping style is a characteristic manner of responding to stressful situations (Endler and Parker, 1994). There are three basic coping styles in humans: task-oriented coping (i.e. focussing on solving the problem or attempting to change the situation), emotion-oriented coping (i.e. responses self-oriented, such as emotional responses or self-preoccupation), and avoidance-oriented coping (i.e. avoiding the stressful situation by seeking social support or distracting oneself with other tasks) (Endler, 1990,2000).

The model also include individual variables, such as trait anxiety (and other traits), vulnerability, physiological arousal, and other biological variables and situation variables, i.e. stressful events, crises, traumas, and physical environments.

These variables can interact with one another and their interaction leads to the perception of threat, which in turn leads to changes in state anxiety (and there is also feedback to both person and situation variables) and ultimately in reactions such as coping responses, defences, illness, behavioural, and biological reactions (Endler, 1997). These reactions also feedback to individual and environmental variables.

From a neurobiological perspective, studies on the effect of stress on anxiety related pathology and depression have mainly focalized on the imbalance of HPA-axis and glucocorticoid receptors induced by prolonged stress, thus this imbalance affects specific neural signalling pathways underlying emotion and results in anxiety and stress (Korte, 2001).

Other studies showed that expositions to a single and unpredictible stressful event is sufficient to induce persistent changes in behavioural and physiological parameters in rats (Koolhaas et al., 1997). Several studies highlighted similar alterations in HPA axis, with the involvement of CRF system, consequent by the exposure to social stress in different species (rat, *Rattus norvegicus*, Plotsky e Meaney, 1993; macaque, *Macaca radiata*, Coplan et al., 1996; baboon, *Papio cynocephalus*, Sapolsky et al., 1997; hamster, *Mesocricetus auratus*, Jasnow et al., 1999; chick, *Gallus gallus*, Sufka et al., 2006). Moreover, studies developed in cynomolgus macaque (*Macaca fascicularis*) (Shively, 1998) demonstrated a correlation between social anxiety disorder and impairment of dopaminergic transmission, and alterations of noradrenergic and serotoninergic systems.

5.1 Anxiety and cognitive efficiency

It has been long hypothesized that anxiety-related behaviour and cognitive processes may interact in a fundamental manner. According to some authors, cognitive dysfunctions are even suggested to be the primary presenting feature of pathological anxiety (Gray, 1990; McNaughton, 1997).

Behaviourally, abnormalities in attentional control are often seen when subjects are presented with threat- related stimuli or distractors thought to be anxiety inducing (Fox and Georgiou, 2005; Koster et al., 2006). Moreover, it has been shown that individuals who are temperamentally anxious show impairments in cognitive tasks, even when they lack any explicit threat-related material. To this end, several studies proved that trait anxiety in humans reduces

working memory (e.g. Leon and Revelle, 1985; Richards et al., 2000; Derakshan and Eysenck, 2009), as well as complex reasoning (e.g., Darke, 1988; Richards et al., 2000; Derakshan and Eysenck, 2009).

Among the first authors, who analysed the relationship between anxiety and cognitive performance (i.e. working memory) are Yerkes and Dodson (1908), who studied the effects in mice of different shock intensities on the rate of learning in a discrimination avoidance task. The Authors showed that when mice were trained in a simple visual discrimination task to avoid shock, their rate of learning improved linearly with an increase in the intensity of the shock. However when mice were trained in a more difficult visual discrimination task, their rate of learning was more efficient with an intermediate intensity of shock than with the highest intensity of shock.

These findings brought to the creation of the so-called Yerkes-Dodson Law, which essentially stated that a high level of motivation can enhance learning on an easy task and impair learning on a difficult task (Yerkes and Dodson, 1908).

Moreover, the relationship between shock intensity and performance on the task is linear (increased shock intensity produced increased performance) for the simple discrimination and nonlinear (an intermediate intensity of shock produced optimal performance) for the complex discrimination.

According to this law, for complex tasks there is an optimum course of performance that describes an inverted U function: at low activation levels are low levels of performance, while gradually with increasing activation, also increases the ability to process stimuli, but an excess of anxiety impairs the performance (Fig.6).

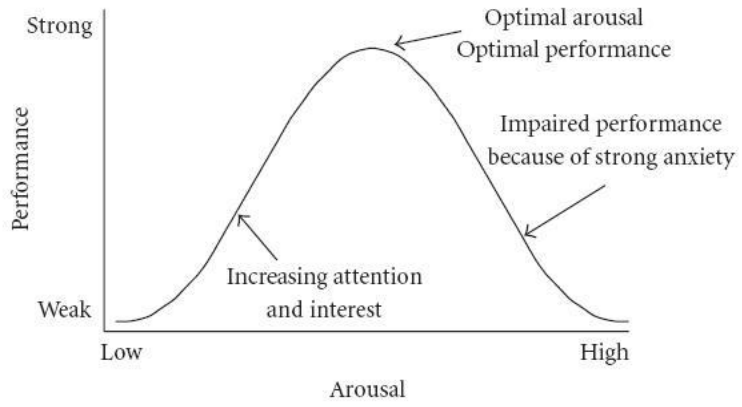

Fig.6 The Yerkes-Dodson law.

From studies in both human and rodents it has also been proved that the relationship between memory performances and circulating levels of glucocorticoids manifest the same inverted-U shape proposed by Yerkes and Dodson. As an example, in their review Lupien and colleagues (2007) reported that long term potentiation (LTP) seems to be optimal when glucocorticoid levels are mildly elevated, whereas significant decreases in LTP was observed after adrenalectomy or administration of synthetic glucocorticoids.

Several theories have been proposed to explain how anxiety may exert its influence on cognitive performance.

Processing efficiency theory (PET), suggests that the effect of negative emotions, such as anxiety, on cognitive performances may be mediated by their effect on working memory (WM), more precisely on the central executive, the component of WM that determines which information are to be made available fro conscious processing by exerting control over voluntary action (Eysenck and Calvo, 1992).

Recently, it has been proposed an updated approach, i.e. attentional control theory (ACT), which contends that anxiety manifests in an impaired attentional control (Eysenck et al., 2007). This theory is founded on the assumption that attention is regulated by two systems: a goal-directed attentional system, governed by expectations, knowledge, and current goals and exemplifies top-

down attentional control, and a stimulus-driven attentional system, sensitive to salient stimuli, and exemplifies bottom-up attentional control (Corbetta and Shulman, 2002).

Anxiety modulates the balance between these two systems, thus, an increasing anxiety leads to "…an increased influence of the stimulus-driven attentional system and a decrease influence of the goal-directed attentional system" (Eysenck et al., 2007). This imbalance is reflected in performance deficits in cognitive tasks.

From a neuroanatomical perspective it has been reported that mPFC may be one of the areas mediating effects of anxiety on cognitive performance.

Indeed, data from several reports indicate that mPFC is implicated in several processing implicit forms of memory, such as temporal order learning, the sequential arrangements of behavioural components, the direction of attention to task relevant behaviour, response selection, spatial and delayed working memory, short term memory, preference judgments, novelty encoding and formation and processing of emotional memory (Wall and Messier, 2000).

The role of mPFC in the processing of anxiety and in controlling attentional responses may also account for ACT theory. Indeed, it has been proposed that approach or withdrawal emotions, i.e. anxiety and fear according to Gray's theory, impair the ability of prefrontal cortex to organize behavior over time, this general functions include maintaining the continuity of motivation, suppression of interference and shifting of strategy (Tormaken and Keener, 1998).

6. Epilepsy

Epilepsy is a neurological condition characterized by a paroxysmal event due to abnormal and hypersynchronous discharges from an aggregate of neurons in the central nervous system (CNS). Epilepsy affects 1% of the general world population, resulting in a condition in which a person has recurrent seizures due to a chronic, underlying pathologic process. Epilepsy affects around

50 million people worldwide, and nearly 90% of them are found in developing areas (WHO Fact sheet N°999).

Epilepsy is a neuronal malfunctioning, many of the studies have been historically focused almost exclusively on the consequences on neuronal alterations, and, in particular, on the unbalance between excitability and inhibition (Holmes, 2005). TLE is often associated with a characteristic pattern of selective and extensive hippocampal atrophy, referred as hippocampal sclerosis (Meldrum and Bruton, 1992). The sclerotic hippocampus is considered to be the source of the electrical events that cause spontaneous epileptic seizures (Spencer, 1998). The indirect evidence that surgical removal of HS produces clinical improvement (Falconer and Taylor, 1968) strengthened the concept that HS itself is an epileptogenic area (Falconer, 1974). However, whether hippocampal sclerosis is the consequence of repeated seizures, or whether it plays a role in the development of the epileptic focus is still debated (Jefferys, 1999). Both clinical and preclinical data suggest that HS can be associated but not necessary for long-lasting epileptic condition.

6.1 Animal model of temporal lobe epilepsy

Temporal lobe epilepsy (TLE) is the most common epileptic syndrome in adult humans (Sanders, 2003); for this reason, the neurobiological bases of TLE have been extensively studied in preclinical research (Zhang et al., 2002), and adequate animal models paralleling human pathology are required. TLE refers to a chronic condition characterized by seizures primarily involving the temporal lobe, despite of the fact that other structures, such as the neocortex, may be the origin of the seizures (Arzimanouglou et al., 2002). In rodents, systemic administration of single dose of pilocarpine, a muscarinic cholinergic agonist, lead to status epilepticus (SE) and, after a seizure-free period, to a chronic condition determined by spontaneous recurrent seizures (SRSs).

Initially, this model has been proposed as sufficiently isomorphic with the human disease (Cavalheiro et al., 1996), but several aspects seem to differ significantly, at least concerning the extent of damage and the incidence of SE, as well as the inflammatory origin (Fabene er al., 2008). In fact, 2/3 of human patients suffering TLE presents hippocampal sclerosis, whereas the remaining 1/3 presents focal limbic lesions. This latter group does not exhibit pronounced segmental neuronal cell loss or concomitant sclerosis (Majores et al., 2004).

We recently demonstrated the occurrence of spontaneous recurrent seizures (SRSs) in rats with preserved hippocampal (and extrahippocampal) morphology and even in absence of status epilepticus (SE) (Navarro Mora et al., 2009).

Pilocarpine-induced non-neural alterations leading to epileptogenesis have been recently more clearly indicated: seizures can induce leukocyte–endothelial interactions (Fabene et al., 2008; Kleen and Holmes, 2008; Ransohoff, 2009), blood–brain barrier (BBB) leakage (Janigro, 2007) and angiogenesis characterized by a poor barrier function (Rigau et al., 2007). The role of other non neuronal cells, such as astrocytes, as critical signaling elements that contribute in the induction of neuronal death following pilocarpine-induced SE has been also clearly demonstrated (Ding et al., 2007).

Furthermore, we have provided evidences that modulating leukocyte–endothelium interaction we can reduce the SRSs frequency up to 60%, even in presence of a severe HS (Fabene et al., 2008).

These considerations indicate that we should carefully interpret the experimental data obtained in animal models of epilepsy and that neuroinflammation has a more important role in the etiopathogenesis of epilepsy than previously considered.

6.2 Epilepsy and Inflammation

Recently, it has been shown that inflammation mechanisms, such as pro-inflammatory cytokines, play a role in the pathogenesis of epilepsy (Vezzani and Granata, 2005). CNS inflammation is

associated with BBB breakdown, and BBB leakage has been implicated both in the induction of seizures and in the progression to epilepsy with chronic seizure generation (Seiffert et al., 2004; Marchi et al., 2007). In addition, BBB opening leads by itself to neuronal hypersynchronization and epileptiform activity mediated by exposure of astrocytes and neuronal cells to blood albumin or potassium ions, respectively (Seiffert et al., 2004; Ivens et al., 2007; van Vliet et al., 2007; Marchi et al., 2007). We have recently demonstrated that leukocyte trafficking mechanisms induce BBB damage leading to seizure generation in animal models of epilepsy (Fabene et al., 2008). The role of immune cells in epilepsy was further supported by the study of Kim and colleagues demonstrating that leukocyte migration through the brain endothelium breaks down BBB and causes severe seizures in an animal model of meningitis (Kim et al., 2009). In support of our work, a recent study showed that epileptiform activity is able to rapidly induce expression of adhesion molecules on brain endothelium (Librizzi et al., 2007) suggesting that each seizure may induce pro-inflammatory mediators able to activate brain endothelium, which in turn may favor the generation of other seizures.

6.3 Epilepsy and Anxiety

A frequent and clinically important comorbid disorder in patients with epilepsy is fear and anxiety (Vazquez & Devinsky, 2003; Beyenburg et al., 2005; Pauli & Stefan, 2009). Up to 50–60% of patients with chronic epilepsy have various mood disorders including depression and anxiety (Beyenburg et al., 2005), and among all types of epilepsy, temporal lobe epilepsy (TLE) is most frequently associated with ictal and interictal fear (Cendes et al., 1994; Feichtinger et al., 2001). The experience of anxiety reported by patients before or in between the occurrence of temporolimbic seizures has been attributed to activation of the amygdala and/or hippocampus (Gloor et al., 1982), which are critically involved in both the pathogenesis of TLE (Lçscher, 1998) and fear-related behavior (LeDoux, 2000).

However, less has been published on how abnormalities of the HPA axis may explain why depressive disorders have the potential to increase the risk of developing epilepsy. Abnormalities of the HPA axis have been identified for a long time in humans with MDDs (Charney et al., 1998), more recently in animal models of epilepsy (Mazarati et al., 2009), and in patients with temporal lobe epilepsy (Zobel et al., 2004). Indeed, high cortisol levels are known to be neurotoxic and thus may play a fundamental pathogenic role in the development of atrophy of temporal lobe structures. Neurons in the paraventricular nucleus of the hypothalamus secrete corticotropin-releasing hormone (CRH), which stimulates the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH, in turn, releases glucocorticoids from the adrenal gland, which have an impact on various brain regions; once in the circulation, they exert an inhibitory effect on the HPA axis. Under normal conditions, the hippocampus and amygdala also inhibit the HPA axis. High levels of CRH and glucocorticoids occur in acute and chronic stress as well as in anxiety, depression and epilepsy. At high concentrations, both hormones have been associated with damage to hippocampal formation. High cortisol levels have been associated with the development of hippocampal atrophy in animal models and humans.

In studies with rats and monkeys, dendrites of pyramidal neurons in the CA3 region retract as a reaction to stress; if the stressful event is short term, these changes are reversible. If long-lasting, the changes become irreversible through a reduction of dendritic branching and loss of dendritic spines that are included in glutamatergic synaptic inputs. Stress-induced secretion of glutamate in the hippocampus has been suggested by Sapolsky et al. (2000) as a potential mechanism of neuronal damage. These investigators suggested that chronic exposure to high glucocorticoid concentrations results in energy depletion by blocking glucose uptake in the neuron, making it more vulnerable to excitotoxicity, such as that mediated by glutamate, which is released in excess after such (and other) insult.

High cortisol serum levels resulting from chronic stress also have been found to interfere with the development of new granule cell neurons in the adult hippocampal dentate gyrus. This effect

is thought to be mediated by a decrease in the secretion of brain-derived neurotrophic factor (BDNF) in the dentate gyrus, pyramidal cell layer of the hippocampus, amygdala, and neocortex (Smith et al., 1995).

6.4 Epilepsy-Stress-Inflammation

Stressful experiences typically have short-lived neuroendocrine and neurochemical effects, but the processes leading to these biological alterations may be sensitized so that later challenges promote exaggerated responses. Audet and collegues demonstrated that as stressors and immunogenic insults have both been associated with inflammatory immune variations within the brain, social stressor would result in augmented corticosterone release and mRNA expression of pro-inflammatory cytokines (for example IL-6) within the prefrontal cortex (PFC). IL-1β and TNF-α expression enhance after the social stres challenge in mice (Audet et al., 2011).

7. Differences between human data and preclinical studies

Animal models are used as "experimental preparations developed in one species for the purposes of studying phenomena occurring in another species" (McKinney, 1984). These models are obviously particularly of help in situations when the impact of stress and/or anxiety cannot be studied in humans because of ethical and other like reasons. However, the choice of which behavioural and/or biological correlates are to be investigated is not easy, since problems with animal models of human psychic disorders include: “(i) the difference between human’s and non-human’s nervous systems; (ii) the difficulty in determining analogous behaviours among species; and (iii) the need in extrapolation of results from animals to humans” (Kalueff and Tuohimaa, 2004). Authors also stated that such difficulties reflect a significant difference in aetiology and complexity of anxious or depressive behaviours. Moreover, it has to be pointed out that the data derived from animal models are valid only to the extend that the models are

valid, and that the severity of the disorder modelled in animals may not be at the same level of the human disorder being modelled (Willner, 1997).

A useful approach to emotions, involving animal research, is the analysis of its possible functional significance, it has been claimed that "important and pervasive human action tendencies, particularly those which occur across a wide range of cultures and specific learning situations, are very likely to have their origin in the functionally significant behaviour patterns of non-human animals" (Blanchard, 1990). It is well known that most of the physiological changes related to anxiety or other emotional states, such as cardiovascular, temperature, respiratory and muscle tonicity changes are present in rodents too. Moreover, several studies investigated neurobiology of anxious and phobic states both in humans and animals (Belzung and Philippot, 2007). However, for anxiety the issue concerning the relationships between human and non-human data is particularly challenging. Indeed, it has been previously mentioned that anxiety is often thought of as a secondary emotion in which cognitive appraisal is strongly involved (Barlow, 2002).

To investigate the appraisal component in animals, a phylogenetic approach to anxiety, which examines the different facets of human anxiety and their presence at different levels of the phylum, has been recently proposed (Belzung and Philippot, 2007).

As an emotion, anxiety can be conceived as an action tendency resulting from specific appraisal of the situation. The concept of action tendency refers, "the inner dispositions (or their absence) of performing certain actions or achieving certain relational changes with the environment. In other words, an action tendency is the activation of a behavioural plan aiming at changing the individual environment relation. Impulses of "moving towards," "moving away," and "moving against" are examples of action tendencies" (Belzung and Philippot, 2007).

These behavioural responses, such as withdrawal from danger, absence of movement and reduction of non-defensive behaviours, are present in all animal species. Moreover, both in

humans and in non-human animals action tendencies could constitute a preparation of the organism.

Regarding the appraisal components it has been proposed a hierarchy of five mechanisms, called stimulus evaluation checks (SECs) (Shrerer, 1999).

The first SEC, "novelty check," looks for potential changes in the pattern of the situation. In animals this component can be detected using habituation; thus, after the repeated exposure of the animal to a new stimulation, the subject will establish that it is inconsequential, and will be able to ignore it. The second is "intrinsic pleasantness check", which evaluates the pleasantness of the stimulus or the situation, on the basis of innate feature detectors or learned associations. This evaluation determines approach or avoidance. This approach-withdrawal mechanism can be found in organisms at all levels of complexity with a different sophistication. The third SEC is the "goal/need conductiveness check", which evaluates the relevance of the stimulus for goals or needs of organism, the stimulus consistency with the state expected and if the stimulus is conductive or obstructive to animal's goals and needs. The "coping potential check" determines the cause of the event and the capacity of the organism to confront and control it. This ability is identifiable in species able to react in different ways, according to the predictability and/or controllability of the stimulus. The last SEC is "norm/self compatibility check", which consiste in evaluating the congruence of the vent and the response with social and individual norms. According to the Authors this may be the only appraisal component, which cannot observed in animals. However, it has been proposed that the presence of the first four SECs could be sufficient for a specie to experience full-blown anxiety (Belzung and Philippot, 2007).

To conclude, it is reasonable to assume that animals experience anxiety and emotional states, that need to be assessed through behavioural and/or physiological measure, which implies several problems regarding data interpretation.

The table (Table 3) represents the core symptoms of different anxiety related disorders, as reported by DSM-IV, and how these symptoms may be modelled in mice, though can be identified in rats as well (Cryan and Holmes, 2005).

Table 3. How symptoms of anxiety disorders, used in DSM-IV, might be modelled in mice (from Cryan and Holmes, 2005).

Symptoms	**How might symptoms be modelled in mice**
Avoidance of places from which escape could be difficult (agoraphobia)	increased avoidance of exposed, well-lit areas
Sudden onset of intense fearfulness, often with respiratory distress and fear of 'going crazy' (panic attack)	Increased flight from a predator
Anxiety provoked by social situations, leading to avoidance behaviour (social phobia)	Low social interaction with unfamiliar conspecific
Anxiety provoked by a specific feared object, leading to avoidance behaviour (specific phobia)	Conditioned taste avoidance
Re-experiencing a traumatic event, leading to increased arousal and avoidance of stimuli associated with the event (post-traumatic stress disorder)	Increased freezing response to fear-conditioned cue or context
Anxiety-provoking obsessions and anxiety-reducing compulsions (obsessive–compulsive disorder)	Increased marble burying and excessive grooming
Difficulty concentrating or mind going blank (generalized anxiety disorder)	Impaired sustained attention
Sleep disturbance/insomnia	Abnormal sleep architecture (measured using electroencephalogy)
Autonomic hyperarousal (tachycardia, blushing, sweating and frequent urination)	Radiotelemetric measurement of heart rate dynamics during anxiety-provocation, such as increased stress-induced hyperthermia
Flashbacks of traumatic events	Impairment in extinction of fear memory
Cognitive bias towards ambiguous or weak threat cues	Increased fear conditioning to partial threat cue
Heightened startle response, particularly in threatening contexts	Increased acoustic startle response and fear potentiated startle response
Separation anxiety	Increased ultrasonic vocalizations in pups separated from their mother
Feelings of losing control or going crazy during a panic attack	Cannot be modelled

Box 1

The issue of animal models

An animal model is a living organism in which normative biology or behaviour can be studied, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon in one or more respects resembles the same phenomenon in humans or other species of animal species (Wessler, 1976).

In the field of psychiatric pathologies, animal models are used in the attempt to reproduce some of the symptoms shown in patients, in order to understand the neurobiological mechanisms that underlie these pathologies and to develop new therapeutic approches. More in general, these models can be used to mirror a human condition, i.e. anxiety, in a way that offers opportunities to better understand its origins, course and/or treatment (Remington, 2009) and its underlying neuronal and neuroendocrinal processes (Van der Staay, 2006).

Other advantages in using animals is the fact that they can be bred, reared, maintained, and observed under standardized laboratory conditions, which allows a better scientific control over environmental influences or provide the basis for specific manipulation either genetically or environmentally.

According to Kaplan (1973) "we may say that a system A is a useful model for the system B if the study of A is useful for the understanding of B without regard to any direct or indirect causal connection between A or B". Two main concepts in the study of behavioural dysfunction using animal models are those referring to *homology* and *analogy*; the first refers to "the relationship of two characters that have descended, usually with divergence, from a common ancestral character " (Fitch, 2000) and therefore to a structural similarity between species, whereas analogy refers to a functional similarity or "the relationship of any two characters that have descended divergently from unrelated ancestors" (Fitch, 2000).

During the years, some fundamental criteria for animal models of psychiatric disorders have been estabilished, in order to consider those models valid (McKinney e Bunney, 1969; Deussing, 2006). From these parameters, the model must:

- be based on a theoretical rationale -*construct validity*-;
- be reasonably analogous to the human pathology in their symptoms -*face validity*-;
- cause of behavioural modifications that can be objectively controlled and monitored -*accuracy*-;
- produce behavioural modifications that can be reversible with the same treatments used effectively in humans -*predictive validity*-;
- be reproducible -*reproducibility*-.

Finnaly all the criteria listed before are applicable to animal tests of emotionality and/or cognition.

8. Assessment of individual differences in rodents

Symptoms	How might symptoms be modelled in mice
Avoidance of places from which escape could be difficult (agoraphobia)	increased avoidance of exposed, well-lit areas
Sudden onset of intense fearfulness, often with respiratory distress and fear of 'going crazy' (panic attack)	Increased flight from a predator
Anxiety provoked by social situations, leading to avoidance behaviour (social phobia)	Low social interaction with unfamiliar conspecific
Anxiety provoked by a specific feared object, leading to avoidance behaviour (specific phobia)	Conditioned taste avoidance
Re-experiencing a traumatic event, leading to increased arousal and avoidance of stimuli associated with the event (post-traumatic stress disorder)	Increased freezing response to fear-conditioned cue or context
Anxiety-provoking obsessions and anxiety-reducing compulsions (obsessive–compulsive disorder)	Increased marble burying and excessive grooming
Difficulty concentrating or mind going blank (generalized anxiety disorder)	Impaired sustained attention
Sleep disturbance/insomnia	Abnormal sleep architecture (measured using electroencephalogy)
Autonomic hyperarousal (tachycardia, blushing, sweating and frequent urination)	Radiotelemetric measurement of heart rate dynamics during anxiety-provocation, such as increased stress-induced hyperthermia
Flashbacks of traumatic events	Impairment in extinction of fear memory
Cognitive bias towards ambiguous or weak threat cues	Increased fear conditioning to partial threat cue
Heightened startle response, particularly in threatening contexts	Increased acoustic startle response and fear potentiated startle response
Separation anxiety	Increased ultrasonic vocalizations in pups separated from their mother
Feelings of losing control or going crazy during a panic attack	Cannot be modelled

9. Animal tests of anxiety

The growing need to understand and treat anxiety disorder and the development of new molecular techniques used in biological research (e.g. creation of transgenic and knockout animals, new anxiolytic drugs, etc.) led in the past 15 years to a drastic increase in the number studies combining molecular tools with behavioural tests of anxiety (see Fig. 7). More than 30

animal models of anxiety are currently used, some of them rely mostly on physiological (e.g., hyperthermia) or endocrine (e.g., plasma corticosterone) responses to stressors; however the majority are based on behavioural testing (Ramos, 2008; Rodgers et al., 1997).

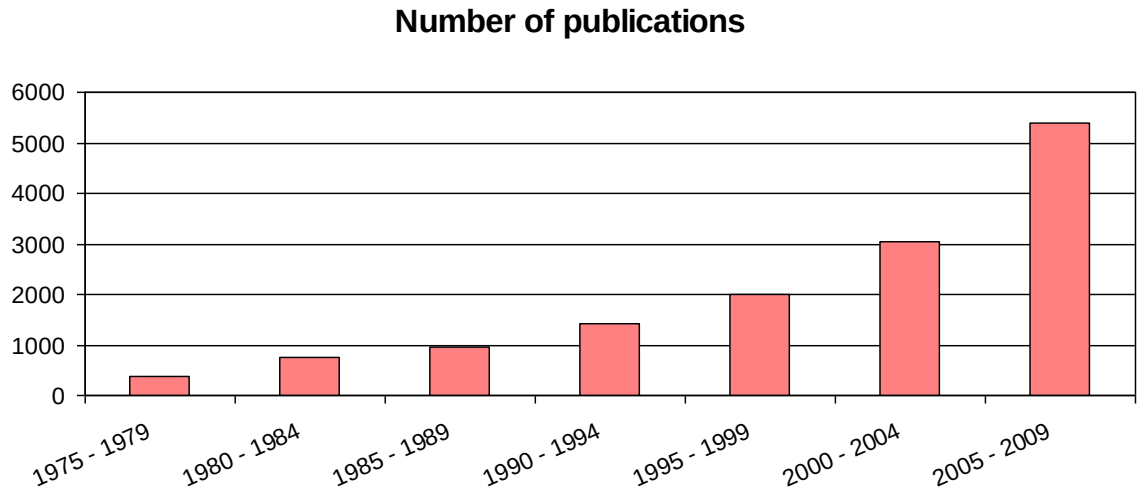

Fig.71 Number of publications. Pubmed search based on the following search syntax: ("anxiety"[MeSH Terms] OR "anxiety"[All Fields]) AND YEAR[dp]. Limits: Species: Animals; Language: English.

Behavioural tests may be classified in conditioned or unconditioned responses to stimuli capable of causing anxiety in humans. Tests involving unconditioned responses reflect a spontaneous behaviour ad present therefore a higher degree of ecological validity. (Table 4).

Since 1934, it has been first conceived the open field (OF) test (Hall, 1934), numerous behavioural tests have been created to assess emotional reactivity in rodents and the majority of them have been used to study potential anti-anxiety agents (Hanson and Nemeroff, 2009).

Most of the tests currently used to assess anxiety are still based on behaviours that depend on body activity and locomotion, which also imply that "a pure measure of emotionality, devoid of non-emotional confounding factors (e.g. motor activity), is unavailable" (Ramos, 2008). For this study with put a particular regard for Elevated Plus Maze (EPM).

Table 4 Commonly used tests and models of anxiety (adapted from Rodgers et al., 1997).

Unconditioned Responses	Conditioned Responses
Elevated plus-maze (Pellow et al., 1985)	Active/passive avoidance task (Bammer, 1982)
Open field (Hall, 1934)	Conditioned emotional response (Kamin, 1963)
Light/dark exploration (Crawley and Goodwin, 1980)	Conditioned taste aversion (Garcia et al., 1955)
Social interaction (File et al., 1976)	Vogel conflict test (Vogel et al., 1971)
Free exploration (Hughes, et al. 1975)	Defensive burying (Pinel and Treit, 1978)
Fear/defence test battery (Blanchard et al., 1986)	Fear potentiated startle (Brown et al., 1951)
Social competition (Blanchard et al., 1995)	Geller-seifter conflict (Geller and Seifter, 1960)
Holeboard test (Boissier and Simon, 1962)	Learned helplessness (Seligman and Maier, 1967)

9.1 Elevated Plus Maze

The Elevated plus maze (EPM) (Fig. 8) paradigm derives from early works on exploratory patterns, which were based on the premise that environmental novelty is able to evoke in rodents both fear and curiosity, creating the typical approach-avoidance conflict (Rodgers and Dalvi, 1997). The test was developed as a result of Montgomery's observation: using Y-mazes comprising different numbers of enclosed and open alleys, the Author noted that rats showed higher levels of exploration of the enclosed alleys and concluded that the avoidance of the open ones was due to the fact that they experienced higher levels of fear (1955).

It has been studied the effect of anti-anxiety drugs and pro-anxiety drugs using an "X-maze" which was raised 70 cm above floor level and comprises two open and two enclosed arms (Handley and Mithani, 1984).

Finally in 1985 Pellow and colleagues validate EPM as a measure of anxiety in the rat and commented upon the merits of this test, as reported by Rodgers and Dalvi (1997) : "(1) the test is fast and simple and does not involve expensive equipment; (2) it is based on spontaneous behaviour and thereby avoids lengthy training, the need of food/water deprivation, and the use of

noxious stimulation; (3) it is able to identify acute anxiolytic effects of benzodiazepine drugs; and (4) it is bidirectional sensitive to manipulation of anxiety". EPM became soon a widely used behavioural assay to measure anxiety-related behaviour in rodents.

The apparatus consisted in maze of four arms in form of a plus: two open arms, and two arms enclosed by walls. The test relies on the conflict between tendencies of rodents to explore a novel area and avoidance of its aversive features that is fear of open and elevated places (File et al., 2004), thus rodents provoked behaviour in the EPM appear to include elements of neophobia, exploration and approach/avoidance conflict, therefore is often referred as an "unconditioned spontaneous behavioural conflict model" (Wall and Messier, 2001). It still remains unclear whether the predominant anxiogenics stimuli is represented by novelty, openness or height, however reducing the height or changing light levels did not increase exploration of the open arms, while adding a clear plexiglas walls along the edge of one of the open arms did. Thus fear of open spaces seems to be the predominant anxiogenics stimulus (Treit et al., 1993). Unlike other behavioural assays commonly used to assess anxiety, this test doesn't rely upon the presentation of noxious stimuli (i.e. electric shock, food or water deprivation) and can be therefore considered an ethological way to value anxiety-related behaviour in rodents (Carobrez and Bertoglio, 2005; Rodgers et al., 1997).

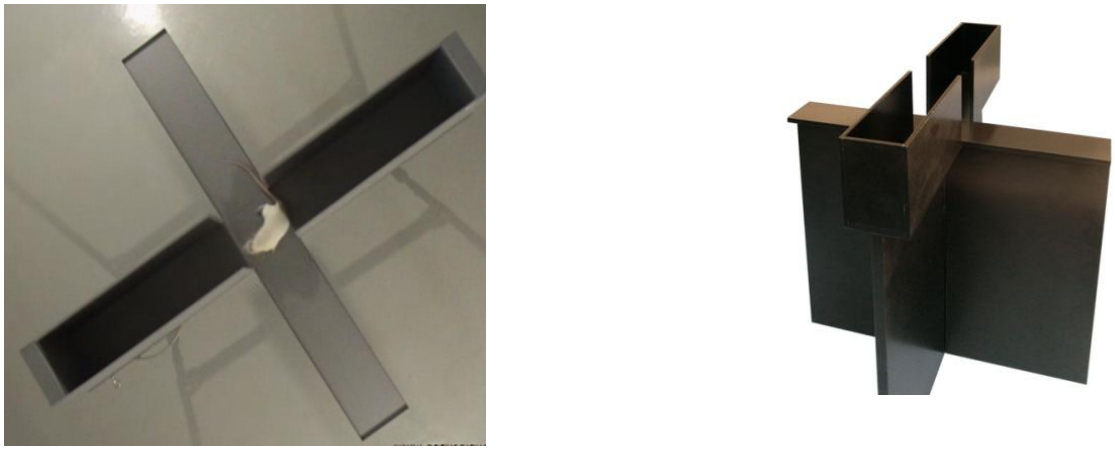

Fig.8 Elevated Plus Maze

EPM has been shown to have good face validity, for instance the anxiety or fear of open spaces seems to be measured with this test; moreover it has construct validity, which is demonstrated by the fact that anxiogenics drugs reduce the time spent on the open arms and anxiolytic drugs increase it (Pellow, 1985).

Finally EPM has predictive validity for other anxiety-related measures, which has been demonstrated for example by Frye and colleagues, who reported that rodents showing an increased open arm activity also displayed increased central square entries in a brightly lit open field (Frye et al., 2000). Furthermore plasma corticosterone is increased with open arms exposure and seems to be positively correlated with risk assessment behaviour in EPM (File et al., 1994; Rodgers et al., 1999).

With the purpose of emphasize the face validity of this model claimed that EPM has to be considered an "ethologically valid animal model of anxiety because it uses natural stimuli that can induce anxiety to human". In facts, the fear of novel, open and bright-lit open arms may resemble, agoraphobia, vertigo and xenophobia (Dawson and Tricklebank, 1995).

10. Animal tests of recognition memory

Memory is generally defined as the ability to store, retain and retrieve past events or information. However, far from being a single, isolated function, memory consist in a complex network of different interrelated functions working together to manage information. Thus, a better definition is that of "memory system", which include several subsystems (Carrillo-Mora et al., 2009).

Declarative memory supports the recollection of facts and events and the encoding of memories in terms of relationships among the elements being learned. The stored representations are flexible and can guide successful performance under a wide range of test conditions (Clark and Martin, 2005).

Recognition memory refers to the capacity to identify a previously encountered item or stimulus and is considered a form of declarative. This ability to accurately recognize an item requires the encoding of the stimulus into memory; after a delay the subject must be able to subsequently discriminate between a novel stimulus and the one that has been previously encountered (Stern and Hasselmo, 2008).

Early work on recognition memory was conducted on monkeys using the delay non-matching to sample task (DNMS). In this task the animal shall firstly displace an object in order to receive food reward, in the so-called sample phase. After a delay the object of the sample phase is presented with a novel object and the animal receives a food reward for displacing the novel object, choice phase (Mishkin and Delacour, 1975).

10.1 Novel Object Recognition

The main assumption at the base of this test is that access to novelty (e.g. an object or an environment) can elicit approach behaviours in animals. This type of behaviour was observed for example in rats that spent significantly more time sniffing a novel object than two familiar objects (Berlyne, 1950). Moreover, it has been demonstrated that rats preferred a familiar stimulus over a novel stimulus only when the environment was familiar, that is after environmental familiarization (i.e. repeated exposure to the environment) (Sheldon, 1969).

The Novel Object recognition test (NOR) (Fig.10) is based on the previously cited studies and was introduced by Ennaceur and Delacour in 1988, in order to assess the ability of rats to recognize a novel object in a familiar environment (Ennaceur and Delacour, 1988).

NOR is a delayed non-matching to sample task, that is it requires to remember a stimulus over a delay in which that stimulus is no longer present (Dudchenko, 2004).

The animal is initially placed in a cage with two identical objects and is allowed to explore them generally for 10 minutes; after a delay the animal is presented with two different objects, one previously encountered (i.e. the familiar object) and a novel one.

Memory performance in the NOR is based on the natural tendency of animal to approach and explore a novel object that have not been paired with a reinforcement stimulus. Moreover, it doesn't involve reference memory components (e.g. explicit rule learning), thus it can be considered a "pure" recognition memory test. The absence of rule learning components also imply a logistical advantage in that the task does not require extensive pre-training to teach the subject the nonmatching rule (Clark and Martin, 2005). Furthermore, the inherent variability introduced during rule acquisition is avoided.

Finally the test doesn't involve positive or negative reinforces (e.g. food, electric shocks); on one hand this facilitates the interpretation of the effects of brain modification on memory, on the other hand it makes NOR comparable to memory tests currently used in humans (Ennaceur and Delacour, 1988). Indeed, NOR can be administered to humans, monkeys and rodents essentially in the same way, humans and monkeys typically view 2-D pictures and rodents are allowed to explore 3-D objects. Several data proved that the behavioural findings have been remarkably consistent across species (e.g. see Nemanic et al., 2004; Pascalis et al., 2004; Zola et al., 2000; Clark et al., 2000; Hammond et al., 2004). Thus, an interspecies comparison is possible.

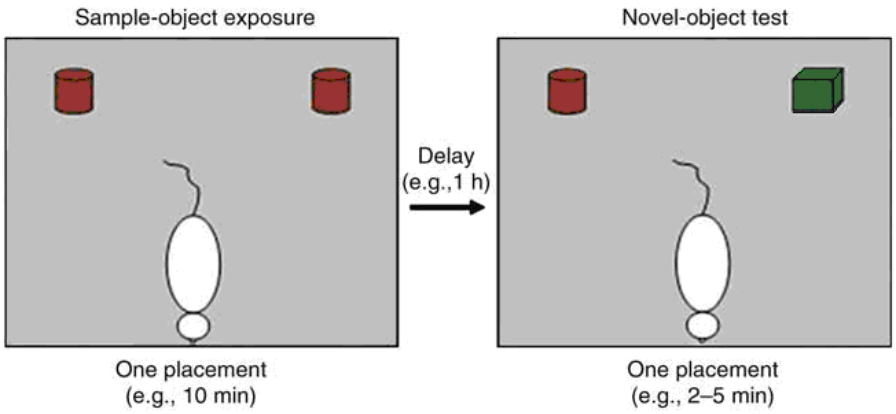

Fig.10 Schematic representation of the Novel Object Recognition test.

11. Animal tests of social stress

It has been demonstrated that social stress has a strong impact on the onset of several neurological and psychiatric disorders (Bjorkqvist, 2001).

Among all animal models of stress, models of social stress have a very important role in the preclinical study of anxiety, being very closed to the principle of "construct validity" (see Box 1).

In fact, adverse events that involve relationship between individuals, that are able to alter stability and adaptation of the subject in the own social environment, can be perceived extremely stressful for the animal.

11.1 Predator Odor Exposure

Predator odor is a stressor of particular relevance to rodents in their natural setting, as an appropriate response may ensure their survival (Blanchard and Blanchard, 2003). Predator odors elicit responses based on their perceived threat to the rodent rather than in response to a physical stimulation (Blanchard et al., 2001, 2003; Day et al., 2004). These responses appear to be innate, appearing in both pups and in adults exposed for the first time (Wallace and Rosen, 2000). The compound 2,5-dihydro-2,4,5-trimethyl thiazoline (TMT), a sulfur-containing odor isolated from fox feces, has been used to induce stress and produce behavioral responses in both laboratory and wild rodents and thus does not require any conditioning or learning to elicit a response (Soares et al., 2003; Morrow et al., 2000; Wallace and Rosen, 2000, 2001). TMT produces reliable fearful responses such as freezing, decreased exploratory behavior and diminished grooming behavior. It also consistently activates the hypothalamic–pituitary–adrenal (HPA) axis resulting in increased serum corticosterone (CORT) levels (Tanapat et al., 2001; Soares et al., 2003).

For many species, olfaction plays a large role in risk assessment (Kats and Dill, 1998). For rodents, the main predators are carnivores, including cats, dogs, mustelids, wolves, and foxes (Gillies and Clout, 2003; Glowacinski and Profus, 1997; Goldyn et al., 2003; Masini et al., 2005). Several studies demonstrate that exposure to the odor of these predators induces species-specific behavioral antipredator responses (Blanchard et al., 1990a; 2003a; Dielenberg and McGregor, 2001). Most of these studies have used feline odors to elicit defensive behaviors, including cat collars, cloths rubbed on cats, cat fur, cat bedding, cat urine, cat feces and soiled cat litter (e.g. Blanchard et al., 1990b; 2003b; Dielenberg and McGregor, 2001; Li et al., 2004; Zangrossi Jr. and File, 1992b). Some other studies have used odors from the red fox (Vernet-Maury et al., 1968). In 1980, Vernet-Maury reported that TMT is the most effective chemical constituent of the fox feces odor for inducing behavioral and autonomic antipredator responses in rats. Since its discovery, TMT has been considered by many to be a specific olfactory cue associated with the red fox. TMT was not found in analyses of the volatile constituents of dog feces (Arnould et al., 1998) or in the anal gland secretions of the dog or coyote (Preti et al., 1976). However, this compound was first isolated from cooked beef (Mussinan et al., 1977), and is also found in wheat flour extrudates (Bredie et al., 2002). Thus, TMT may not necessarily be a specific predatory stimulus to rodents, although it could represent an ethologically-relevant odor.

EXPERIMENTAL RESEARCH

Differences in cognitive functions and emotional states have been widely studied in non-human animals by comparing the performance of different strains and genetically selected rodents in learning and memory tasks (see for example Bert et al., 2004; Nguyen et al., 2003; Sik et al., 2003; Ferguson and Cada, 2004; Holmes et al., 2002). All these data showed that animals, who consistently exhibit high levels of anxiety, display poor learning and memory abilities.

However, although from an evolutionary perspective studying individual differences in outbred animal strains is closer to existing natural population, research in these animals is less common, probably due to the wider variability in the results obtained.

Indeed, few studies tested outbred animals of the same strain, gender and age and in all of these the sample was relatively small (Schwarting et al., 1998; Ho et al., 2002).

Tests to assess spatial or recognition memory performance, such as Novel Object Recognition (NOR), are widely used in many studies investigating cognitive abilities in rodents Alzheimer disease's models (e.g. Lawlor et al., 2007; Abbas et al., 2009; Peng et al., 2010) and lesioned rodents (e.g. Broadbent et al., 2009; Davis et al., 2010) but anxiety level of the animals tested has been rarely assessed.

Indeed, it has been recently pointed out that "there is a traditional dichotomy between "emotional" domains (such as anxiety and depression) and "cognitive" domains (such as memory and learning) in behavioural neuroscience" (Kalueff and Murphy, 2007).

The aim of this study is to help overcome this dichotomy.

We firstly intended to provide an evidence of the huge interindividual differences in anxiety levels in naïve Wistar rats and, secondly, to demonstrate how they can affect a widely used cognitive test. For the same reasons we intended to evaluate if interindividual differences in trait

anxiety in C57Bl/6J mice can reflect a difference in the susceptibility to the onset of a neurological disease, the temporal lobe epilepsy, and eventually the progress of the pathology.

It has been chosen a social stress test, specifically the predator odor exposure, with the purpose of mime an ecological condition of stress, closer to the natural and wild conditions in which rodents can be in nature. We used TMT (2,4,5-trimethylthiazoline), a synthetic compound extracts by anal glands of fox (and that can be found in its feces), commonly used for this type of studies.

12. Anxiety and Physiological Processes: Evaluation in Rats

Rat represent one of the most used species in biomedical research. In the scientific research is mostly used *Rattus norvegicus*. In nature rat is a night animal, commensal and omnivorous; it is able to adapt to the environmental perturbations.

Rat, by nature, is a social animal, particularly is a colonial animal: the social structure of a population of rats is based on a hierarchical order, in which is possible to identify dominant subjects and subordinate subjects.

Rats of the present study belong to Wistar strain, a strain of albino rats, that we chosen for their peculiar features of docility, their homology in behavioural response and in susceptibility to stress between different subjects of the same strain.

13. Anxiety and Pathological Processes: Evaluation in Mice

Mouse also represent one of the most used species in biomedical research. In the scientific research is mostly used *Mus Musculus*. Most of the behavioural characteristics in nature of mice are common with rats.

The study of the influence of anxiety on a pathological process, the temporal lobe epilepsy, lead us to choose a mouse model instead a rat model, because of the strong effort that this kind of

model needs. Our choice allowed us to keep and observe a larger population of epileptic animals, but also it will allow us to continue the future research in this field with genetically modified mice, since C57Bl/6J is the background strain for any kind of genetical modification.

MATERIALS AND METHODS

All the procedures and protocols of these experiments received authorization from the Italian Ministry of Health, and were conducted following the principles of the NIH Guide for the Use and Care of Laboratory Animals, and the European Community Council (86/609/EEC) directive.

14. Anxiety and Physiological Processes in Rats: Experimental Design

182 male Wistar rats, were housed two per cage 2 weeks before the beginning of the experiment, in order to habituate them to the experimental environment.

Firstly EPM was performed to screen their anxiety-like behaviour to provide information about the basal anxiety level. After the testing session body weight of all animals was measured and animals were then placed in individual cages (42.5 x 18.5 x 26.5 cm).

The testing session lasted three days; 20 animals were tested each day in order to test animals in the same day period (i.e. 9.00 – 13.00 h). Novel Object Recognition test was performed 10 days after EPM was finished, in order to allow animals to return to their pre-EPM anxiety level. Successively, a subgroup of animals (n=60), belonging to different profiles of basal anxiety, were tested in NOR during a 3 days testing session, 20 animals each day, in the same day period (i.e. 9.00 – 13.00 h).

14.1 Subjects and housing

182 two-months-old male Wistar rats (Harlan Laboratories, Milano, Italy), weighting 250-275g at the beginning of the experiment, were used for EPM testing, 60 of them were later submitted to the NOR test.

Subjects were housed in transparent polycarbonate cages (42.5 x 18.5 x 26.5 cm), with a metal grid as a cover, with with food and water *ad libitum*.

Cages were changed once a week and animal were handled in those occasions; both cages changes and handling were performed by the same experimenters. All the behavioural tests have been run in dedicated rooms, equipped by soundproofed walls (three different levels of lead-sheet in each wall), 17/h air changes, constant temperature (22°±2° C), independent air conditioning and and with a 12h/12h light/dark cycle (dark phase: 07.00 – 19.00 h). Thus animals were tested during their period of activity.

Furthermore, the ceiling and the walls of the EPM room are black-painted, and no extra-maze cues were present once the behavioural test was running. Testing was performed always by the same experimenter.

15. Behavioural Tests

15.1 Elevated Plus Maze

The EPM is commonly used to evaluated the state anxiety in many species of laboratory animals. The apparatus used in our laboratory for albino rats is made of black polycarbonate and consists of four arms, each 50 cm long and 10 cm wide; there are two open arms, without any wall, crossed with two closed arms, with 40 cm high walls. Each arm of the maze is attached to a black polycarbonate leg 50 cm long (Fig.11).

The test consisted in a single 5 minutes long session in which the animal was firstly placed at the centre of the maze; the centre of the maze and the open arms are considered “open portion” of the apparatus. Animals were then let free to explore the arms.

After one subject has been tested and before testing the following one, the maze was cleaned with paper towels and diluited ethyl alcohol.

A video recording system was used to register behavioural testing, after which data were scored by the experimenter using software The Observer XT (Noldus Information Technology, The Netherlands).

For each subject the number of entrances in the open and in closed arms and in the centre and the time spent in each arm and in the centre were recorded. An arm entry was counted when all four paws of the rodent were in the considered arm.

Time spent in the open arms and in the centre (OT=open time) is to be considered a measure of the animal's level of anxiety, while the frequency of entries in the various arms (TE=total arm entries) is an estimate of the rat's locomotor activity.

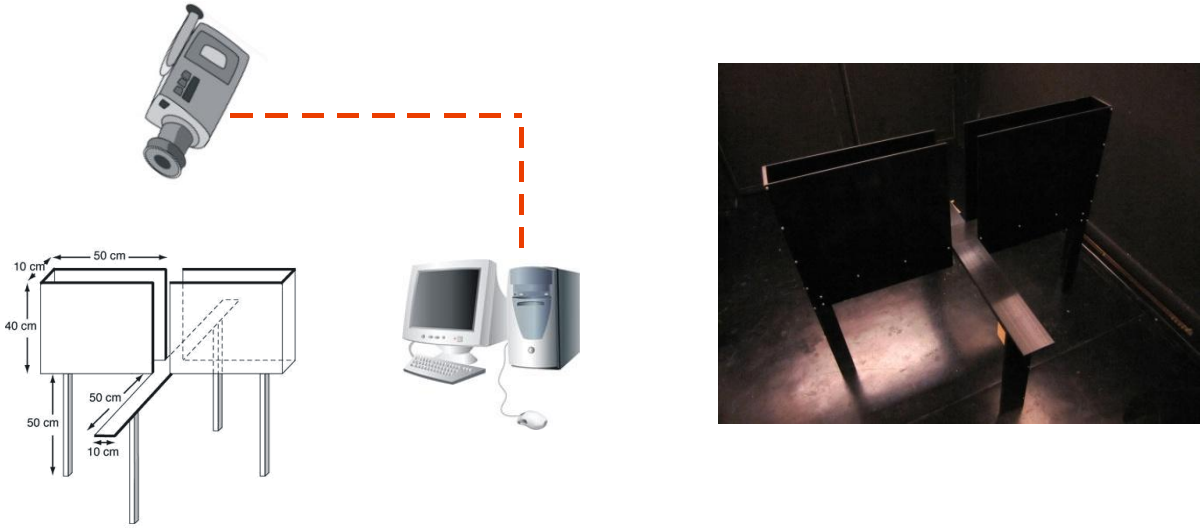

Fig.2 Schematic representation of the EPM apparatus and the recording system and the EPM apparatus used in our laboratory.

15.2 Novel Object Recognition

Novel Object Recognition test was performed using a set of four PhenoTyper chambers (Noldus) made of transparent polycarbonate (45 x 45 cm) (Fig.12a). The floor of the chamber was covered with dark brown sawdust, to allow the detection of the animals with a video-tracking system.

Two different sets of objects were used, familiar objects consisted in green plastic cubes (4 x 4 cm) (Fig. 12b), whether novel objects consisted in wooden balls (4,5 cm diameter), attached to wooden squared basis (4 x 4 cm) (Fig.12c).

a) 

b) 
c) 

Fig.12 (a) A PhenoTyper chamber and the objects used in the NOR task: **(b)** familiar object and **(c)** novel object.

The NOR task comprised a familiarization phase, a delay and the test phases. The task was preceded by an habituation phase lasting 10 minutes, during which the animal was let free to explore the apparatus without any object in it.

During the familiarization phase (T1), the apparatus contained two identical objects (the familiar objects) placed in a symmetrical position about 10 cm away from the wall, opposite to which the rat was placed. The rat was always placed in the apparatus from a hole on one wall of the box, facing the centre of the apparatus.

This trial lasted 10 minutes; after that the rat was put back in its home cage.

Subsequently, after a delay interval lasting 5 minutes, the rat was put back in the apparatus for the test phase, or novel trial (T2). During T2 the box contained two dissimilar objects, the familiar one and a new one (the novel object). This trial lasted 5 minutes.

A video recording system placed over each box was used to register behavioural testing; the times spent exploring each object, the frequency in approaching each object and the distance moved during the first and the second trial were recorded for each subject. The data were scored using Ethovision software (Noldus Information Technology, The Netherlands) (Fig.13a).

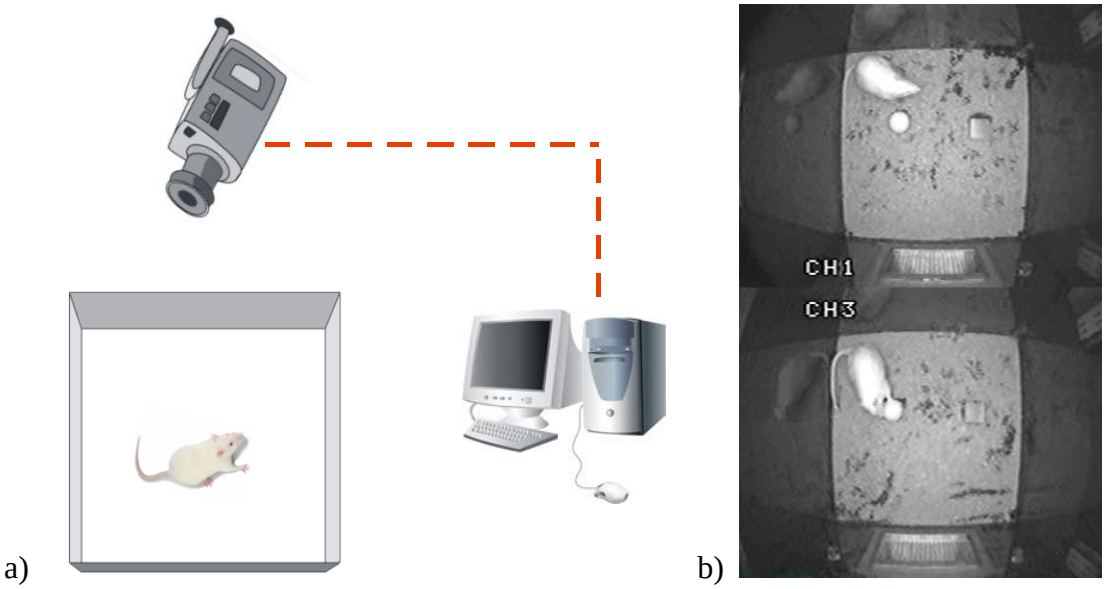

Fig.13 (a) Schematic representation of NOR apparatus (during the test phase) and **(b)** screenshot from the video recorder, rats exploring the objects during the test phase.

In order to avoid the presence of olfactory trails the objects were thoroughly cleaned after each trial. Moreover, a third copy of the familiar object was used during the test phase, in order to eliminate the possibility that the subject recognized the familiar object because of a scent-mark from the familiarization phase (as opposed to recognizing its visual characteristics). The objects are secured to the arena floor so the subject cannot displace them during exploration.

In addition, locations of the novel object (right or left) was randomized between the subjects to reduce potential biases due to preferences for particular locations or objects.

The basic measure was considered the total time spent by rats in exploring the different objects during T1 and T2. Exploration of an object was defined as entering with the nose in an area of 10,5 x 10,5 x 10,5 x 10,5 cm around the objects.

Other measure calculated were the number of approaches each animal made to the objects, discrimination difference, i.e. the difference between time spent exploring the novel object and the time spent exploring the familiar one during T2, and the average duration of an approach,

namely the total time spent exploring an object divided the number of approaches to the same object.

16. Anxiety and Pathological Processes in Mice: Experimental Design

150 male C57Bl/6J mice were housed four per cages 2 weeks before the beginning of the experiment, in order to habituate them to the experimental environment.

Firstly EPM was performed to screen their anxiety-like behaviour to provide information about the basal anxiety level. After the testing session body weight of all animals was recorded, and animals were returned in their home cages.

After two weeks, the same animals were treated with pilocarpine, a muscarinic cholinergic agent, in order to obtain a widely animal model of temporal lobe epilepsy. After the latent phase of the disease, mice were observed by the experimenter for 4 hours/day (from 12:00 p.m. to 4:00 p.m.) in their pilocarpine-induced spontaneous recurrent seizures, a behavioural parameter of chronic epilepsy.

35 of all the epileptic mice were individually tested in the Predator Odor Exposure test, in a session of 15 minutes, in which animals were recorded and observed in their behaviour.

Right after the session test, animals were returned in their home cages and observed for other 2 hours by the experimenter, in order to evaluated their behaviour after the odor exposure.

16.1 Subjects and housing

150 eight-weeks-old male adult C57Bl/6J mice (Harlan Laboratories, Milano, Italy), weighting 20-25 g at the beginning of the experiment, were used for EPM testing, were later submitted to the pilocarpine-induced status epilepticus, then were exposed by Predator Odor Exposure test.

Subjects were housed in transparent polycarbonate cages for mice, with a metal grid as a cover, with food and water *ad libitum*.

Cages were changed once a week and animal were handled in those occasions; both cages changes and handling were performed by the same experimenters. All the behavioural tests have been run in dedicated rooms, equipped by soundproofed walls (three different levels of lead-sheet in each wall), 17/h air changes, constant temperature (22°±2° C), independent air conditioning and and with a 12h/12h light/dark cycle (dark phase: 07.00 – 19.00 h). Thus animals were tested during their period of activity.

Furthermore, the ceiling and the walls of the EPM room are black-painted, and no extra-maze cues were present once the behavioural test was running. Testing was performed always by the same experimenter.

17. Behavioural Tests

17.1 Elevated Plus Maze

The apparatus of Elevated Plus Maze used in our laboratory for C57Bl/6J black mice is made of white polycarbonate and consists of four arms, each 30 cm long and 5 cm wide; there are two open arms, without any wall, and two closed arms, with 25 cm high walls. Each arm of the maze is attached to a white polycarbonate leg 50 cm long. For protocol see Paragraph 15.1.

17.2 Pilocarpine model

Systemic administration of pilocarpine, a muscarinic cholinergic agent, is widely used in mice in order to mime temporal lobe epilepsy. In particularly, pilocarpine injection induce an acute event of pathology, named *Status Epilepticus* (*SE*), in which the first epileptic convulsion and continuous seizure activity appear, usually lasting 1-2 h; a subsequent phase of pathology, named *silent period*, in which epileptogenesis generates and animals don't show any kind of pathological behavior or seizure; finally the *chronic phase* of the pathology, in which animals develope spontaneous recurrent seizures (SRSs) during the rest of life.

C57BL/6 mice were pretreated with methyl-scopolamine (1 mg/kg, i.p., Sigma Aldrich, Germany) to minimize peripheral muscarinic effects. Thirty minutes later, mice received an injection of pilocarpine (300 mg/kg, i.p., Sigma Aldrich, Germany) diluted in 0.01 M phosphate-buffered saline, pH 7.4 (PBS). Systemic administration of pilocarpine induced in mice with continuous seizure activity usually lasting 1–2 h. Behavioral observations of pilocarpine-induce seizures during SE were evaluated according to a modified version of Racine scale using categories 1–5, with 5 being the most severe. Then after a latent (seizure-free) phase of 1–2 weeks, mice go on to develop chronic epilepsy characterized by spontaneous convulsions. All mice that developed SE also developed spontaneous seizures. SE mice were observed by the experimenter every day for two weeks, for 4 hours (from 12 p.m. to 16 p.m.) during the activity phase of their circadian rhythm. The frequency of epileptic convusions was monitored online by the experimenter.

17.3 Predator Odor Exposure

Predator odor exposure, is widely used in order to induce behavioural stressful response in mice. 2,4,5-Trimethylthiazoline (TMT, Contech Entrerprises Inc., Canada), the most effective chemical constituent of the fox feces odor, is used like predator odor for mice.

35 C57Bl/6J mice, belonging to the population of epileptic mice testing before to the EPM, were tested. Mice were subjected individually to a session of predator odor protocol; subjects were put into an novel empty cage containing the odorant stimulus (a glass plate with a filter paper soaked with 35 μl of TMT) for 15 minutes; control animals were exposed to a no odorant stimulus (saline). All sessions were video-recorded and videos were observed and analyzed offline by the experimenter, in order to evaluated the manifestation of epileptic convulsions. After the session, each animal were put again in its home cage.

Fig.14 Predator Odor Exposure setup.

18. Drugs and sostances

For Pilocarpine model we used:

- Methilscopolamine: 1 mg/kg, dissolved in 0.01 M phosphate-buffered saline, pH 7.4 (PBS), and administrated intraperitoneally.
- Pilocarpine: 300 mg/kg, dissolved in in 0.01 M phosphate-buffered saline, pH 7.4 (PBS), and administrated intraperitoneally.

For Predator Odor Exposure we used:

- 2,4,5-Trimethylthiazoline (TMT): 35 µl in a filter paper.
- Saline: 35 µl in a filter paper.

RESULTS

19. Anxiety and Physiological Processes

19.1 Elevated Plus Maze Results

Given that three samples of animals were tested separately, we first checked for the homogeneity of the samples, according to their OT (time spent in the open arms and in the centre).

Both the test of homogeneity of variances (Levene statistic=0.528, p=0.591) and the one-way ANOVA ($F_{2,179}$=1.493, p=0.227) demonstrate that in the three samples the variances are homogeneous.

Total arm entries (TE) was also evaluated as a measure of locomotor activity. We performed the test of homogeneity of variances (Levene statistic=0.698, p=0.499) and the one-way ANOVA ($F_{2,179}$=0.407, p=0.666) also on this measure to assess that variances and means of the three samples were equal.

Given these results we considered the entire data pool (n=182) for the subsequent analysis.

According to their OT value animals were divided in three groups, corresponding to the subdivision of the population with the tertiles [1] high anxiety (HA) group; [2] medium anxiety (MA) group; [3] low anxiety (LA) group (Fig. 15).

TOTAL DURATION IN OPEN ARMS + CENTRE EXPLORATION

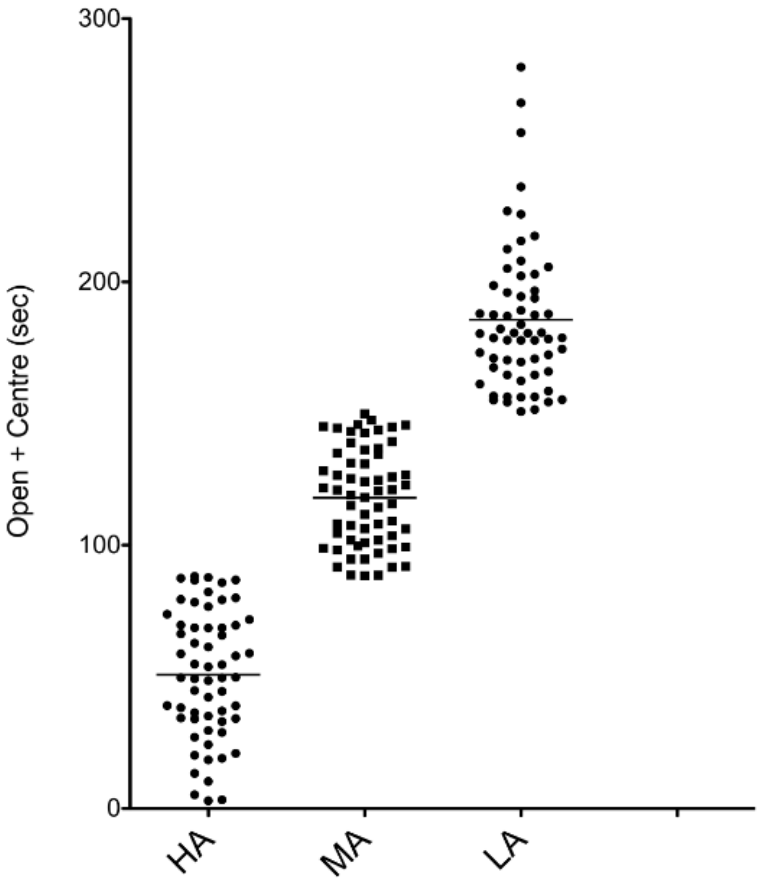

Fig.153 Distribution of the 182 subjects tested in the EPM test according to their OT (time spent in open arms and centre).

The Gaussian nature of the data distribution was further assessed the using Kolmogorov-Smirnov test (statistic= 0.049; p=.2) (Fig.16).

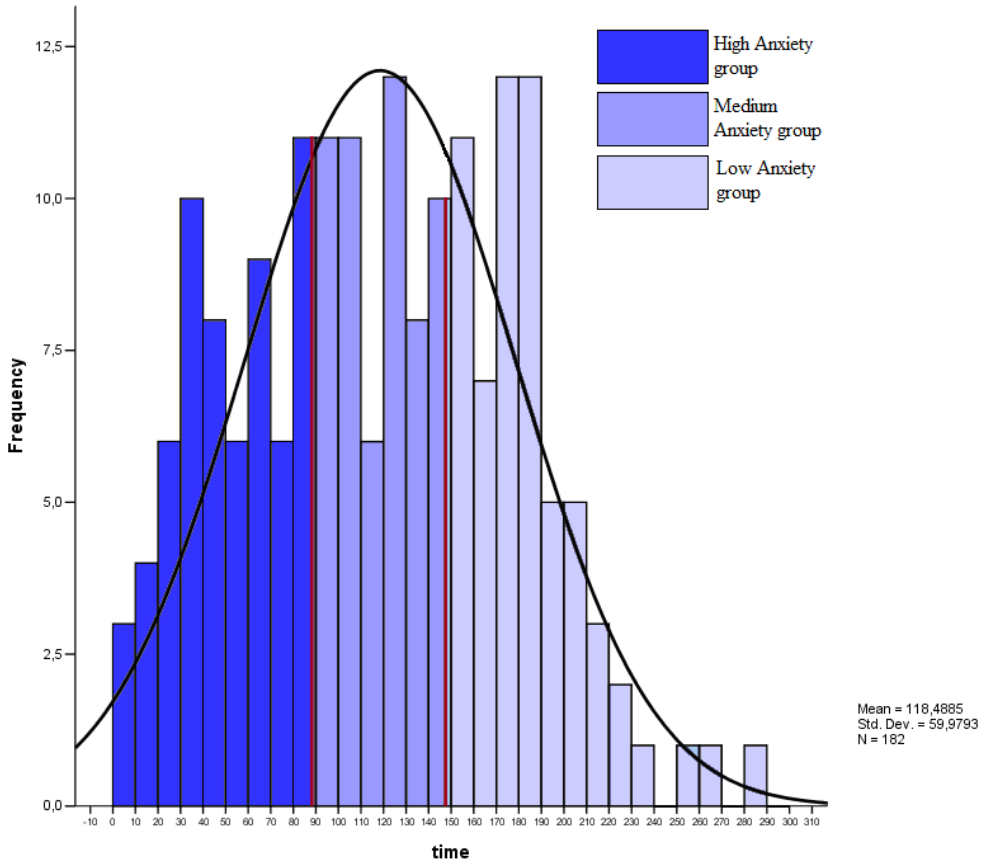

Fig.16 Histogram representing on the x-axis the time spent in the open arms (clustered in 10 seconds intervals) and on the y-axis the number of subjects spending a certain amount of time in the open arms and in the centre.

Moreover, univariate ANOVA performed on the measure of TE between the three groups revealed a significant difference ($F_{2,179}$=24.234, p=0.000).

Bonferroni's post hoc tests revealed that the difference were significant between the HA group and the MA group (p=0.00) and between the HA and LA groups (p=0.00), but not between the MA and the LA group. (See Fig.17)

TOTAL NUMBER OF ARMS AND CENTRE ENTRIES

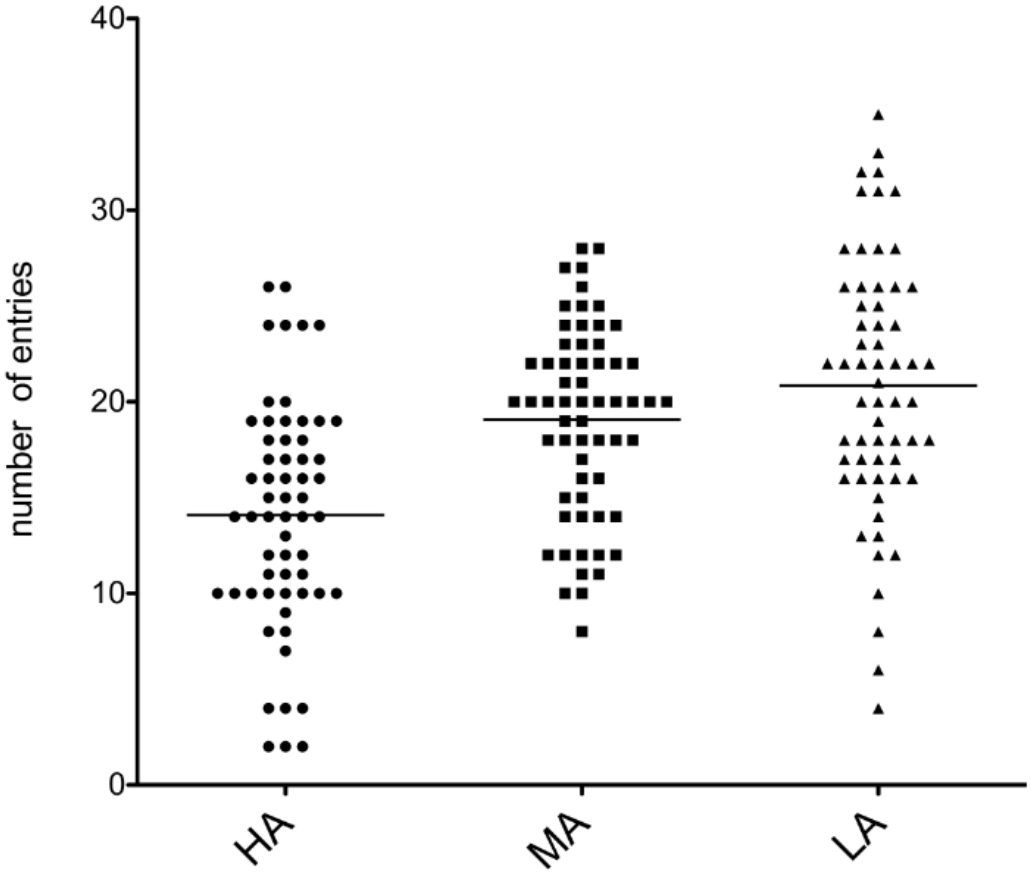

Fig.17 Means of the total number of entries in any arms by each group.

19.2 Novel Object Recognition Results

60 male Wistar rats that were previously scored with the EPM test were tested with NOR; 19 subjects belonged to HA group, 15 to the MA group and 26 to the LA group.

To prevent potential biases due to preferences for particular locations (i.e. right or left) we calculated for each animal the difference between time spent exploring the right object and time spent exploring the left object during T1 and we exclude from subsequent analysis of T2 data all animals, that differed ±1,5 DS from the mean. Overall 7 subjects were excluded, 3 belonging to HA group and 4 to LA group.

No differences were found in the total time spent exploring both objects in T1 between the three groups.

Regarding the time spent exploring the novel and the familiar object in T2, the paired sample t-test revealed a significant preference for the novel object in the MA and LA groups (t_{14}=2.54, p=.02; t_{21}=2.33, p=.03) and no preference was found in the HA group (Fig.18).

DURATION OF OBJECTS EXPLORATION DURING THE NOVEL TRIAL

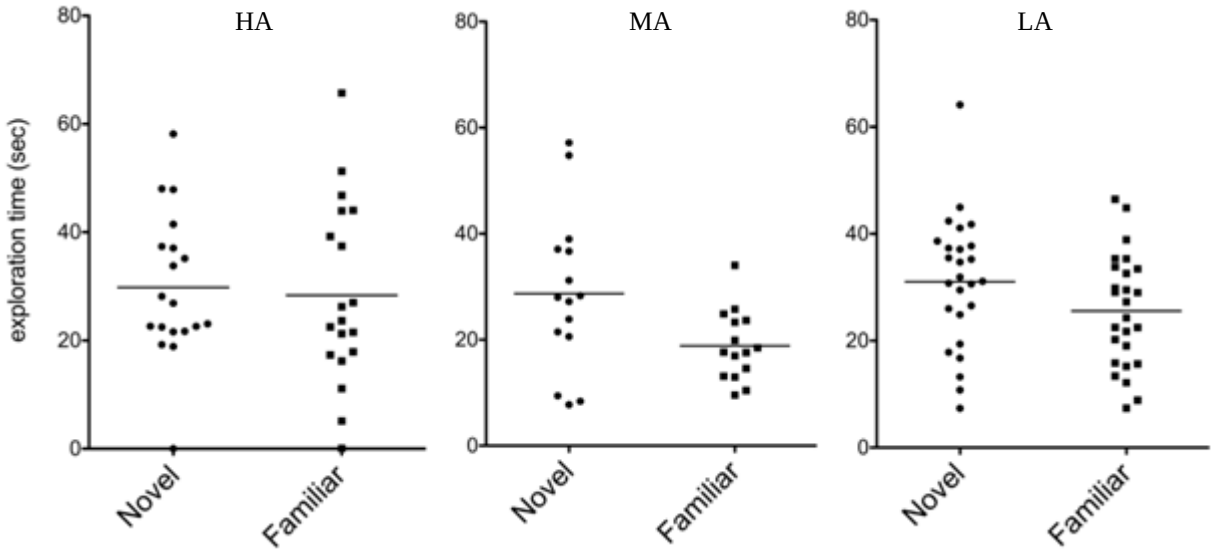

Fig.18 Means of the duration of approaches, i.e. the time spent by subjects of each group exploring the two objects during T2.

No differences were found in the paired-sample t-test on the frequency of exploration of each object in any group.

The presence of a significant difference in the exploration time of the familiar object between the groups was confirmed by the univariate ANOVA ($F_{2,50}$= 3,983, p=0,025) and Bonferroni's post hoc test, which was significant for the difference between HA and MA group (p=.02) (Fig.19).

DURATION OF EXPLORATION OF THE FAMILIAR OBJECT DURING THE TEST

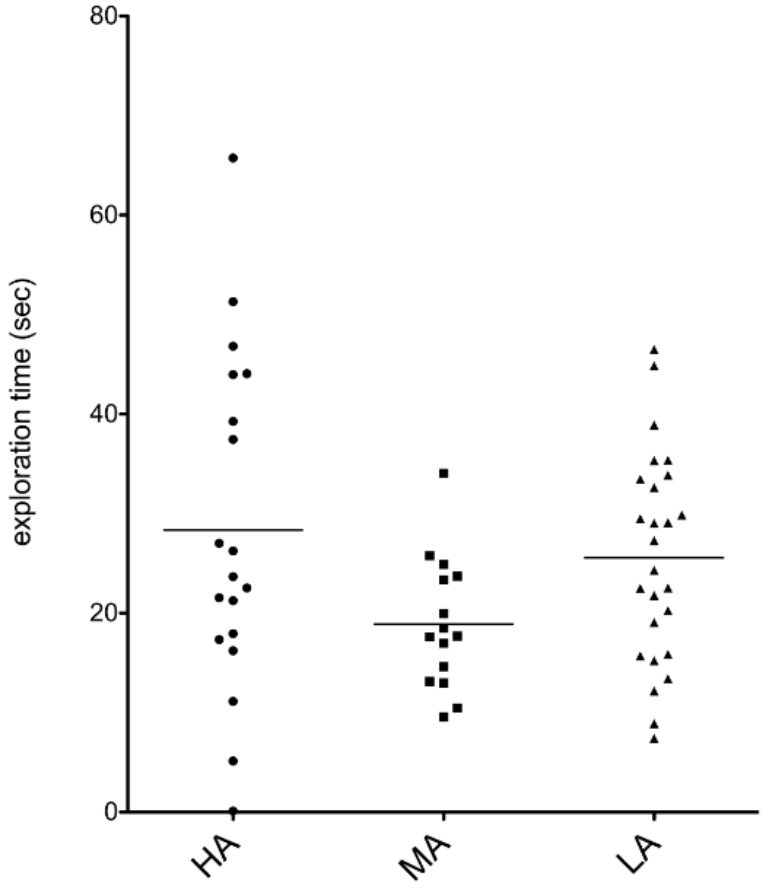

Fig.19 Mean of the time spent by each group exploring the familiar object during T2.

No differences were found between the groups in the exploration of the novel object.

Moreover, no differences were found between the groups in the frequency of exploration of both the familiar and the novel objects.

To have a unitary view of the two variables, i.e. duration and frequency, we also evaluated the average duration of an approach, thus duration of the exploration divided by frequency.

Significatively differences emerged between average duration of novel object approach and familiar one only in the MA group (t_{14}=3.05, p=.00).

No differences were found between the novel and the familiar object in the HA and LA groups (Fig. 20).

AVERAGE DURATION OF OBJECTS APPROACH

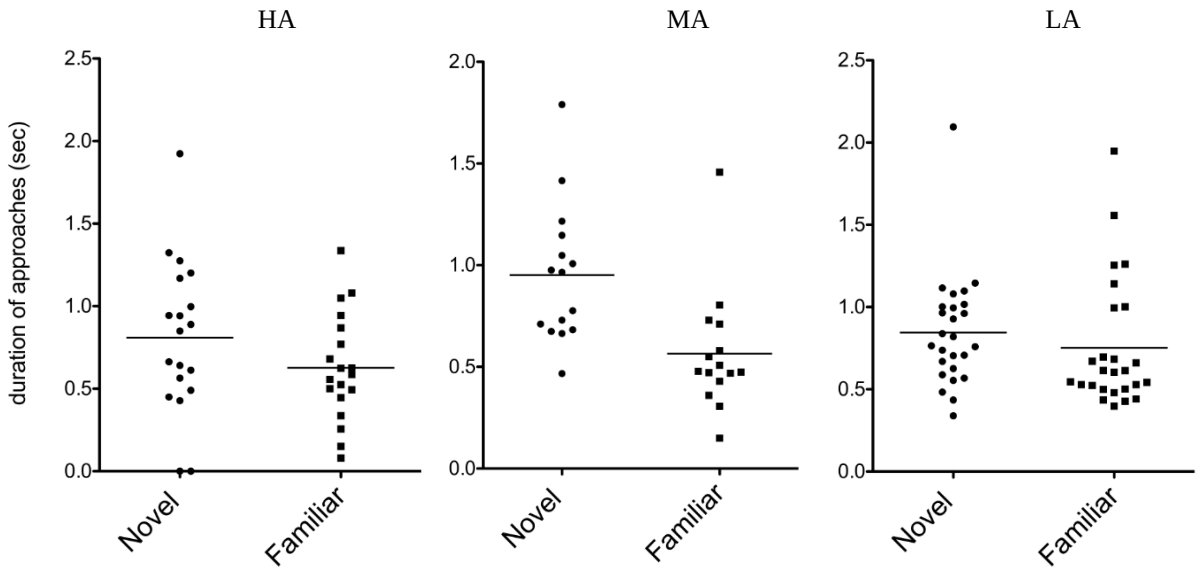

Fig.40 Average duration of novel and familiar object's approach in the three groups during T2.

Discrimination difference (d), i.e. the difference in exploration time between novel and familiar object in T2, was also assessed, as a measure of the discrimination between the familiar object and the novel object. Object recognition is reflected by a positive discrimination score value.

MA and LA groups reported a positive mean score value, whether HA group a negative score value (Fig.21). However the paired sample t-test shows only a tendency to significance (p=.073) for the comparison between HA and MA groups.

RECOGNITION DIFFERENCE

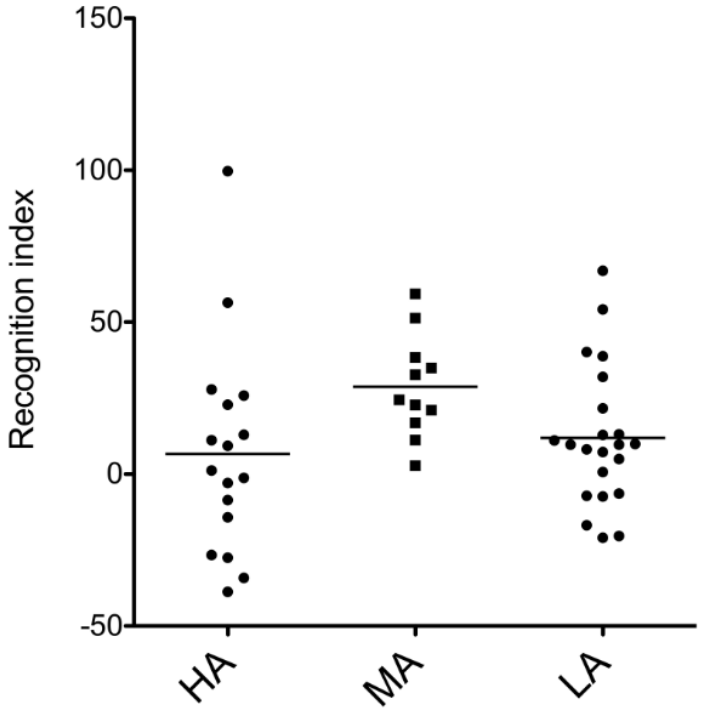

Fig.51 Mean of recognition difference value of each group.

20. Anxiety and Pathological Processes

20.1 Elevated Plus Maze Results

150 mice were screened in the Elevated Plus Maze in order to obtain their trait anxiety level. As previously related for the population of rats, time spent in the open arms and in the centre (OT) of the maze was evaluated. According to this parameter, we checked for the homogeneity of the samples, that resultated homogeneous (Levene statistic=0.843, p=0.433).

Animals were divided in three groups, corresponding to the subdivision of the population with the tertiles [1] high anxiety (HA) group; [2] medium anxiety (MA) group; [3] low anxiety (LA) group (Fig. 22).

TOTAL DURATION IN OPEN ARMS + CENTRE EXPLORATION

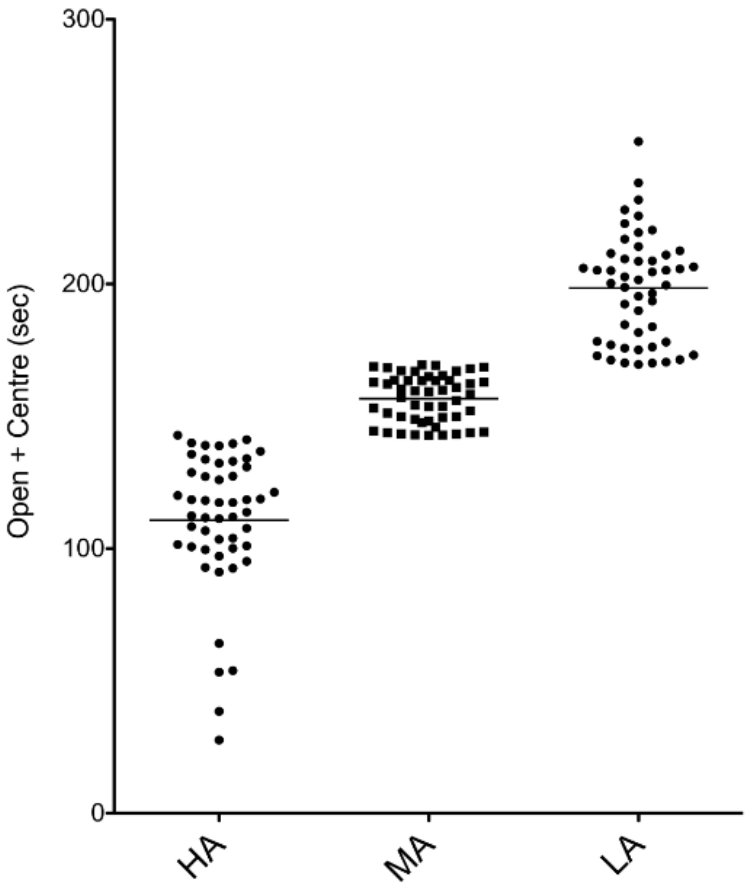

Fig.22 Distribution of the 150 subjects tested in the EPM test according to their OT (time spent in open arms and centre).

20.2 Pilocarpine Model of Epilepsy Results

The occurance of *status epilepticus* (SE) in comparison to the different traits anxiety was evaluated right after the pilocarpine administration. A number of 107 mice, in the population of 150 pilocarpine-treated subjects, developed SE (SE mice), whereas 43 mice did not develop SE and they were called resistant mice (NO SE mice). More precisely, we observed 39 SE mice and 11 NO SE mice in the High Anxiety trait group (HA group); 32 SE mice and 18 NO SE mice in the Medium Anxiety trait group (MA group); and 36 SE mice and 14 NO SE mice in the Low Anxiety trait group (LA group) (Fig. 29). No differences in the Chi-Square test were found

between the number of SE mice and NO SE mice, in comparison to different traits anxiety (p= 0,299).

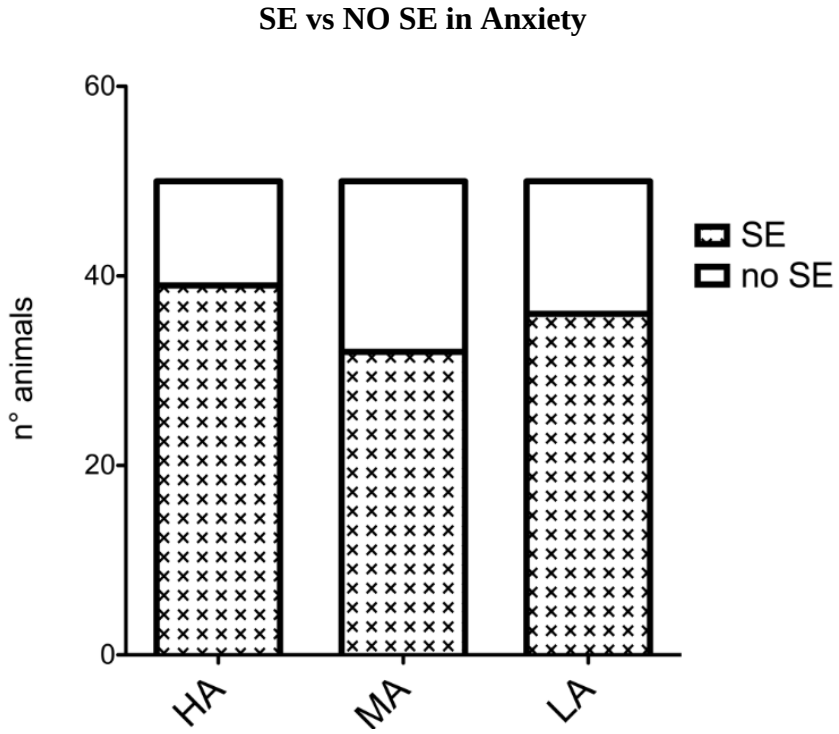

Fig.23 Number of mice that developed status epilepticus (SE mice) and not (NO SE mice) in comparison to their trait anxiety.

Considering SE mice population, we observed 8 SE survival mice, 14 SE mice died right after the manifestation of status epilepticus, and 17 SE mice died some days after the manifestation of status epilepticus (during the silent period) in the HA group; 14 SE survival mice, 8 SE mice died right after the manifestation of status epilepticus, and 10 SE mice died some days after the manifestation of status epilepticus (during the silent period) in MA group; 13 SE survival mice, 12 SE mice died right after the manifestation of status epilepticus, and 11 SE mice died some days after the manifestation of status epilepticus (during the silent period) in LA group (Fig. 24). No significant differences in the Chi-Square test were found between the number of SE survival mice, SE died right after mice and SE died days after mice, in comparison to different traits anxiety HA, MA and LA groups (p= 0,309).

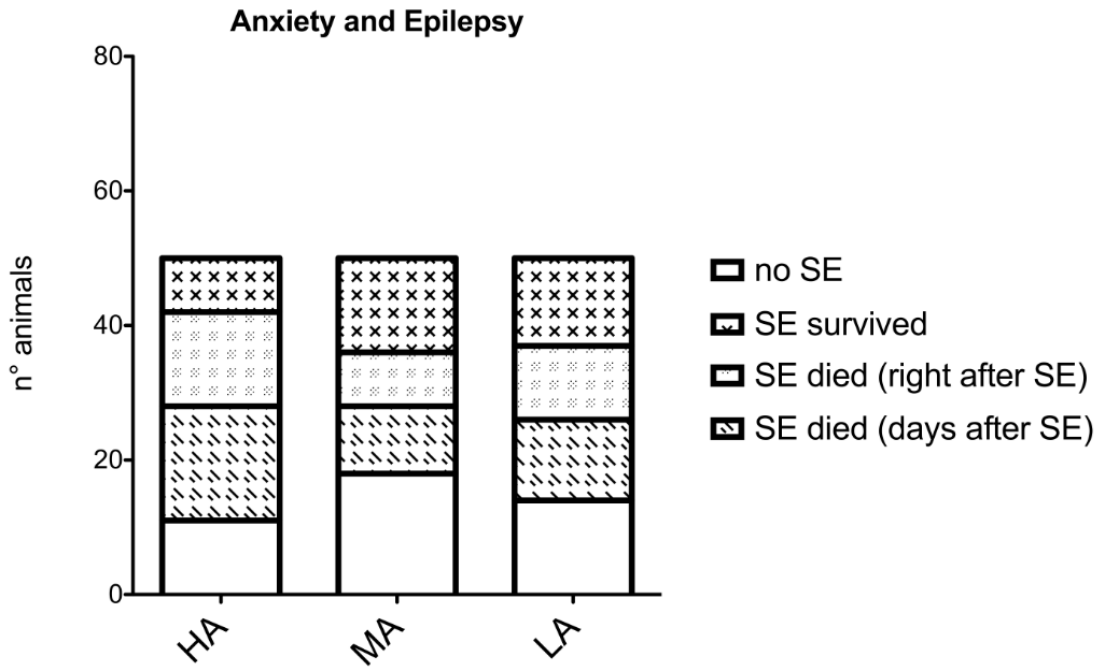

Fig.24 Different response to pilocarpine injection in mice belonging to the three different trait anxiety levels.

After the silent period of pathology, animals entered in the chronic phase of the illness. Considering only the survival mice, we observed that all mice that developed SE (SE mice) developed spontaneous recurrent seizures (SRSs), whereas mice that did not develop SE (NO SE mice) did not develop SRSs and did not show any kind of pathological behaviour. NO SE mice did not continue the study. Frequency of seizures were monitored and the mean of seizures per group were analysed. Mice of HA group showed a mean of 3,25 seizures; mice of MA group showed a mean of 2,71 seizures and mice of LA group showed a mean of 2,38 seizures (Fig. 25). We did not observe any significant difference between the mean of SRSs and the level of trait anxiety, as the one-way ANOVA ($p= 0,653$) and Bonferroni's post hoc test (HA vs MA $p= 0,535$; HA vs LA $p= 0,865$; MA vs LA $p= 0,329$) confirmed.

Mean of SRSs vs Anxiety

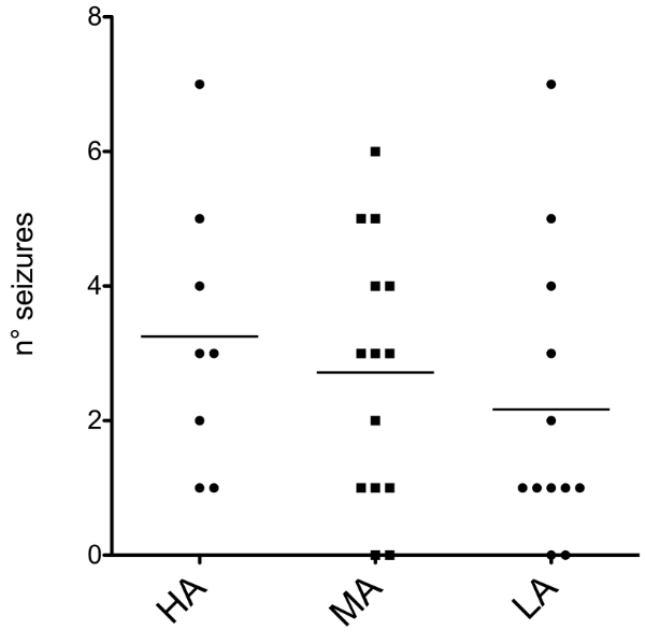

Fig.25 Correlation between the mean of the number of seizure and trait anxiety.

After two weeks of behavioural observation of seizures, SE mice were divided into two groups and tested in the Predator Odor Exposure Test. 18 mice were exposed to TMT predator odorant stimulus (TMT mice), and 17 mice were exposed to saline (CONTROL mice), for 15 minutes. After that session, animals returned in their home cage and they were observed again for other two hours by the experimenter, in order to monitored the frequency of epileptic convulsions. During the Predator Odor session, animals did not show seizures. During the subsequent observations, 1 TMT mouse of HA group showed a seizure, 2 CONTROL mice of MA group showed a seizure, and 1 TMT mouse of LA group showed a seizure, but no significant differences were found.

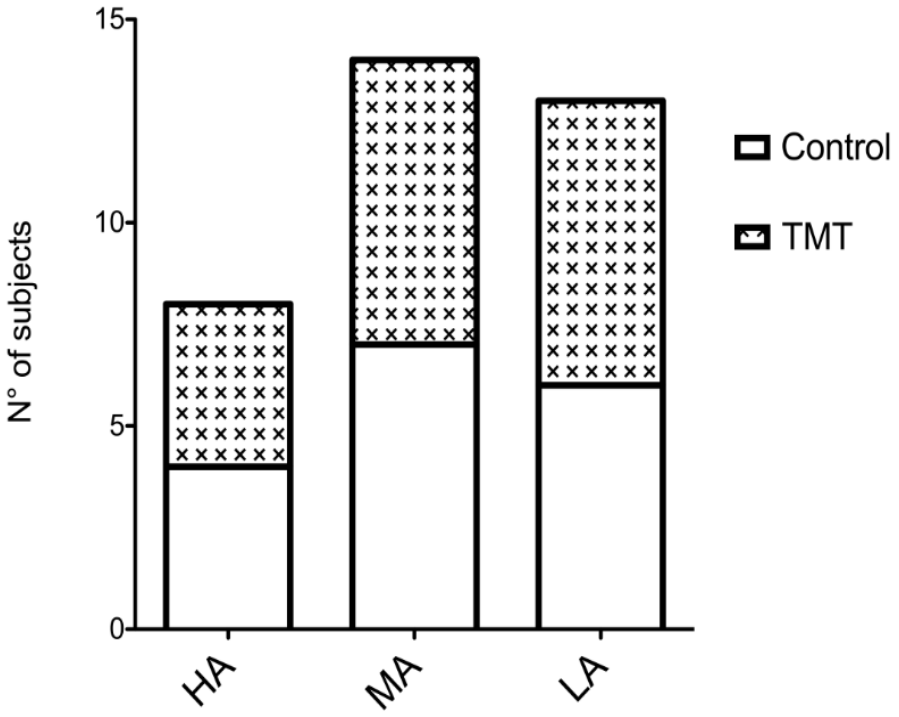

Fig.26 Number of seizures, in the two hours after the predator odor exposure, of SE mice in comparison with their trait anxiety.

DISCUSSION

In this project we firstly intended to conduct a population study on anxiety. Furthermore, we investigated whether behavioural and cognitive profiles of male Wistar rats in a Novel Object Recognition test are related to individual differences in the Elevated Plus Maze. Finally we asked whether individual differences in trait anxiety can affect the susceptibility on the onset of temporal lobe epilepsy of male C57Bl/6J mice, and eventually the progress of pathology in term of worsening of illness, measuring behavioural parameters.

First and foremost, EPM results provided strong evidence for a huge variability in anxiety level in naive animals. Rats classified as displaying an elevate anxiety proneness (HA rats) spent on average less than 17% of time in the open arms, whether animals belonging to LA group more than 60%. Moreover, rats from HA group displayed less locomotor activity if compared to the other groups. This data may be due to the tendency of HA rats to enter in closed arms and remain there and/or the presence of freezing responses.

These results are consistent with those reported by other EPM studies conducted on naïve rats, which have already shown that rats, although identical in strain, sex and age can differ systematically in their behavioural response to the maze (Ho et al., 2002; Schwarting et al., 1998).

However, by far such a wide range of subjects, i.e. 182 animals, has never been tested. Thus, not only we proved further demonstrations that behaviourally relevant differences in anxiety levels can be observed even within a sample of rats identical in age, gender and strain; but also the size of our group may help in giving a complete picture of possible individual differences in anxiety levels.

Interestingly, comparing the results with data derived from testing HAB (High Anxiety Behaviour) and LAB (Low Anxiety Behaviour) rats, i.e. selected strains for extreme trait

anxiety, with EPM, it emerges that the performance of these strains is similar to that of the HA and LA groups we identified in our study. It has been reported that LAB rats spent more than 60% time in open arms, whether HAB rats less than 20%, very similar results were obtained by Landgraf (reported by Salomé et al., 2002).

It must be taken into account the presence of a great variability in performing behavioural tests between different laboratories.

However, it is clearly demonstrate that animal researches on basal anxiety levels can be conducted using naïve animals, instead of selected strains, minimizing all the other confounds.

In facts, it has been pointed out that the study of selected strains implies several potential confounds. For example they reported that, whereas HAB rats, show a positive relationship between hippocampal volume and anxiety, NAB (Normal Anxiety Behaviour) rats, i.e. normal rats, display a negative relationship (Kalish et al., 2006). Authors claimed that, because of the co-segregated biological differences, these animals also differ in depression, locomotion and exploration.

It has been reported that individual differences in EPM behaviour in this sample can predict behavioural outcome in other tests of anxiety, such as object burying and active avoidance paradigms, but not in models of depression, such as forced swim test (Ho et al., 2002).

A challenging issue is the one regarding the distinction between trait and state anxiety in animal testing. Our intent was to have a measure of anxiety proneness in rats, thus, trait anxiety.

Tests for animal models such as Elevated Plus Maze, Open Field and Light-Dark Box compare animals with an anxiety provoking situation. Therefore, they are considered as modelling state anxiety.

Except for studies on selected strains, the only test that has been proposed as an animal model of trait-anxiety is the free exploratory paradigm (Griebel et al., 1993). In this test, animals are given the opportunity to freely move around within an environment containing both familiar and novel parts. As the animals have a choice between novelty and familiarity, it is expected that

individuals with low trait anxiety would exhibit a preference for novelty whereas high trait anxiety subjects would prefer familiarity.

However, this test is not completely free from anxiety provoking stimuli. The presence of a novel environment can be considered as potentially threatening. Indeed, even the Authors claimed that this approach allows the evaluation of neophobic responses.

If we consider trait anxiety as a permanent disposition without a specific stimulus to elicit anxiety response, this is not the case of free exploratory paradigm.

Therefore, current behavioural tests only measure state anxiety and are thus considered inadequate for modelling a persistent human conditions (Lister, 1990). In addition, as previously noticed, genetic models imply several confounds.

Thus, in animal research on anxiety it could be useful to considered emotional states and traits just as two sides of the same coin (Lazarus, 1991).

An anxious individual would be anxious more often and more intensely than others. Moreover, A-trait was originally defined as a predisposition to perceive non dangerous circumstances as threatening, and to respond to these "with A-state reactions disproportionate in intensity to the magnitude of the objective danger" (Spielberger, 1966).

Consequently, to measure anxious trait we need to assess how often and intensely the rats experience anxious state. A high trait anxiety would correspond to an inner disposition to react more anxiously in anxiety-related tests, when compared to the other animals (Ramos, 2008).

NOR data clearly demonstrate that individual anxiety levels significantly affect cognitive performance. Indeed, the measures of duration of each object exploration, average duration of approaches on the total duration of the test and discrimination difference were concordant, showing that MA and LA rats were able to discriminate between the novel and the familiar object, whereas HA rats failed to do so and spent the same amount of time exploring both.

Moreover the measure of average duration of object approaches during the test phase revealed that a clear discrimination between the novel and the familiar object occurred only in MA rats. These results suggest a trend similar to the one proposed by Yerkes and Dodson (1908). Indeed both excessively high and low anxiety seem to impair recognition memory.

It has to be point out that this data might reflect both a pure memory impairment or an impairment mediated by attentional deficits. Indeed, anxious individuals appear to have a greater tendency to explore the environment and such exhaustive scanning augments their capacity to be distracted by peripheral events and reflects an inability to maintain attention focused on a particular stimulus (Mathews et al., 1990; Shapiro and Lim, 1989).

Moreover, it is possible to explain HA group failure in objects discrimination. Indeed, it has been assumed that anxiety may act modulating the interaction between goal-directed attentional system and a stimulus-driven attentional system (Eysenck et al., 2007). Goals can be considered "representations that help to control behavior and bias how information is processed" (Gray, 2001). Anxiety seems to lead to a decreased influence of goal-directed system, i.e. detection of novelty, on behaviour and increased influence of the stimulus-driven attentional system, which implies an exploration of both objects in the same manner.

Another problem in the interpretation of results may arouse from the fact that the status of the novel object may be confused. Sometimes it has been used for assessing anxiety, and sometimes for assessing novelty seeking behaviour (Ennaceur et al., 2009). However, the main difference we found was related to the duration of the familiar object exploration, thus, no avoidance behaviours toward the novel object, which may reflect anxiety-like behaviour, were detected.

It has been already noticed that in rodents, NOR task has become particularly popular also to investigate neural basis of recognition memory.

However, by now the findings are rather mixed. For example the question regarding hippocampus involvement remains unsolved and discrepancies have been found (Broadbent et al., 2009).

As the relationship between anxiety and cognition has been extensively investigated in humans and several researches have been carried out in non-humans, one may expect this variable to be taken into account in all studies assessing memory in rodents as an explanation of interindividual variability and as a possible confound.

However, in their review on pharmacology and neurobiology of novel object recognition, it has been reported some possible explanations for discrepancies in data obtained with NOR between studies carried out in different laboratories. It is interesting to note that anxiety and/or emotional levels of the subjects are not mentioned as possible confounds (Dere et al., 2007).

To conclude, one of the first questions asked in studies on animal emotionality and affective behaviours should be "can my findings be a result of merely altered memory or learning?" (Kalueff and Murphy, 2007); we demonstrated that the inverted question should be asked too.

Regarding the population study in mice, in order to evaluate the effect of trait anxiety in a neurological disease in preclinical research, we needed to turned our attention from rat model to mouse model. This is because mouse is the most appropriate species to the development and the study of temporal lobe epilepsy. Moreover, the C57Bl/6J strain, that we used in our study, is the genetic background for any model of pathology, in particular for the genetically modified animal models. This can gave us the main reason to use mice for the second experiment of the study.

There is a strong evidence for a huge variability in anxiety level in naive mice of the same strain, gender and age;even in this case, our results are consistent because a population of 150 animals all together has never been tested and so the size of our group may help in giving a complete picture of possible individual differences in anxiety levels.

Our results showed that there is no differences between mice belonging to different trait anxiety group and their susceptibility to the epilepsy. However HA group of mice had a major number of subjects that developed status epilepticus in comparison to the other groups of mice (HA: 39

SE/50 total mice; see Fig.23); this tendency can give an idea of the strong impact that trait anxiety can have on animals.

Moreover, if we observe the mean of the number of seizures, that mice of each group showed in the chronic phase of the illness, and their level of trait anxiety, no significant differences were observed. However, in mice that spent less time in the open arm and in the centre of the EPM, the HA group, we observed a mean of 3,25 seizures, that is higher than the mean of seizures of MA group (2,72 seizures) and the mean of LA group (2,38 seizures).

Regarding the possible effect of the stressful stimulus, the predator odor exposure, on the progress of the illness, we did not find differences in the number of seizures after the exposure of TMT odor between the groups HA, MA and LA. These results can be explain by the indication that TMT induces freezing and potentiates the analgesia expressed in the presence of a conditioned fear stimulus (Bolles and Fanselow, 1980; Fendt and Fanselow, 1999, Walf and Frye, 2003). Freezing behavior, analgesia and inhibition of pain could be explained like a protection of subjects to a dangerous situation (i.e. predator exposure), in which animals must be concetrated to escape and to save themself.

In the same way, many studies have been demonstrated, over the past 25 years, that TMT stimulates autonomic and behavioral changes in rodents and induces increase in corticosterone and adreno-corticotropin hormone release in naive animals (Day et al., 2004; Morrow et al., 2000; 2002). These demonstrations provide the relevance of this model in the induction of stress behaviours, as we observed in our mice. The authors highlighted the activation of the hypothalamic-pituitary-adrenal-axis. This excessive activation of HPA axis can leand to an increased vulnerability of neuronal cells in hippocampal and frontal lobe structures, that are involved in the pathogenesis of epilepsy. The opportunity for us to monitor once again each subject, exposed to predator odor test, in the EPM test, in order to compare their trait anxiety to their state anxiety induced by TMT, was failed because pilocarpine induces some alterations in vision mechanisms; animals were not be able to perform the test in the apparatus.

Finally, we scheduled the evaluation of hormonal parameters by analysis of blood samples, and molecular evaluation of inflammatory parameters, such as dosage of cytokines, chemokines and adhesion molecules by the analysis of the brain of subjects; a technical problem in our laboratory facilities, in particular the breakdown of the freezer containing the all the samples, caused the defreezing and so the lost of all our samples. Future prospective is to reproduce this study, in small scale, in order to complete the work with molecular analysis.

In conclusion, we claim the need to consider interindividual differences in emotionality (e.g. anxiety) in general, and the need to assess anxiety level while studying rats cognitive abilities. It will be possible to include it as a covariate in the statistical analysis, in studies that schedule behavioural factors, in order to avoid interpretative errors dued to this hidden variable.

FREQUENT PROCEDURAL MISTAKES IN EXPERIMENTAL ASSESSMENT

Discrepancies in literature on behavioural assessment of emotionality and/or cognition in animals may be due to procedural differences or mistakes. For example an assumption that is frequently made is that all species/strains will display the same behaviour and/or pharmacological response to experimental manipulations (Hogg et al., 1996). However, there are several evidences of the presence of intra-strains differences (e.g. Walsh,1980, Van Lier et al., 2003; Ceballos et al., 2006). For example in the original validation of EPM, it has been reported that baseline levels of anxiety-like behaviour were different between Lister and Wistar rats (Pellow et al., 1985).

Other factors that may influence animal testing are connected with housing, cleaning and laboratories facilities.

An important factor, which may alter behaviour in several tests, especially those assessing anxiety, is social isolation. Different studies demonstrate that isolated rats are more stressed than group-housed rats (e.g. Hatch et al., 1963, Brain and Benton, 1979, Sharp et al., 2002a).

Husbandry procedures, such as cage cleanings and general health checks, are regularly carried out practice in animal laboratories. It has been shown that handling of the subjects and alteration of the cage environment may disturb animals, for example by the disruption of odour cues which are necessary for social communication and recognition (see Abou-Ismail et al., 2008).

Indeed, handling and cleaning procedure have been shown to be stressful for rats and induce short term-memory changes in a range of behavioural, physiological, endocrinological and immunological stress indicators, e.g. heart rate, arterial blood pressure, and body temperature (Sharp et al., 2002a, 2002b).

It has been pointed out that, given these common procedure appears to be stressful over short term, the point in the circadian cycle at which are applied may be important. Indeed, in many laboratories a light inverted cycle is not used, thus cage cleaning results in being carried out during the light phase of the animal, i.e. inactive period, and may result in sleep disruption and additive stress (Abou-Ismail et al., 2008)

Light-dark cycle also influences behavioural performances in the various tests, such as a water tank social interaction test in mice (Nejdi et al., 1995)

Moreover, it is important to note that prior testing and/or manipulations may exert a deep influence upon behavioural tests. Acute stressors have been reported to be influential on the behaviour exhibited by animals on the EPM; for example, electric shock, forced swim, surgical stress and saline injection all enhance anxiety. Similarly, immobilization, social defeat, and exposure to cat, cat odour, or conspecific odour reduce the exploration of the open aspects of the maze (see Hogg et al., 1996).

Finally, Dere and colleagues reported that a marked variability has been found comparing different studies using NOR and this discrepancies might be due to “(a) differences in animal housing conditions, (b) the rodent strains used, (c) the age and sex of the animals, (d) the time of day at which the experiments were performed, (e) the dimensions, shape and the illumination of the apparatus in which object recognition was tested, (f) whether rich spatial and contextual cues

were present during testing, (g) the type of objects used, (h) the duration of sample and test trials, (i) whether a sample trial exploration criterion was used or not, (j) the length of the inter-trial interval, (k) the degree of experimental pre-experience of the animals, (l) whether animals were subjected to multiple or single object recognition tests, and (m) the measure used to infer object recognition" (Dere et al., 2007).

FREQUENT INTERPRETATIVE MISTAKES IN LITERATURE

An approach of subjective experience in animals is offered by cognitive ethology (Griffin, 1976) and psycho-ethology. Animal's subjectivity is dependent on the meaning of environmental cues for each individual, constructed from interrelations between the subject and its worlds, through perception and action (Von Uexküll, 1956; Nagel, 1974). Therefore, it has been pointed out that the interpretation of behaviour should not be based on quantitative analysis of separate items, but rather on a global analysis taking into account the context in which items have been expressed (Rodgers et al., 1997; Allen, 1998).

However, interpretation of behaviour, especially spontaneous behavior, in affective terms may not be straightforward. For example approach behaviour may be interpreted as a defensive reaction toward threatening or dangerous stimuli, or a response to valued and positively reinforcing stimuli (Paul et al., 2005).

Differences in exploratory behaviour have been interpreted as caused by changes in the animal's cognitive appraisal or risk assessment (Rodgers and Cole, 1994), in the likelihood of potential predatory attack (Dawson and Tricklebank, 1996) or in the probability of agonistic contact with a territory holding dominant conspecific (Hendrie et al., 1996).

Animals of the same strain manipulated in the same way and tested through the same paradigm but by different research teams often express very differently in their behaviour (Crabbe et al., 1999). Thus, behaviour cannot be considered a linear phenomenon.

Inter-individual phenotypic variations should be been attributed to the sum of genetic and environmental effects. A parallel could be draw between human personality and the behaviour of an individual animal, both are influenced by genes, environment and their interactions (Bouchard and Loehlin, 2001). Thus, "ideally, when submitting a rodent to an anxiety test, a picture of at least one facet of its 'personality' should be obtained." (Ramos, 2008).

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