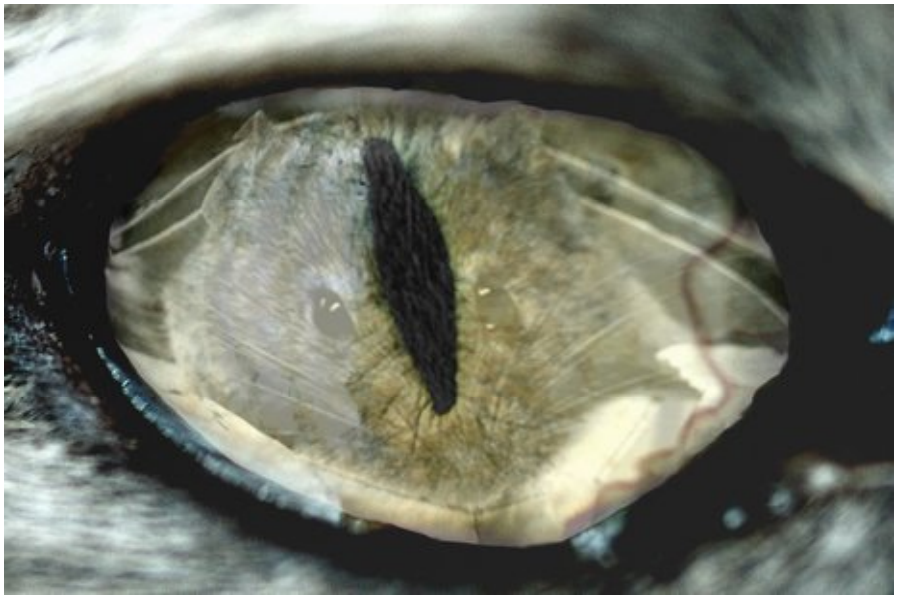
ROLE OF *NTRK3* IN THE EXTINCTION OF FEAR MEMORIES AND IN STRESS-COPING: STUDIES IN A MOUSE MODEL OF PANIC DISORDER



Doctoral dissertation

Alejandro Amador Arjona

Centre for Genomic Regulation

PhD Programme in Health and Life Sciences

Pompeu Fabra University

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A mis padres,

Enrique y Carmen

This Doctoral Dissertation has been supported by a 'Formación de Personal Investigador (FPI)' fellowship from the Spanish Government (BES-2004-3972) during the period 2004-2008.

Furthermore, behavioural and molecular biology laboratories infrastructure and materials used have been financed by Jerome Lejeune Foundation (R5046-1), Science and Technology Ministry (SAF-2001-1231, SAF-2004-02808 and GEN2003-20651-C06-03) and European Community (CEC DOCE QoL-2001-3-15 November 2000-Quality of Life).

PRESENTATION

The overall goal of our research in the group of neurobehavioral phenotyping of mouse models of disease (CRG) is the understanding of the role of putative candidate genes for human complex genetic diseases that impair the structural elements connecting the neurons with consequences on brain cognitive systems.

The aim of this thesis has been the understanding of the role of neurotrophic receptor TrkC in human panic disorder (PD) pathophysiology. Previously, our laboratory demonstrated that transgenic mouse model overexpressing TrkC (TgNTRK3) shows phenotypic alterations similar to PD (Dierssen et al, 2006), which makes it feasible to propose as a mice model for PD. Briefly, validation of the model revealed that transgenic mice show anxiety/panic phenotype, sensibility to clinically effective pharmacological agents and neurobiological mechanisms homology similar to human PD responses.

PD patients show an inability to correctly identify and possibly store fear-related information (Berksun, 1999), which propose alterations in brain regions that encode for the emotional properties of sensory stimuli and that interact with neural systems involved in attention, executive control and memory, key elements in the etiopathogenesis of PD. Moreover, PD patients experience anxiety and fear in reaction to an even/experience, which can be perceived as an external (real threat) or internal stressor, that might cause stress system dysfunctionality by increased and/or prolonged secretion of stress hormones. The contexts in which PD syndromes developed implicated a prominent role for stress and trauma (Faravelli, 1985), suggesting a possible area of causal overlap wit post-traumatic stress disorder (PTSD), another anxiety illness that often includes panic attacks.

The objectives of this thesis were to study the role of TrkC in the pathophysiology of PD through affecting neurobiological systems involved in stress and fear responses, such as hypothalamic-pituitary-adrenal (HPA) axis and limbic system. Disturbation of these systems would cause innapropiate physiological and behavioral responses, and unleash to the psychiatric pathology.

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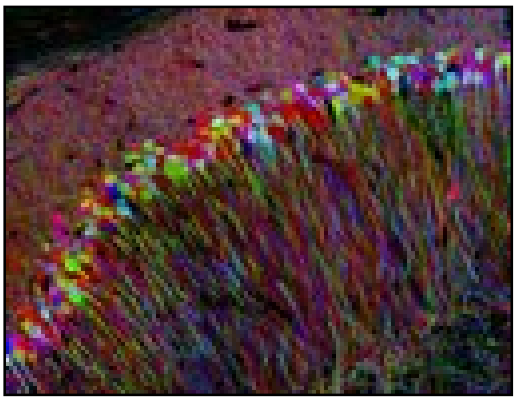
INTRODUCTION



Portion of the hippocampus from a 'Brainbow' transgenic mouse. The multicolor 'Brainbow' labeling reveals neurons of the CA1 area and their dendrites (pointing downward). Confocal microscopy by Tamily A. Weissman (Jeff W. Lichtman lab, Harvard University). Reproduced with permission of the copyright owner.

1. ANXIETY DISORDERS AND PANIC DISORDER: GENERAL ASPECTS

PD, agoraphobia and social phobia are a heterogeneous group of behavioral alterations that are responsible for a substantial burden of suffering and economic costs (Greenberg et al, 1999). PD occurs in 2-3% of the population and is associated with medical and social morbidity, including increased risk for major depression and cardiovascular illness (Zaubler and Katon, 1996). Prevalence studies have shown consistently an excess of PD in female individuals, a modal age of onset in late adolescence or early adulthood, and strong associations with both agoraphobia and major depression (Weissman et al, 1997). Moreover, PD is a stress-related disorder currently encountered in clinical settings (Holsboer et al, 1992; Roy-Byrne, 1992).

Twin studies propose a strong genetic contribution to the pathogenesis of PD with an estimated heritability of up to 48% (Hettema et al, 2001). A variety of acute treatments are effective. However, etiology is unknown, and both long-term course and management of associated morbidity remain problematic. The military contexts in which PD syndromes developed implicated a prominent role for stress and trauma, suggesting a possible area of causal overlap with PTSD, another anxiety illness that often includes panic attacks.

PD is defined by the fourth edition of Diagnostic and Statistical Manual of Mental disorders (DSM-IV) and the International Classification of Diseases 10 (ICD-10) as a disorder that requires the presence of recurrent sudden and unexpected panic attacks, along with worry about the possibility of future panic attacks or development of phobic avoidance (i.e., staying away from places or situations in which the individual fears could elicit a panic attack). Panic attacks are perceived to be uncontrollable and are accompanied by somatic (Stein et al, 1992), affective (Zhang and Barrett, 1990) and cognitive alterations (Clark, 1986). A panic attack is a sudden and unexpected surge of extreme fear accompanied by major neurovegetative changes, which lasts for about 20 min. The extreme form of avoidance is known as agoraphobia, in which case the patient is afraid of leaving home unaccompanied, becoming incapacited for most social requirements.

A PA is a rapid increase of emotionally intense anxiety (expressed as fear/discomfort, as defined by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition [DSM-IV] criteria) that peaks within 10 minutes. Additionally, at least 4 of 13 associated symptoms must co-occur for the event to be qualified as a panic attack. These symptoms can be divided into psychological and somatic. Psychological symptoms include fear of dying, dissociation (derealization/depersonalization), and concerns of behaving inappropriately (loosing control or "going crazy"). Somatic symptoms include cardiac (palpitations, chest pain, dizziness), respiratory (shortness of breath, choking, paresthesias, and hyperventilation), gastrointestinal (nausea), motor (trembling), and autonomic (sweating, chills) symptoms (APA 1994).

Family and twin studies of PD and phobic disorders have provided consistent evidence that these phenotypes are familial and heritable (Horwath et al, 1995). PD is one of the most impressive stress-related disorders currently encountered in clinical settings (Holsboer et al, 1992; Roy-Byrne,

1992). The aetiology of PD is of relevance to this particular study. PD invariably imparts anticipatory anxiety (Markowitch et al, 1989; Roth et al, 1992) and is accompanied by autonomic activation and hypervigilance (Gorman et al, 2000). In the majority of cases, PD is also associated with agoraphobia.

Panic results partly from the individual's inability to develop adequate coping strategies in response to an unexpected stressor; concomitantly, a significant increase in the activity of the HPA system can be observed (Breier et al, 1987).

2. PANIC DISORDER: POSSIBLE AETIOLOGY

Even though several factors contributing to the development of PD have been identified, our understanding of the aetiology and pathophysiology of anxiety disorders, including PD, is still limited, several reasons account for this: a) genetic and social predisposing factors, and b) the neural circuitry involved in the regulation of anxiety and panic is complex (Figure 1), and it is difficult to attribute the development of PD to pathology of a discrete brain area or system, including various brain regions such as the cerebral cortex, hippocampus, amygdale, hypothalamus and the brainstem.

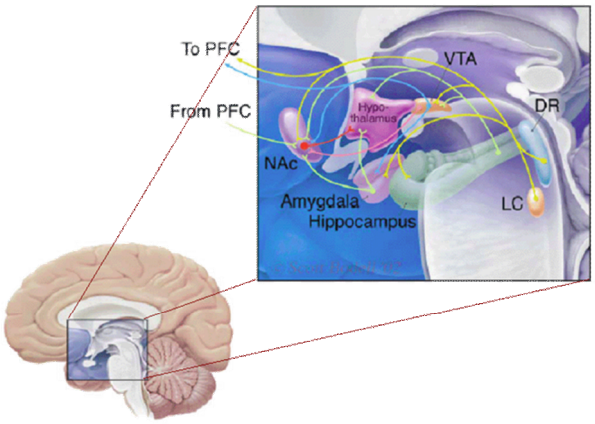


Figure 1. Neural circuitry of anxiety.
The figure shows a highly simplified summary of a series of neural circuits in the human brain, which may contribute to anxiety symptoms. Apart of the cortical areas such as hippocampus and prefrontal cortex (PFC), there are several subcortical structures implicated in reward, fear, mood and motivation, which are critically involved in anxiety, such as the locus coeruleus (LC), amygdala and hypothalamus. The figure shows a subset of interconnections between these brain regions, and their innervation by monoaminergic neurons. noradrenergic neurons of the LC and serotonergic neurons of the dorsal raphe (DR) innervate all the regions shown in the figure. The ventral tegmental area (VTA) provides dopaminergic input to the nucleus accumbens (NAc), amygdala, PFC, and other limbic structures.

Reproduced from Nestler et al (Neuron 2002; 34:13-25).

It is noteworthy that no single hypothesis, model, or "panic circuitry" can possibly account for hetereogeneous clinical presentations, variations of panicogen responsivity, the frequent complexity of treatment response, or the variable clinical progression of PD and its related complications.

2.1 Brain regions with a key role in the aetiology of panic disorder

Since many years ago, an altered noradrenergic activity within the locus coeruleus (LC) has been postulated as the starting point of the neuroanatomical model in PD (Figure 2) (Gorman et al,

2000). This model connects the three components of the disease (the acute panic attack, anticipatory anxiety, and phobic avoidance) to three concrete sites in the central nervous system (CNS) (i.e., the brainstem, the limbic system, and the prefrontal cortex (PFC)). Hypothalamic corticotropin-releasing hormones (CRH) neurons stimulate and modulate noradrenergic activity (Nisticò and Nappi, 1993) and act as a mediator in stress-induced increase in the LC neuronal firing rate (Valentino et al, 1991), on the other hand, noradrenaline (NA) stimulates CRH release in the hypothalamus (Gold, 1990).

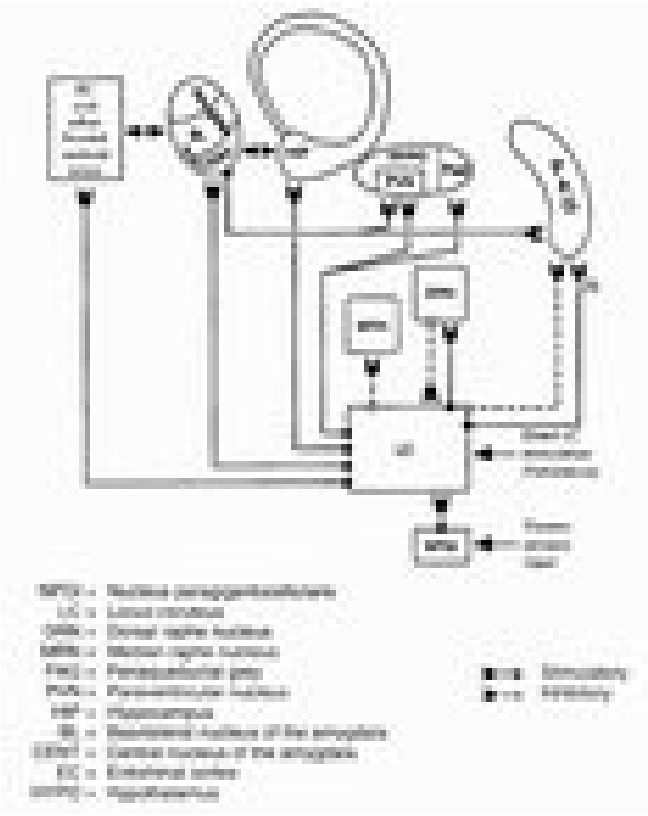


Figure 2. The LC contains the highest concentration of NA-producing neurons in the brain and sends projections to diencephalic and forebrain regions believed to play an important role in mediating fear, such as the amygdala, BNST, entorhinal cortex, PAG, paraventricular nucleus of the hypothalamus (PVN), lateral hypothalamus, thalamus and nucleus tractus solitarius (NTS). The medullary nucleus paragigantocelluaris (NPGi) sends excitatory input to the LC.

Reproduced from Coplan et al (Biol Psychiatry 1998; 44:1264-1276).

A variety of studies have raised the possibility that other brain regions in addition to the LC, that include those in or near the dorsal periaqueductal gray (dPAG), medial hypothalamus and amygdala, which are capable of inducing intense states of fear and/or terror (Coplan et al, 1998) and are associated to the 'fight or flight' response (Nashold et al, 1974). The PAG has been postulated to be activated by numerous unconditioned aversive stimuli (like an unexpected panic attack) and its stimulation provokes subjective fear of dying. Fear is also a conditioned response linked with one or more cues, and is thought to derive from stimulus-induced amygdala nuclei (LeDoux et al, 1990). Elaboration of chronic or anticipatory anxiety and expectation of negative events is mediated by bed nucleus of stria terminalis (BNST) (Davis, 1992). Finally, amygdalohippocampal pathway has been proposed to serve in determining the magnitude of threat from a conditioned aversive stimulus (Deakin and Graeff, 1991).

Structural neuroimaging studies using magnetic resonance imaging (MRI) and computed tomography (CT) have found reduced volumes of temporal lobe (Uchida et al, 2003; Vythilingam et al, 2000) and an enlargement of frontal lobe in PD patients (Wurthmann et al, 1997). Moreover, the volume of brainstem gray matter, comprising regions associated with the ascending reticular system, LC, dorsal raphe, substantia nigra and ventral tegmental area (VTA), was increased in PD (Protopopescu et al, 2006). Increased ventral hippocampal and decreased PFC volumes were also observed. These results are in accordance with previous work describing the brainstem involvement in PD (Gorman et al, 2000). Moreover, De Cristofaro et al. (1993) found that in comparison with healthy controls, PD patients exhibited reduced regional cerebral blood flow (rCBF) in the hippocampal area. Thus, PD patients have shown an altered hippocampal metabolism.

In recent years, a growing number of investigations on the pathogenesis of PD have been focused on the amygdala, a nuclear complex located within the temporal lobe. The amygdala is thought to play an important role in assigning emotional and motivational valence to sensory inputs (LeDoux, 2000). In PD, altered amygdala functions are suggested by in vivo structural imaging reports, showed by a smaller amygdalar volumes in PD patients than healthy controls (Massana et al, 2003). Recent studies have revealed that PD patients exhibit significantly higher levels of glucose uptake in the amygdala and other regions associated with the fear network in basal conditions (Sakai et al, 2005). Moreover, PD patients that showed improvement after cognitive-bahvioural therapy (CBT) decreased glucose utilization in most of these fear-related regions (Sakai et al, 2006).

3. IMPAIRED INFORMATION PROCESSING IN PANIC DISORDER

A general hallmark of PD patients is the misinterpretation of somatic sensations and the exhibition of dysfunctional cognition such as widespread catastrophic thinking (Clack, 1986). These symptoms suggest abnormalities of sensory and cognitive information processing. In this work, we were interested to evaluate two types of information processing, sensorimotor and emotional, in TgNTRK3 mice.

The mechanisms involved in the filtering of sensory information and attention focusing, can experimentally measured by startle reflex. Concretely, prepulse inhibition (PPI) is used as an experimental measure of sensory and cognitive information-processing mechanisms that are deficient in a number of neuropsychiatric disorders, such as schizophrenia or PD (Ludewig et al, 2005), and are characterized by a loss of the normal ability to suppress or gate irrelevant sensory, motor or cognitive information.

Much of our understanding of the neural systems that respond to emotinal information processing has come from studies using Pavlovian fear conditioning. Briefly, conditioned responses, such as freezing behaviour, are engaged through association of two stimuli, leading to the formation of a learned association.

3.1. Sensorimotor information processing in panic disorder

As previously described, PD patients show abnormalities in sensory information processing (Ludewig et al, 2002, 2005). This results supports the hypothesis that PPI deficits could reflect an inability to suppress or gate information in PD. The startle response is defined as 'an immediate reflex response' to sudden, intense stimulation' and is widely used as neurophysiologic measures of information processing. PPI, which consists to the suppression of the acoustic startle reflex when a low-intensity prepulse precedes a high-intensity startle pulse, is a normal unlearned suppression of the startle reflex.

Briefly, intense acoustic stimuli elicit the startle response, a coordinated muscle contraction from the eyelids to the extremities, with the likely purpose of faciliting the flight reaction and/or to protect the body from a sudden attack. Small changes in the sensory environment prior to the startle stimulus, called prepulses, can modify the magnitude of the acoustic startle response (ASR). Because impaired PPI is found in humans in some neuropsychiatric disorders, including schizophrenia, there is great interest in the mechanisms mediating PPI.

Patients with squizophrenia show impaired sensorimotor gating, expressed as a reduction in PPI, and reduced startle habituation (Braff and Geyer, 1990). PPI can be attenuated by administration of dopamine receptor agonists, such as apomorphine, and the dopamine releaser, amphetamine (Geyer et al, 2002). This effect can be reversed by dopamine receptor antagonists such as haloperidol and clozapine (Mansbach et al, 1988). The attenuation of PPI by dopamine agonists has been proposed as an animal model of the deficits in sensory-gating processes in schizophrenia, associated with dopaminergic hyperactivity.

The ASR is mediated by a pathway located in the ponto-medullary brainstem that has been extensively studied in rats (Davis et al, 1982; Koch and Schnitzler, 1997). Based on the short latency of the ASR in rats it was assumed that the primary neuronal pathway is composed of a small number of neurons connected serially by chemical synapses, and that this pathway is located near the primary auditory pathway. Consequently, all of the ASR circuits proposed so far include synaptic relays in the cochlear nuclear complex, in the nearby reticular formation and in cranial and spinal motorneurons (Yeomans and Frankland, 1996). These brainstem areas are modulated by a multitude of higher brain centres, including the medial PFC (mPFC), VTA, hippocampus, amygdala and nucleus accumbens (Koch, 1999) (Figure 3).

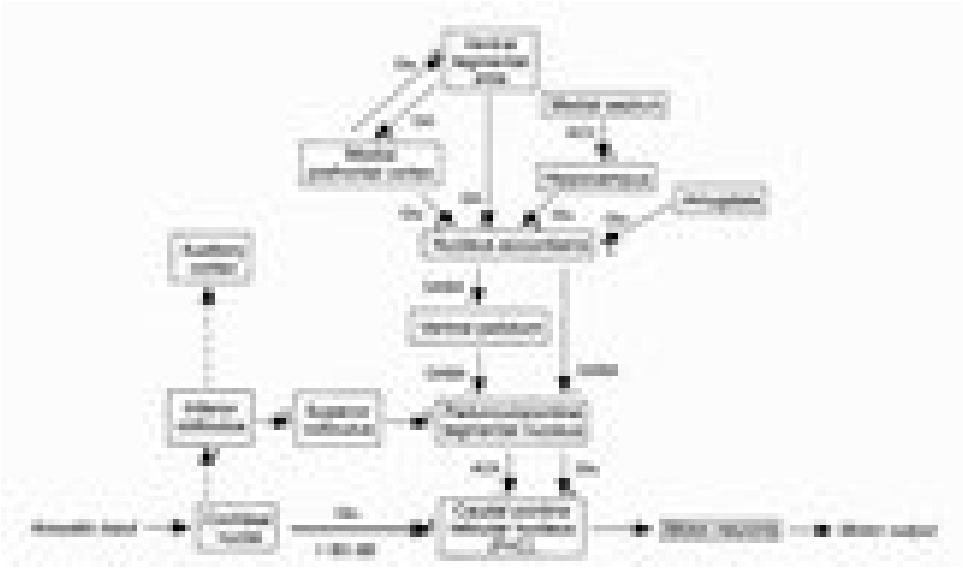


Figure 3. A hypothetical circuit mediating prepulse inhibition (PPI). In this model it is postulated that a corticolimbic-striatal circuitry modulates the primary PPI circuit via a projection to the pedunculopontine tegmental nucleus. The presumed physiological activity of the different projections is symbolized as + (excitatory) and – (inhibitory). ACh, acetylcholine; DA, dopamine; GABA, γ-aminobutyric acid; Glu, glutamate.

Reproduced from Koch et al (Behav Brain Res 1997; 89: 35-49).

The anatomical basis of deficient PPI in PD is unclear and remains to be determined. However, brain regions that are implicated in a 'fear network' in PD, in particular the amygdala, which is a critical region of dopaminergic innervation, which modulate cortico-striato-pallido-pontine circuits in PPI (Ludewig et al, 2005).

3.2. Emotional information processing in panic disorder

Fear learning is considered to be highly adaptative response to aversive events that ensures survival in changing and novel environments (LeDoux, 1995). A major goal of preclinical psychiatric research has been to identify the neural substrates underlying the pathophysiology of fear and anxiety disorders, such as PD. In this regard, the baseline state in PD is characterized by mild to moderate levels of chronic anxiety, termed 'anticipatory anxiety'.

The fear response, termed the "fight or flight" reaction by Walter Cannon, is present across species (Cannon, 1914). This fear response in the laboratory can be studied by the classic fear conditioning, that is a variation on Pavlov's experiments (Pavlov, 1927). In terms of the physiologic and behavioural responses, the fear conditioning, is probably our best current animal model for understanding the neurocircuitry relevant to pathologic anxiety, and in particular to PD.

Animal models have focused attention on the amygdala as a potential site of dysregulation in anxiety disorders (Shekhar et al, 1999), and hyperactivity within the amygdala could play a role in panic. The amygdala can also influence hypothalamic activity and cortisol release (Rubin et al, 1966). Excessive reactivity of the amygdala could contribute to panic and lead to enhanced HPA axis reactivity. However, amygdalar output is a consequence of complex inputs from multiple regions, including cortex (Shekhar et al, 1999), involving a cortical/limbic circuit. mPFC is a strong candidate

in this modulatory circuit, since it is known to process emotional salience and to provide inhibitory input to the HPA axis (Diorio et al, 1993).

To understand the mechanisms of fear memory consolidation in humans (or animals), a one-trial avoidance learning model has been widely modelled, in which it is established a conditioned response by learning to avoid danger in just one trial. Fear conditioning in animals involve the pairing of an innocuous sensory stimulus, such as a particular tone, with a noxius or aversive stimulus, such as an electric shock to the foot. The association takes place in seconds, and the onset of of memory consolidation can be determined with precision (Izquierdo and McGaugh, 2000). The initially innocuous stimulus is termed the conditioned stimulus (CS), whereas the noxius stimulus is termed the unconditioned stimulus (US). Then, the animal learns that the tone predicts the shock, and when the tone (CS) alone is subsequently presented, the animal exhibits a characteristic multifaceted response, termed the conditioned response (CR) (Bolles, 1970). Physiologic aspects of this conditioned response include increase in heart rate, blood pressure, and respiration, along with changes in the HPA axis. Behavioural aspects of the conditioned response include flight and freezing. With these changes, the animal is prepared to deal with the predicted impeding danger. However, although fear responses serve an evolutionary valuable function in protection from potential dangers, they may also be maladaptative in that any contextual stimulus can become associated with recurrent fear and anxiety.

3.2.1. The neurocircuitry of fear

Sensory information encoding the CS (the tone) and the US (the foot shock) travels to the thalamus, which is the major synaptic relay station for information reaching the cerebral cortex. Lesion, tract tracing, and electrophysiological studies collectively suggest that fear conditioning involves the transmission of sensory information about the CS and US to the basolateral nucleus of the amygdala (BLA), where they converge and become associated (LeDoux et al, 1990). The BLA is a storage site for affective information, particularly fear-related memories (LeDoux, 2000), and has been implicated in fear acquisition and consolidation.

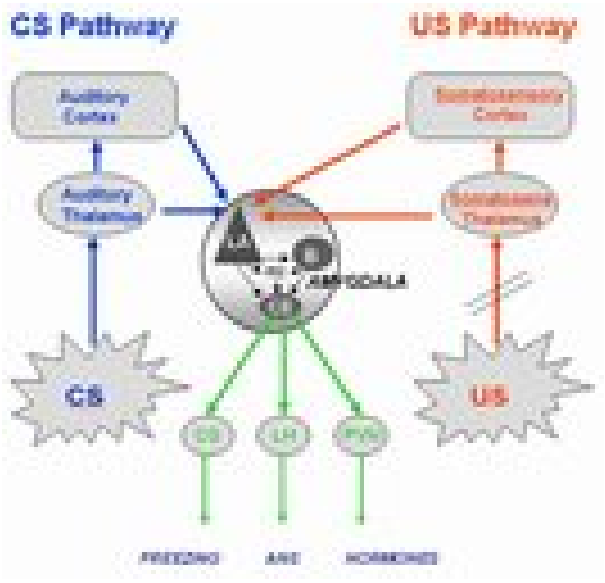


Figure 4. Neural circuit of fear conditioning. Pathways processing the CS (auditory pathways) and US (pain pathways) converge in the lateral nucleus of the amygdala (LA), which receives CS and US inputs from both thalamic and cortical areas. The LA communicates with the central nucleus of the amygdala (CE or CeA) both directly and by way of basal sunleus of the amygdala (B) and intercalated masses (ITC). The CE connects with brainstem and hypothalamic areas that control the expression of fear responses, including freezing behaviour (mediated by the central gray, CG), autonomic nervous system (ANS) responses (mediated by the lateral hypothalamus, LH), and hormonal responses (mediated by the paraventricular hypothalamus).

Reproduced from Joseph E. LeDoux (Scholarpedia 2007; 2(7):1806).

Immediately downstream from the BLA are two structures, the central nucleus of the amygdala (CeA) and the BNST (Figure 4), which project in turn, to areas that mediate the specific signs and symptoms of fear, producing an integrated fear response. The CeA is thought to play the role of an output conduit and also contributes not only to the behavioural changes in fear expression but also in learning and consolidation of fear conditioning (Wilensky et al., 2006).

3.2.2. Consolidation of fear memories

To understand fear memory cognition, it is important to understand how a learned response becomes a long-lasting memory. Consolidation relies on molecular events in the CA1 hippocampal subregion that resemble those involved in the CA1 long-term potentiation (LTP), and it also requires equivalent events to occur with different timings in the BLA and the entorhinal, parietal and cingulated cortex (Izquierdo and McGaugh, 2000). Many of these steps are modulated by monoaminergic pathways, such as NA or dopamine, related to the perception of and reaction to emotion, which at least partly explains why strong and resistant consolidation is typical of emotion-laden memories.

Molecular studies of the memory process suggest that the conversion from a labile short-term memory into enduring and fixed traces, i.e. consolidation into long-term memory (LTM), involves protein synthesis (McGaugh, 2000). Consolidation requires a sequence of specific molecular processes in the hippocampus and elsewhere that culminate in gene transcription and de novo protein synthesis (Figure 5).

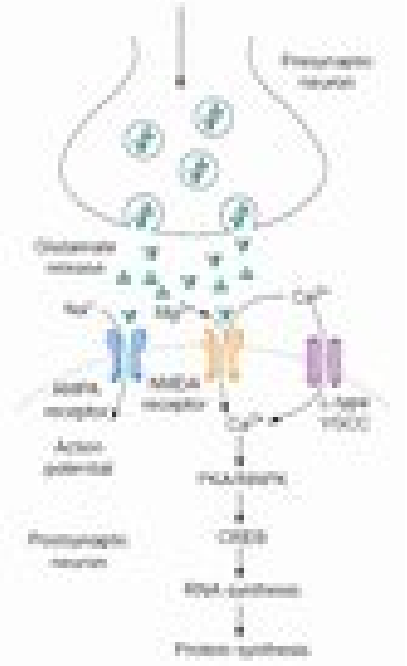


Figure 5. Biochemical and Imolecular basis of amygdala L-LTP and fear memory consolidation. L-LTP involves the presynaptic release of glutamate and Ca^{2+} influx into the postsynaptic cell through either NMDA receptors or L-type VGCCs, the increase in intracellular CA2+ leads to the activation of protein kinases, such as PKA and ERK/MAPK. Once activated, these kinases can translocate to the cell nucleus where they activate transcription factors such as CREB. The activation of CREB by PKA and ERK/MAPK promotes CRE-mediated gene transcription and the synthesis of new proteins that are critical for the ultrastructural and/or functional changes that underlie L-LTP.

Reproduced from Schafe et al (Trends Neurosci 2001; 24:540-546).

Evidences from specific receptor blockade or enzyme inhibitors into a given brain structure have shown that the CA1 region of the hippocampus is essential for consolidation of fear conditioning (Izquierdo and McGaugh, 2000). However, the hippocampus has bidirectional links with the septum, the entorhinal cortex and with other regions of the cortex and the amygdala.

3.2.3. Fear memory retrieval

Retrieval is dynamic and serves to incorporate new information into preexinsting memories (Suzuki et al, 2004). Recently, memory retrieval has attracted much attention because it has been found that inhibition of protein synthesis before or immediately after memory retrieval impairs the previously consolidated memory (Nader et al, 2000; Suzuki et al, 2004). The expression of fear memories can be diminished using two approaches. First, extinction training involves repeated nonreinforced re-exposure to the CS and results in a new memory being formed (CS-no US) (Bouton and Bolles, 1979). Secondly, the reconsolidation of a previously acquired fear memory, which is required to restabilize the memory after its reactivation through retrieval or CS re-exposure, can be disrupted to impair later fear memory expression (Eisenberg et al, 2003).

Protein synthesis dependency has been observed for both consolidation and reconsolidation (McGaugh, 2000). Because reconsolidation and extinction are dissociable both temporally and anatomically, this raises the possibility that the two processes are also dissociable biochemically. For example, pharmacological blockade of either cannabinoid receptor 1 (CB1) or L-type voltage-gated calcium channels (LVGCCs) blocked extinction but not reconsolidation. In contrast, blocking N-methyl-D-aspartate (NMDA) receptors or protein synthesis disrupts both reconsolidation and extinction.

The impairment of short- and long-term extinction processes caused by the inhibition of LVGCCs indicates that protein synthesis mechanisms downstream LVGCCs function are required for the stability of the extinction memory.(Suzuki et al, 2004). Moreover, a role for CB1 receptors in long-term extinction suggests a general role of CB1 in extinction of fear memories by selective inhibitory effects on local inhibitory networks in the amygdala (Marsicano et al, 2002).

3.2.3.1. Extinction of fear memories

Extinction refers to the repeated presentation of the CS in the absence of the US with which it was previously paired. Following a series of CS presentations, the acquired fear CRs that were established during the preceding acquisition phase gradually diminishes (Bouton and Bolles, 1979). Insights into the anatomical and biochemical basis of the extinction of conditioned fear have directly informed possible future treatments for anxiety disorders (Walker et al, 2002). Empirical support shows that extinction does not completely destroy the original CS-US association, and suggests that leaves the CS-US association intact (Rescorla, 1996). Advances to understanding neural circuitry of extinction comprise brain region seizures and protein synthesis inhibitors approaches. In this regard, destruction of the ventral mPFC, which consists of infralimbic and prelimbic cortices, blocks recall of

fear extinction (Morgan and LeDoux, 1995), indicating that mPFC might play a crucial role in the storage of long-term extinction memory.

Recent data engages the idea that extinction is not only 'inhibitory new learning' of a context-no shock association, but also involves at least some 'unlearning' of the preexistring context-shock association, supporting the idea that memory retrieval makes preexisting memory labile via protein degradation processes in order to update or reorganize the memory with new information (Lee et al, 2008).

Extinction research has been largely based on work with laboratory rodents. However, it remains an open question as to whether these findings can be generalized to humans. There are differences in brain anatomy between mice on the one hand and humans on the other. Moreover, humans are endowed with a cognitive system that is able of a symbolic and propositional analysis of conditioned experiences (Lovibond and Shanks, 2002). However, given that research has established comparable findings in human and nonhuman animals, and due to the clinical relevance of the topic, the translation of animal extinction research to human samples has received more attention lately, and extinction of the conditioned fear can be viewed as a laboratory analog for the study of exposure treatment for anxiety disorders, such as PD (Bouton et al, 2001).

The inhibition of protein synthesis in prolonged reexposures to the CS in the absence of the US, blocks the formation of the new extinction memory, leaving expression of the original memory unchanged. However, reconsolidation theories are controversial. Although some studies have shown a crucial process of post-retrieval mechanisms in LTM, other studies have failed to disrupt memory after retrieval (Cammarota et al, 2004), questioning the conclusion that retrieval results in a new phase of stabilization.

3.2.3.2. Reconsolidation

After consolidation, retrieval of a memory trace can induce an additional labile phase that requires an active process to stabilize memory after retrieval (Misanin et al, 1968). Despite its name, 'reconsolidation' is not a simple reiteration of consolidation; rather is thought that is a process distinct from consolidation, although overlap in both its function (storage) and underlying mechanisms (protein synthesis) does exist (Dudai, 2006).

De novo protein-synthesis is considered a hallmark of the consolidation process, required to render structural cellular changes permanent. For example, several transcription factors have been implicated in memory reconsolidation (Figure 6). Briefly, the roles of cAMP response element-binding (CREB), ELK1 and nuclear factor- κB (NF-κB) in reconsolidation are consistent with their previously demonstrated roles in the initial consolidation of memory (Tronson and Taylor, 2007). However, within the hippocampus, a double dissociation has been reported between brain-derived neurotrophic factor (BDNF), which is selectively required for consolidation, and the transcription factor ZIF268, which is selectively required for reconsolidation (Lee et al, 2004).

The storage hypothesis of reconsolidation makes several important predictions for psychiatric disorders. Pathological memories made labile by reactivation could be susceptible to disruptions, and if memory can be disrupted during a retrieval-induced labile period, there is a possibility that disruption of reconsolidation could be particularly efficacious in the treatment of strong, intrusive memories in disorders such as PTSD, phobias and PD (Cetonze et al, 2005).

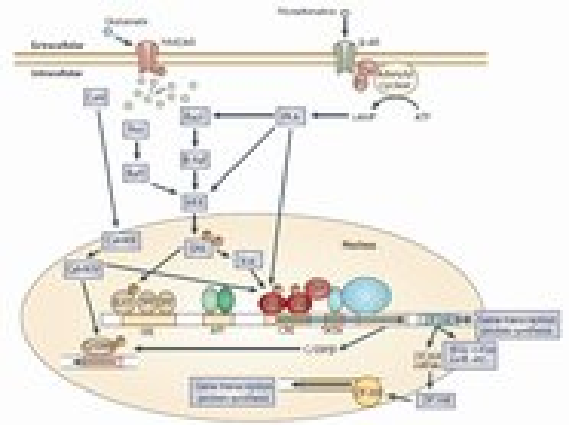


Figure 6. Molecular mechanisms of memory reconsolidation. This figure integrates findings from many individual molecules that have been identified as being required for memory reconsolidation. Possible therapeutically relevant neurotransmitter targets include β-adrenergic receptors (β-AR) and NMDA receptors, which molecular signalling cascades have been implicated in reconsolidation.

Reproduced from Tronson and Taylor (Nat Rev Neurosci 2007; 8:262-275).

3.2.4. Neurotrophins are involved in fear learning processes

Neurotrophins are attractive candidates as a molecular mediators of learning and memory, and may play a key role in the formation and/or storage of fear memories. Although neurotrophins are involved in synaptic plasticity, a definitive role for these molecules in behavioural models of learning and memory has been difficult to demonstrate. However, recent studies have implicated neurotrophic factors signalling pathway, and more concretely BDNF/tropomyosin kinase receptor B (TrkB) system, in fear memory consolidation. Although hippocampal BDNF is required for the late phase of LTP (L-LTP) (Messaoudi et al, 2002) as well as for mnemonic processes, its involvement in acquisition, consolidation, and reconsolidation is far from being clear. Growing evidences suggest that BDNF and its receptor, TrkB, may play a critical role in amygdala-dependent learning due to specific changes in BDNF gene expression after paired stimuli using the Pavlovian fear conditioning (Rattiner et al, 2004). In this regard, recent evidences have shown that BDNF is not only necessary, but sufficient to induce a late postacquisition phase in the hippocampus essential for persistence of LTM storage (Bekinschtein et al, 2008). In accordance with this affirmation, $BDNF^{+/-}$ null mice showed an impaired contextual learning (Liu et al, 2004).

Less is known about the role of neurotrophin-3 (NT-3)/TrkC system in learning and memory. Previous studies suggest that conditional NT-3 mutant mice do not show abnormal fear consolidation (Akbarian et al, 2001), however, in this experiment, mice were repeatedly exposure to electrical footshocks, which differ from our procedure.

4. STRESS AND PANIC DISORDER

Affective disorders display pathological mood and affect, such as the mood and anxiety disorders. Patients suffering from anxiety disorders, experience anxiety and fear in some or other form in reaction to an event/experience, which can be perceived as an internal stressor or can be a real threat (external). Anxiety disorders are of the most common psychiatric disorders and include PD, social phobias, obsessive-compulsive disorder (OCD), PTSD and generalized anxiety disorder (GAD).

When the stress response is chronically activated, the stress system may lead to a number of disorders that are the result of increased and/or prolonged secretion of CRH and/or glucocorticoids (Table 1). One of the most stressful anxiety disorders is PD, as classified by the DSM IV (APA 1994) and by the ICD-10 (WHO 1992).

Table 1. Disorders with increased or decreased HPA axis activity.

4.1. Stress as an evolutionary response

Life exists through maintenance of a complex dynamic equilibrium, termed homeostasis, that is constantly challenged by intrinsic or extrinsic, real or perceived, adverse forces, the stressors. Stress produces complex physiologic and behavioral responses, which if excessive, inadequate or prolonged, may affect behavior, personality development and physiologic functions, such as reproduction, growth, metabolism, circulation and the immune response (Chrousos & Gold, 1992). When the individual is having excessive or inadequate adaptative responses and suffering adverse consequences for long states of time, it is termed allostasis.

The stress system receives and integrates a repertoire of emotional, neurosensory, cognitive and peripheral somatic signals that arrive through different pathways. The activation of the stress

system leads to physical and behavioral changes that are remarkably defined in their qualitative presentation, and defined as the stress syndrome. Due to the adaptative and time limited profile of these changes, they improve the chances of the individual for survival.

Behavioral adaptation includes increased alertness, vigilance and arousal, improved cognition; enhanced analgesia; focused attention; and inhibition of vegetative functions, such as feeding and reproduction. Parallely, physical adaptation mainly consists to an energy redirection and vital substrates availability to the CNS and the stressed body sites to cope with the stressful stimulus, such as an increase in respiratory rate, cardiovascular tone and intermediate metabolism (lipolysis, gluconeogenesis), whereas immunity, growth reproduction and digestive function are inhibited (Chrousos, 1997).

Richard Lazarus studied the intra-individual differences in front of the same stressful situation, that could affect in a different way to different people. There are some situations that are stressors for some persons but not to the others, and it is not possible to establish a simple casual relation between stressful stimulus and stress response. In that way, Lazarus proposed the existence of motivational and cognitive factors that could be different between individuals. He emphasized the relevance of cognitive evaluation of the situation (appraisal) that determines the emotional value, and the coping strategies that are engaged to face up to the situation (Lazarus 1993).

The impact of traumatic life events is often measured by how well people appear to cope with the experience. This suggests that there are psychological control processes that can buffer the noxious consequences of stress. Interesting factors are the control capacity and the predictability of the individual from the stressful situation. The control capacity is refered to the individual competence to diminish or end the intensity, duration or frequency of the aversive stimulus (Weiss, 1972), and it is a strong modulator of the HPA axis activity. The predictability is refered to the individual's capacity to predict some aspects of the stimulus, such as the beginning, the intensity, etc. (Abbot et al, 1984). An example of the relevance of both factors were the experiments done by Cover et al, (1973) in rats, in which the animals were exposed to an active avoidance test for 10 days. These rats reduced both the number of shocks received (learning to escape from the situation) and the corticosterone levels through the experiment, giving rise to a relation between number of shocks and corticosterone. Nevertheless, exposure to the active avoidance test for more days did not reduced the number of shocks received, but animals had reduced corticosterone levels, suggesting an increase in the control capacity and predictability, which could reduce the HPA axis activation.

There are psychological factors that modulate the HPA system. Novelty or social isolation can activate the HPA axis, whereas social support can bufferi the activating effects of other types of challenge (Levine, 2000). A number of previous studies report on the positive effect social interactions have on health and well-being (Cohen, 1988). Social support or affiliation appears to dampen the HPA stress response (Kirschbaum et al, 1995; Thorsteinsson and Jamea, 1999). Certain behavioural and physiological constructs have been proposed as indicators of beneficial social buffering against stress. Despite its apparent importance little is known about the precise

physiological mechanism involved in positive social interactions buffering the stress response (Cohen, 1988; Uchino et al, 1996).

Extensive research has shown that at least two alternative behavioural strategies to cope with environmental stimuli can be distinguished in several species including humans, rats and mice (Ursin and Olff, 1993a; Koolhaas et al, 1999). One is the 'active' coping style (or fight-flight) and the other is the 'passive' coping style (or conservation-withdrawal). Active coping is associated with higher sympatho-adrenal activity and lower HPA reactivity, whereas passive coping is characterised by predominant parasympathetic activity and higher HPA reactivity (Koolhaas, 1994). The main difference between the two coping strategies is likely the degree of behavioural plasticity or flexibility (Koolhaas et al, 1999), which may have implications for a differential susceptibility for stress-related disorders. This thesis will focus on differences in baseline and stress-induced and functioning of the HPA system in TgNTRK3 mice that show distinctly different behavioural coping strategies.

4.2. Predisposing factor for the onset of panic disorder: Stress

The way animals, including humans, cope with stressful events shows a considerable individual variation, which may underlie a differential vulnerability to the development of stress-related pathologies. PD patients usually suffer other co morbid psychiatric pathologies and show a higher vulnerability to stress. In fact, stress is one of the most important predisposing factors to unleash this anxiety disorder. In addition to unexplained physical symptoms, negative or stressful life events may facilitate the appearance of PD (Faravelli, 1985; Manfro et al, 1996). In retrospective studies, as many as 80% of patients with PD report exposure to life stressors in the year prior to diagnosis, and a vast majority believe that these events contributed to the onset of the PD (Manfro et al, 1996; Roy-Byrne et al, 1986a). Furthermore, symptom severity has been correlated with negative life events, including interpersonal conflicts, physical or health-related problems, and trouble at work (Lteif and Mavissakalian, 1995). The presence of chronic life stressors may also worsen the course of PD (Wade et al, 1993), while a recent personal loss or separation appears to elevate the risk of depression co morbid with PD (Roy-Byrne et al, 1986a).

The role of life experiences in the aetiology of PD has been widely studied (DeLoof et al, 1989), and stress is thought to be prominent in the aetiology and course of PD. In fact, PD patients have an increased tendency to report life events in general (Lteif & Mavissakalian, 1996) which causes impediment to evaluate the causal role of life events in PD, because it suggests that live events preceding the onset of PD fail to provide comparison for the time period of onset. Moreover, life events may interact with other risk factors such as family history of depression in precipitating episodes of panic (Manfro et al, 1996).

Life events research indicates that a majority of panic patients experienced stressful events, such as separation an interpersonal conflict, prior to their panic attacks, which per se are repetitive

stressful events (Faravelli, 1985; Roy-Byrne et al, 1986a; Silove, 1987), although not necessarily 'traumatic' or life-threatening, according to DSM-IV.

4.3. Stress response: The hypothalamic-pituitary-adrenal axis

The HPA axis activity has an intrinsic rhythm, producing high stress hormone levels during the transition from sleep to activity and lower levels as the sleep phase again approaches, that is call dark-light cycle. However, HPA axis is activated in response to stress. The principal regulator of the pituitary-adrenal axis is the hypothalamic CRH, which stimulates the secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary. CRH has a potent synergistic factor in the secretion of ACTH but with very little secretagogue activity on its own, the arginine-vasopressin peptide (AVP) (Gillies et al, 1982). In the hypothalamus, CRH and AVP have a positive reciprocal interaction stimulating the secretion of the other, and both are secreted in the portal system in a circadian, pulsatile, and highly concordant fashion (Horrocks et al, 1990; Veldhuis et al, 1990). In addition, other factors, such as lipid mediators of inflammation, various cytokines and angiotensin II are secreted and act on the hypothalamic, pituitary, and/or adrenal components of the HPA axis and potentiate its activity.

In addition to its effect as a pituitary hormone, CRH has direct effects as a neuromodulator and neurotransmitter within the CNS (Smith el al, 1986; Dieterich et al, 1997). studies have demonstrated that intracerebroventricular (ICV) injection of CRH produces heavy behavioral effects in animals which resemble certain symptoms of panic, such as increased startle response (Swerdlow et al, 1986) and stress-induced freezing behavior (Kalin et al, 1988). CRH neurons are densely concentrated in brain regions that have been hypothesized to be involved in the pathogenesis of mood and anxiety disorders including the forebrain (PFC, amygdala and BNST) and brainstem (e.g. LC, parabrachial nucleus) (Swanson et al, 1983; Merchenthaler, 1984; Sawchenko and Sawchenko, 1985).

The adrenal cortex is the main target of ACTH, which regulates adrenal androgen and glucocorticoid secretion by the zona reticularis and fasciculate, respectively, and participates in the control of aldosterone secretion by the zona glomerulosa. Other hormones, cytokines and neuronal information from the autonomic nerves of the adrenal cortex may also participate in the regulation of cortisol secretion (Andreis et al, 1992; Bornstein and Chrousos, 1999).

The final effectors of the HPA axis are the glucocorticoids. These hormones are pleiotropic, and exert their effects through their ubiquitously distributed intracellular receptors (Munck et al, 1984). Two different glucocorticoid receptors coexist in the CNS, the mineralocorticoid receptor (MR) that responds to low concentrations of glucocorticoids, and the glucocorticoid receptor (GR) that responds to both basal and stress concentrations of glucocorticoids and mediates negative feedback control of CRH and ACTH. In the absence of hormone binding, the glucocorticoid receptors resides in the cytoplasm of cells and forms a large multiprotein complex consisting of the receptor polypeptide, two molecules of hsp90, and other proteins (Bamberger et al, 1996). Upon hormone binding, the

receptor dissociates form the complex and translocates into the nucleus, where it binds as homodimer to glucocorticoid-response elements (GREs) located in the promoter regions of target genes to regulate their expression positively or negatively. The activated receptor can also modulate gene expression by interacting with other transcription factors, such as NF-κB and activating protein-1 (AP-1) (Bamberger et al, 1996).

Glucocorticoids play a main role in the regulation of basal activity of the HPA axis, and in the termination of the stress response by acting at the hypothalamus, the pituitary gland, and extrahypothalamic brain regions. The hippocampus and the efferent pathway linking it to the paraventricular nucleus of the hypothalamus (PVN) of the hypothalamus also play an important role on the regulation of negative feedback (Cullinan et al, 1993). Such negative feedback to terminate the stress response serves to limit the duration of the total tissue exposure of the organism to glucocorticoids, thus minimizing the immunosuppressive, lipogenic, catabolic and antireproductive effects of stress hormones. The feedback inhibition occurs via multiple feedback loops – fast/rate sensitive, intermediate and slow/delayed feedback (Liberzon et al, 1997).

Under stress and conditions of non-stress, the HPA hormones maintain homeostasis and well being of the brain and the body (Sapolsky et al, 2000). They facilitate information processing and adaptation so that the body is prepared if the same stressor is encountered again (de Kloet, 1991; de Kloet et al, 1998; Sapolsky et al, 2000). Thus, these adaptative processes help the individual to respond to the ever changing environment (McEwen, 2000). However, when exposed to prolonged stressors for extended periods of time, an individual may develop pathological consequences (Sapolsky, 1992), since excess glucocorticoids can have damaging effects (Sapolsky and Meaney, 1986).

The adrenal gland is an essential stress-responsive organ that is part of both the HPA axis and the sympatho-medullary system. Previous work has evidenced that chronic stress-induced adrenal gland alterations, such as adrenal enlargement and increased basal plasma corticosterone despite normal plasma ACTH levels. This enlargement in adrenal weight is characterized by a hyperplasia and hypertrophy in specific adrenal subregions, and is associated with increased maximal corticosterone responses to ACTH (Ulrich-Lai et al, 2006). These chronic stress-induced changes in adrenal growth and function may have implications for patients with stress-related disorders, such as depressed patients and victims of suicide (Nemeroff et al, 1992).

4.3.1. Brain region activation after stress

Activation of endocrine hormones can be caused by psychological or a physical stress. Maximal secretion of corticosteroids is approximately 15 to 30 min after exposure to a brief stressor (Ursin and Olff, 1993b; Richter et al, 1996) and reaches baseline levels 60 min after exposure (de Kloet et al, 2005). It appears as though different types of stressors are directed though different neural pathways in order to stimulate the release of CRH. Systemic stressors have a direct threat to the body, and are probably channelled directly to the PVN via brainstem catecholaminergic projections. Stressors that require cognitive processing and interpretation appear to be relayed through limbic

forebrain structures (Herman and Cullinan, 1997). During development it is hypothesised that as different neural pathways mature, these regulate the stress response of the neonate to differential stressors (Sawchenko, 1991; Rosenfeld et al, 1992; Kovacs and Sawchenko, 1996). In addition to these neuronal pathways other brain pathways have been proposed to converge at the PVN in order to stimulate the secretion of CRH (Sawchenko, 1991).

While c-Fos mapping has some limitations (for example, cells not expressing c-Fos cannot be assumed to be completely inactive) (Hoffman and Lyo, 2002), there is no evidence that any other immediate early genes (IEGs) are better than c-Fos to characterize neuronal activation all over the brain. The data suggest that different types of stressful stimuli have both common and divergent activational properties. For example, the parvocellular PVN, which contains the cell bodies responsible for initiation of HPA secretion cascade, shows c-Fos activation regardless of stress type (Sawchenko et al., 1996). Similarly, the dorsal vagal complex, which contain NAergic cell groups controlling HPA activation, and several PVN-projecting hypothalamic nuclei show c-Fos protein expression after exposure to swim, restraint, footshock, hemorrhage or interleukin-1β (IL-1β) injection (Cullinan et al, 1995; Day and Akil, 1996; Sawchenko et al, 1996). In contrast, the lateral septum, medial divisions BNST and MeA show c-Fos activation following swim, restraint, social stress or footshock but not IL-1β injection, whereas the central amygdaloid nucleus and lateral subdivisions of the BNST show induction following IL-1β and hemorrhage but not swim, restraint or footshock (Cullinan et al, 1995; Day and Akil, 1996; Sawchenko et al, 1996).

The LC plays a major role in behavioral arousal in response to novel or stressful stimuli. It gives rise to divergent efferent pathways that provide the major source of NA to the forebrain (Sawchenko and Swanson, 1982) and is involved in arousal and in response to novel stimuli. Stress increases the firing rate of LC NAergic neurons as well as levels of NA and tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of catecholamines. As a consequence, chronic stress increases the magnitude of NA release in response to a novel stressor examined whether stress might also affect neurotrophin mRNA levels in the LC. Elevated NA activity enhances anxiety-like behavioral responses and inappropriate activation of the LC, may participate in the exaggerated stimulus-responsiveness and increased emotionality seen in patients with stress or anxiety disorders (Goddard and Charney, 1997).

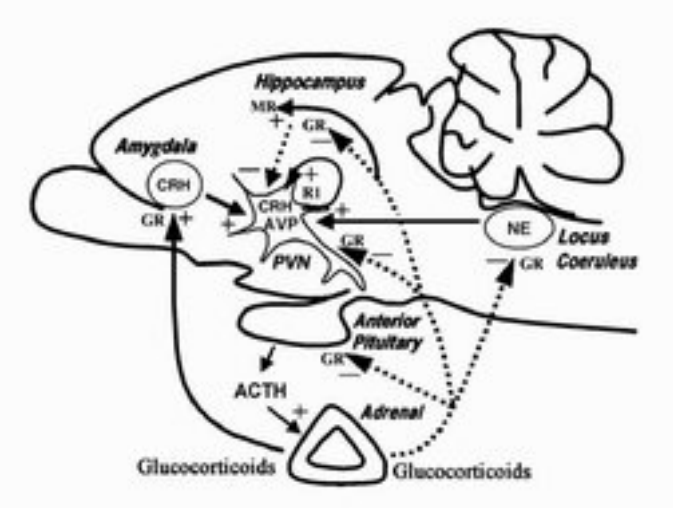


Figure 7. Multiple feedback loops activating CRH systems.

Reproduced from Makino et al (Pharmacol Biochem Behav 73: 147-158).

NA- and CRH-containing neurons are activated during the exposure to stressful stimuli (Figure. 7). Several studies have shown an increase in NA activity during stress and a depletion of stored NA as a consequence. Stress is associated with the activation of HPA axis, which is initiated by secretion of CRH in the median eminence region of the hypothalamus by CRH-containing neurons whose are located in the PVN.

PVN CRH neurons and the LC-NA system innervate mesolimbic and mesocortical components of the dopaminergic system and are activated during stress (Roth et al, 1988). The mesolimbic system consists of dopaminergic neurons of VTA, which innervate the nucleus accumbens, and plays a principal role in reinforcement/reward/motivational phenomena and in the formation of the central dopaminergic reward system. The mesocortical system also consists of dopaminergic neurons of VTA, which send projections to the PFC, and is involved in cognitive functions and anticipatory phenomena. Interestingly, activation of the PFC is associated with inhibition of the stress system (Diorio et al, 1993).

CRH/AVP and LC-NA neurons reciprocally innervate and are innervated by pro-opiomelanocortin (POMC) neurons of the arcuate nucleus of the hypothalamus (Calogero et al, 1988b). Activation of the stress system stimulates hypothalamic POMC-derive peptides, such as β-endorphin and α-melanocyte-stimulating hormone (α-MSH), which reciprocally inhibit the activity of both of the central components of the stress system, produce analgesia through projections to the hind brain and spinal cord, where they inhibit ascending pain stimuli.

The amygdala-hippocampus complex is activated during stress primarily by ascending catecholaminergic neurons in the brainstem, and by glucocorticoids, the end-product of the HPA axis, but also by inner emotional stressors, such as fear, which is processed in the amygdala (Gray, 1989). The amygdala can stimulate central components of the stress system in response to emotional stressors. The hippocampus is involved in tonic and stimulated inhibitory effects on the activity of the amygdala, PVN and LC-sympathetic system.

4.4. The hypothalamic-pituitary-adrenal axis reactivity in panic disorder patients

At the hormone level, PD is often accompanied by an overactivity of HPA axis and an inability to develop adequate coping strategies in response to an unexpected stressor (Goldstein et al, 1987). Studies in PD patients about HPA axis activity (Table 2) have been controversial but tend to show normal daytime (Cameron and Nesse, 1988; Brambilla et al, 1992; Holsboer, 1992) or slightly elevated baseline levels of plasma cortisol in panic patients (Roy-Byrne et al, 1986b; Abelson and Curtis, 1996; Goldstein et al, 1987; Charney and Bremner, 1999; Wedekind et al, 2000; Marshall et al, 2002) and elevated nocturnal basal cortisol secretion (Abelson and Curtis, 1996; Abelson et al, 1996; Bandelow et al, 2000b). PD patients as well as normal individuals are confronted with various more or less stressful events during the day, whereas during the night, the difference between patients and controls reaches significance. However, as cortisol increases during REM and secretory

activity is stimulated by nocturnal awakening could explain the differences reported (Abelson et al., 1996). Interestingly, circadian HPA axis alterations in PD are modulated by illness severity and treatment seeking behavior (Abelson et al, 1996). Studies with non-stressful methods, such as urinary free cortisol (UFC) or salivary samples, showed that cortisol were found to be normal (Uhde et al., 1988) or elevated only in patients with complicated PD (those with agoraphoba or secondary depression) (Lopez et al., 1990) or suffering severe PD (Wedekind et al., 2000) when compared with healthy controls, under baseline conditions.

Cortisol is elevated in anticipation of panic attacks in the laboratory, but not during the attack (Coplan et al., 1998), which distinguishes panic attacks from normal fear reactions, may be due to attenuated subjets' anxiety by the presence of medical personnel. However, Bandelow et al, (2000a) reported that during spontaneous panic attacks there was a subtle but significant elevation of salivary cortisol levels in PD patients, in a naturalistic setting. Moreover, under conditions of acute mild stress, significantly higher cortisol levels in PD patients have been found compared to healthy controls (Stones et al, 1999).

Recent studies have reported an increase in plasma ACTH concentrations before pharmacological treatment (Brambilla et al, 1992; Abelson et al, 1996), and reduced ACTH/cortisol ratios in the more frequent panickers (>2/week) suggesting an increased adrenal sensitization to ACTH lead by more chronic, central overdrive (Abelson et al, 1996). Although investigators have failed to observe enhanced cerebrospinal fluid (CSF) levels of CRH in PD (Jolkkonen et al, 1993; Fossey et al, 1996). CRH concentrations in CSF provide CRF activity information, but do not provide information concerning the specific regional changes in synthesis, storage or release of this peptide or the relative contributions of hypothalamic and non-hypothalamic secretion to the overall CSF concentration.

CRH challenge studies in PD patients have given inconsistent results. In the early studies, PD patients showed a blunted ACTH release after CRH challenge (Roy-Byrne et al, 1986b; Holsboer at al, 1987). However, experiments done by Brambilla et al., (1992) showed normal responses. And in recent studies, PD patients showed a lightly enhanced ACTH response (Rapaport et al, 1989; Curtis et al, 1997). It has been proposed that negative feedback resulting from elevated plasma cortisol levels, due to acute reactivity of patients to the preparations for CRH administration, might account for blunted ACTH responses to CRH. This would be consistent with the impression that PD patients are specially sensible to minor environmental challenges (Roth et al, 1992; Leyton et al, 1996).

Dexamethasone suppression test (DST), which consists in the administration of dexamethasone, a exogenous steroid that provides negative feedback to the pituitary to suppress the secretion of ACTH, revealed that nearly one third of PD patients had cortisol non-suppression (Kopp et al, 1989; Coryell et al, 1991); however, other DST studies have reported normal negative glucocorticoid feedback (Kellner and Yehuda, 1999), suggesting that abnormal DST in some PD patients could be attributed to causes other than panic attacks or agoraphobia (Curtis et al, 1982), such as depression disorders.

In particular, depressed patients show an attenuated ACTH response to CRH (Gold et al, 1984), and continue to exhibit this response to CRH if depressed patients are pretreated with high doses of dexamethasone (Von Bardeleben and Holsboer, 1989), the DEX-CRH test. Individuals with PD exposed to the DEX-CRH test show a higher response than healthy controls, but smaller (Schreiber et al, 1996) or similar magnitude (Erhardt et al, 2006) compared with ACTH and cortisol response in major depressive disorder (MDD). Indeed, as PD does not show chronic hypercortisolism, as there is in MDD, the blunted ACTH response to CRH in PD may occur because of a less pronounced desensitization of pituitary CRH receptors secondary to hypothalamic CRH hypersecretion than that in depression, as well as an altered negative feedback inhibition.

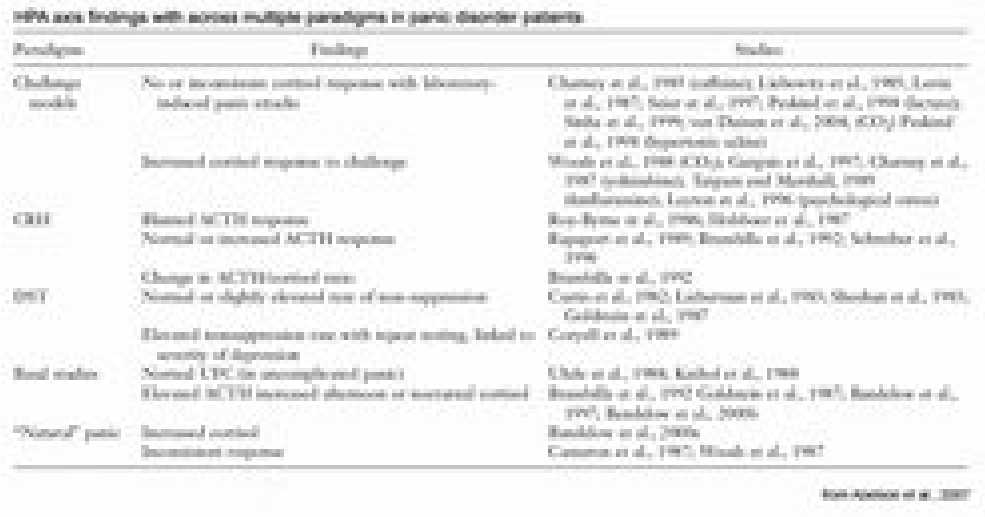


Table 2. HPA findings in PD patients.

The assumption that HPA axis hyperactivity is a not a simple state marker in PD that resolves with pharmacological treatment is corroborated by different studies. An example of effective medications in the treatment of PD are the benzodiazepine receptor agonists, that consistently attenuate both stress- and drug-induced HPA activation, assessed with alprazolam and diazepam in nonhuman primates (Kalogeras et al, 1990) and PD patients (Lopez et al, 1990; Roy-Byrne et al, 1991; Abelson et al, 1996). Inhibition of the HPA axis may be an epiphenomenon of effective drug treatments and not a necessary concomitant of efficacy. In addition, some studies have reported that measures of HPA activity in PD predicted treatment response (Coryell et al, 1989, 1991; Abelson and Curtis, 1996), which raise the possibility that something about HPA axis dynamics marks a more severe disorder or some other characteristic of individuals that makes recovery more difficult.

Finally, clonidine treatment, an alpha-2 receptor agonist, in PD patients induced a significantly greater percentage cortisol decrease versus controls (Coplan et al, 1995), suggesting a hyperresponsivity to clonidine in PD patients, and an uncoupling of the NAergic system and the HPA axis. However, fluoxetine-dependent clinical improvement did not normalize such greater maximal percentage of cortisol decrease

In conclusion, the results obtained in HPA system studies show that a significant percentage of PD patients have a distressed HPA system regulation. Consequently, PD may be considered to be an illness that is characterized by only a "subthreshold" dysfunction of the HPA system.

In PD, brain structures involved in hyperarousal, perception or external and internal stimuli and cognitive judgement, could be of primary importance of HPA axis regulation. In this context, amygdala, hippocampus and PFC would have major significance, brain areas that modulate the HPA axis, rather than sustained CRH sensitivity and disturbances of feedback mechanisms at the hypothalamic level. As CRH release seems to be not altered (Fossey et al, 1996), there is more likely an imbalance of central CRH pathways not in general but only in distinct brain areas, which are involved in the endogenous network of fear such as the hippocampus and the amygdala (Gorman et al, 2000)

5. NT-3/TRKC SYSTEM

NT-3 is a member of a wider family of polypeptides collectively termed the neurotrophins, which includes nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-4/5 (NT4/5) and neurotrophin-6 (NT-6), which share approximately 50% amino acid sequence identity, but have a distinct range of neurotrophic activities (Barde, 1989). Neurotrophins regulate growth, differentiation, and survival of neurons (Lindsay et al, 1994; Lewin and Barde, 1996), as well as in some non-neuronal cells (Tessarollo, 1998). The neurotrophins are non-covalently linked homodimers of two highly basic 120-residue long polypeptide chains. Whilst all neurotrophins bind with equal affinity to a low-affinity glycoprotein transmembrane receptor, $p75^{LNGFR}$ (Chao, 1994), the major signal-transducing components appear to be the transmembrane tyrosine kinases, which exhibit ligand-dependent activation, i.e. $p145^{TrkC}$ (TrkC) binds NT-3. Unlike NGF or BDNF, NT-3 can to some extent also bing and activate non-cognate TrkA and B receptors, although the physiological relevance of these interactions is unclear (Clary and Reichardt, 1994).

The TrkC loci encodes a variety of receptor isoforms, in addition to the full-length tyrosine kinase receptors. Although various kinase-deficient TrkC receptor isoforms have been identified over the years for TrkC genes, only two truncated isoforms, which are known as TrkC NC1 (Tsoulfas et al, 1993; Palko et al, 1999) and TrkC NC2 (Menn et al, 1998), and TrkCic158 (Valenzuela et al, 1993), are believed to play important roles in vivo. The cytoplasmatic tails of these truncated receptors are encoded by separate exons, which are evolutionary conserved. Their protein products are present in both the embryo and in the adult animal, and their expression is dynamically regulated during development (Escandón et al, 1994; Menn et al, 1998). To date, the main function attributed to the kinase-deficient truncated isoforms is inhibition of the kinase-active receptor isoforms, which is achieved by acting as a dominant-negative inhibitor of the full-length receptor or by a ligand-sequestering mechanism, which limits the neurotrophic factor available to bind the kinase-active receptor (Tessarollo, 1998; Huang and Reichardt, 2001). However, the high degree of sequence conservation of the intracellular domains of truncated receptors among species suggests the

potential for other functions, such as interaction with cytoplasmatic adaptor proteins and activation of signalling pathways (Hapner et al, 1998).

To date, no molecules have been linked truncated TrkC NC1 receptors to intracellular signalling pathways. Moreover, there are no data on direct biological functions, per se, although it has been reported that TrkC NC1 with p75 can induce neural crest cell differentiation, and in animal models of glaucoma truncated TrkC NC1 is overexpressed concomitantly with retinal ganglion cell death (Hapner et al, 1998; Rudzinski et al, 2004).

Several lines of evidence suggest that characteristic axonal and dendritic morphologies throughout the nervous system are determined, in part, by local patterns of expression of neurotrophins and neurotrophins receptors (McAllister et al, 1997; Lentz et al, 1999). For example, transfection of explant cultures of dorsal root ganglia (DRG) neurons with TrkC kinase receptor showed enhanced elaboration of major axonal processes, whereas the truncated isoform reduced elaboration of major processes and increased branching (Ichinose and Snider, 2000).

5.1. NT-3/TrkC signalling cascade

NT-3 plays a critical role in the development and survival of mammalian neurons. The binding of NT-3 to TrkC results in the autophosphorylation of five tyrosine residues (Kaplan and Stephens, 1994). Three of the tyrosines (674, 678, 679) or (705, 709, 710) probably play a role in activation of the kinase towards exogenous substrates. Phosphorylation of tyrosine 485 (or 516) allows the phosphotyrosine-binding domain of Shc to interact with TrkC, resulting in tyrosine phosphorylation of Shc and its consequent interaction with the Grb2-Sos complex.

This association then stimulates the activation of the Ras__Raf-1__mitogen-activated protein kinase (MAPK) kinase__MAPK cascade. The phosphorylation of tyrosine 789 results in the additional binding of phospholipase Cγ (PLCγ) via its SH2 domain. PLCγ is then phosphorylated and activated. PtdIns 3-kinase is also activated, although the mechanism by which this occurs is not clear. In summary, there may be at least three pathways by which NT-3 could activate the MAPK cascade: one via Shc__Grb2-Sos__Ras__Raf-2, another via PLCγ__diacylglycerol__PKC__Raf-1 and a further poorly characterized pathway involving PtdIns 3-kinase.

5.2. NT-3/TrkC role in neurogenesis, differentiation, survival and plasticity

Neurotrophins play a prominent role in the development od the vertebrate nervous system by influencing cell survival, differentiation, and cell death events (Levi-Montalcini, 1987; Lewin and Barde, 1996). Previous studies have revealed that TrkC, as well as TrkB, are essential for the development of peripheral nervous system (PNS). However, it is hypothesized that neurons of the CNS may have access to other neurotrophic factors that can compensate for these receptors in vivo (Silos-Santiago et al, 1997).

Neurotrophins also exhibit acute regulatory effects on neurotransmitter release, synaptic strength, and connectivity (Thoenen, 1995; Bonhoeffer, 1996). In addition to promoting axonal and dendritic branching, neurotrophins serve as chemoattractants for extending growth cones in vitro (Gallo et al, 1997). Mutations in Trk neurotrophin receptor function lead to deficits in survival, axonal and dendritic branching, LTP, and behaviour (McAllister et al, 1999; Minichiello et al, 1999).

NT-3, unlike BDNF, does not have neurotrophic activity on central cholinergic or dopaminergic neurons (Knusel et al, 1991). Later studies, have reported that NT-3 can enhance the proliferation of embryonic hippocampal precursors (Collazo et al, 1992), and promotes the survival of TrkC-expressing DRG neurons (Ip et al, 1993).

5.3. NT-3/TrkC expression in central nervous system

Neurotrophins can only exert their programmed trophic roles in those cells that express their cognate signalling receptors. The spatial and temporal expression pattern is determinant to analyze its implication the ontogeny of the vertebrate nervous system.

The first expression of NT-3 has been observed at embryonic day 11 (E11), and displays a dramatic increase in NT-3 transcripts. NT-3 has a temporal pattern of highly expression in the developing brain which decreases in the adult (Ernfors et al, 1990). The analysis of NT-3 expression in spinal cord (E12), cerebellum (first 3 weeks after birth) and hippocampus (E17-postnatal day 0) showed a remarkable correlation between high levels of expression of this neurotrophin with active neurogenesis and the initial formation of axons. In the adult brain, NT-3 mRNA has been predominantly detected in the cerebellum and in the hippocampus, predominantly in the vast majority of dentate granule cells as well as in subpopulations of pyramidal neurons located in CA1 and CA2 (Maisonpierre et al, 1990; Emfors et al, 1990).

The rat TrkC locus encodes a number of differentially spliced isoforms which lack the cytoplasmatic tyrosine kinase domain and others that exist as splice variants of variable length [with 14 (TrkC.ki14), 25 (TrkC.ki25) and 39 amino acid inserts (TrkC.ki39)] (Lamballe et al, 1993; Tsoulfas et al, 1993; Valenzuela et al, 1993). TrkC is widely expressed in the developing and adult nervous system. In mouse embryos, the TrkC transcripts are first observed in the telencephalon and spinal cord at embryonic day 9.5 (E9.5), and from E10 through birth, TrkC has a dynamic pattern that involves CNS and peripheral nervous system (PNS). TrkC expression appears to correlate with axon outgrowth and organogenesis. Between E11.5 and E13.5, TrkC is expressed in the auditory system, the cerebellum, and the tectum. In intermediate embryonic stages (E13.5), TrkC is expressed in various regions of the CNS, such as caudatoputamen, septal nuclei, cerebellum, and brainstem. In later embryos (E15.5-E17.5), two additional sites begin to display TrkC expression, the germinal trigone of the developing cerebellum (initial site of granule cell generation) and the hippocampus (pyramidal cells are born and differentiate from the CA1 through the CA3 regions). As observed in the younger embryos, expression of TrkC correlates both spatially and temporally with cytogenetic gradients of cell growth. In the adult mouse, TrkC transcripts are heterogeneously

distributed throughout the brain, with highest levels in limbic and diencephalic structures, such as cerebral cortex, hippocampus, thalamus and hypothalamus. In the hypothalamus, elevated TrkC expression is detected in the dorso- and ventromedial nuclei located adjacent to the third ventricle (Lamballe et al, 1994).

In the PNS, at E11.5, trkC expression is first detected in the DRGs, where the axons begin to project to target organs (Rugh, 1990), and then its expression decreases and by E17.5 trkC-expressing cells are distributed solely in the periphery of the ganglia. Comparison of this expression pattern with that of NT-3 mRNA expression (Scheeterson and Bothwell, 1992) indicates a significant analogy between TrkC and NT-3 expression in the embryo.

In the adult brain, TrkC mRNA is expressed both in the pyramidal layers of the hippocampus and in the granule layer of the DG, but apparently more prominently in the latter (Hassink et al, 1999). Levels of TrkC mRNA in these areas do not seem to change overtly from P1 to adulthood.

NT-3 and TrkC transcripts colocalization, e.g. adult hippocampus and cerebellum, raises the possibility that trophic effects of this system is produced by paracrine and/or autocrine mechanism. Another main point is the colocalization of TrkC with other Trk receptors. The expression of TrkB and TrkC shows significant areas of overlap, but their spatial and temporal expression differ. Both genes are expressed in cerebral cortex and in the pyramidal cells of the hippocampus. However, TrkC expression is significantly more striking than that of TrkB.

HYPOTHESIS AND OBJECTIVES

1. HYPOTHESIS

The correct development and function of CNS is critical for brain health of the organism. Early or chronic stress causes prominent alterations in brain function, and affects the expression of neurotrophic factors in limbic brain regions involved in the regulation of mood and cognition. Recent evidences have opened the idea that in complex organisms, an altered expression of certain neurotrophins by stress could be involved in the onset and pathophysiology of most psychiatric disorders, such as depression, squizophrenia or anxiety disorders. It is hypothesized that altered levels of neurotrophic factors could contribute to the atrophy and cell death of these regions, including the hippocampus and prefrontal cortex, which would produce a malfunction in limbic-related areas, and as a consequence, a precipitation or worsening of psychiatric illnesses. We were interested in panic disorder pathophysiology, which is a stress-related disorder and is characterized by an altered cognitive processing of emotional information. Although little evidence has been found supporting a neurotrophic role in PD, recent data has revealed that NT-3/TrkC signaling might play a key role in limbic system morphology and function. Therefore, we suggest that NT-3/TrkC system is involved in PD pathogenesis.

For these reasons, we propose that an altered expression of TrkC could participate in the pathophysiology of panic disorder through affecting neurobiological systems involved in stress and fear responses, such as HPA axis and limbic system. Disturbation of these systems would cause innapropiate physiological and behavioral responses, and unleash to the psychiatric pathology.

2. OBJECTIVES

The main objective in the work of this doctoral thesis lie to determine the role of *NTRK3* gene in emotional cognition and stress response processes that underlies PD. To this end, we used a genetically modified mouse model of *NTRK3* overexpression, which was validated as a model of PD. Here, it is characterized the effects produced by the increase of *NTRK3* expression in the CNS, focusing in neural alterations that might influence changes in cognitive processes involved in coping strategies. Moreover, it is studied the mechanisms that underlie in these processes by different approaches, 1/physiologically, measuring the HPA axis response, 2/brain activation, analyzing the activation pattern to a stress stimulus, 3/cellular and gene expression profiling, characterizing key brain regions in cognitive processes, and 4/pharmacologically, studying neurotransmitters function.

The specific objectives are:

1. To specify the *NTRK3* involvement in the cognitive processes related with the pathophysiology of PD, evaluating cognitive-behavioral changes in the mouse model of Ntrk3 overexpression and its relation to phenotypic alterations in PD.
2. To characterize cellular changes, such as cellularity and gene expression, caused by the increased expression of *NTRK3*, in brain regions that are involved in emotional memory formation and storage.
3. To elucidate the alterations in the HPA system produced by the increased expression of *NTRK3*, underlying abnormal stress response.
4. To dissect the abnormal function of different brain regions through the increased expression of *NTRK3*, by acute exposure to a stressful stimulus.

MATERIAL AND METHODS

Mouse exposed to immobilization (IMO) in board. Photography by Alejandro Amador-Arjona (Mara Dierssen lab, CRG).

1. ANIMAL MODEL

The experiments were performed in a mouse model genetically modified with overexpression of *NTRK3*, encoding for the high affinity receptor for neurotrophin 3, TrkC (TgNTRK3).

As previously described (Dierssen et al, 2006), transgenic mice were generated by standard pronucleus microinjection on a hybrid B6/SJL-F1J genetic background. The construct contains the human *NTRK3* cDNA, which shows a high homology sequence with mouse *Ntrk3* cDNA, and is directed by the human *PDGFB* chain promoter (Resnick et al, 1993) which drives efficient and specific expression in the brain (Sashara et al, 1991). In order to exclude positional effects, we used two different lines of this mouse model with insertion of the transgene in different chromosomes. Each transgenic line carries different copy numbers of the transgene (line 35, 2 copies and line 69, 5 copies). The mouse colony was maintained by crossing transgenic male mice with hybrid B6/SJL-F1J female mice. The non-transgenic littermates of TgNTRK3 mice served as controls.

DNA from tail biopsies was used for genotyping routinely by PCR analysis using the primer pairs: *NTRK3* human/forward 5'-CTGTTTGACGAAGTGAGTCCC-3' and *NTRK3* human/reward 5'-TCCAGTGACGAGGGCGTG-3'. Hybrid founder were backcrossed extensively in order to attenuate littermate's genetic differences. All experiments were performed in mice from the F18-F24 generations.

Same sex littermates were group-housed (4-5 animals per cage) in standard macrolon cages (40 x 25 x 20 cm) under a 12-h light/dark schedule (lights on 7:00 a.m. to 7:00 p.m.) in controlled environmental conditions of humidity (60%) and temperature (22 ± 2ºC) with food and water supplied *ad libitum*. Adult TgNTRK3 (Dierssen et al, 2006) and wild type male littermates (2-7 months of age) from different litters were used for these studies to avoid female estrous cycle variations.

All experimental procedures were approved by the local ethical committee (CEEA-PRBB), and met the guidelines the local (Spanish law 9/2003, and Catalan law 5/1995), European regulations (EU directive nº 86/609, EU decree 2001-486) and the Standards for Use of Laboratory Animals nº A5388-01 (NIH). The CRG is authorized to work with genetically modified organisms (A/ES/05/I-13 and A/ES/05/14).

2. BEHAVIOURAL CHARACTERIZATION

All the behavioural studies were conducted for both lines under basal conditions or after exposure to chronic environmental stress by the same experimenter in an isolated room. The order of the experimental test scheduled from less to more aversive to reduce the influence of each test on the other and standarized handling protocols (each animal was handled 3 min per day during four-six days) were administered before the initiation of the experiment. Nevertheless, animals that showed behavioural stress responses (fur alterations, injuries, lost of weight, etc.) were ruled out from the sample. Tests were performed in a different room spatially isolated from the animal rooms. Tests

started 2 hours after the lights switch on. Behavioural experimenters were blinded as to the genetic status of the animals.

On the other hand, due to the high biological variability found among subjects in hormonal and behavioural tests, it was necessary to use higher number of animals than in other behavioural protocols. In this thesis we used a number of animals greater than 10 per group.

The behaviour exhibited by each animal in the course of each behavioural test session was videotaped using a remote-controlled video camera placed vertically.

2.1. Anxiety-like behaviour

Modified Hole-Board Test

The *modified hole board* (mHB) paradigm is a test that explores unconditioned behaviour and was designed to investigate exploration in rodents (File & Wardill, 1975; Lister, 1990), but it also allows to evaluate a variety of other behavioural parameters, such as locomotor activity and anxiety-like behaviour (Ohl & Fuchs, 1999; Ohl et al, 1998). Figure 1 shows schematic drawings of the mHB.

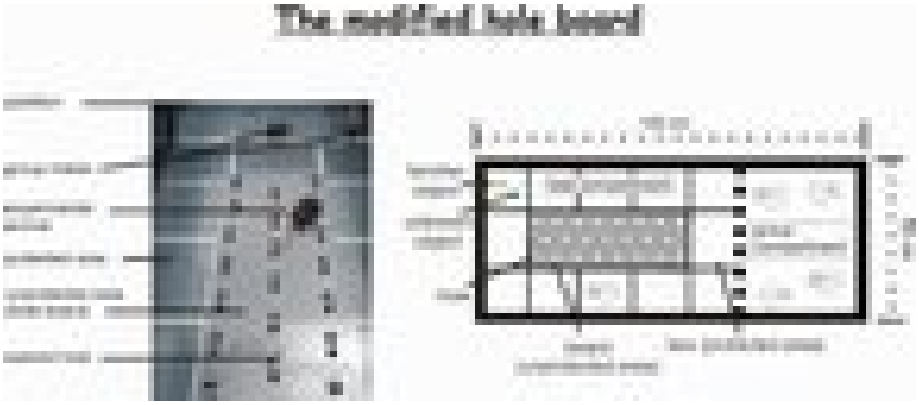


Figure 1. Schematic representation of a modified hole board.

The apparatus consisted of an opaque grey PVC board (60 x 20 x 2 cm) with 23 holes (1.5 x 0.5 cm) staggered in three lines that were covered by removable lids of the same material. The board was placed in the middle of an opaque dark grey PVC box (100 x 50 x 50 cm), thus representing the central area of an open field. The outer area was divided into 12 equally sized quadrants (20 x 16 cm) by lines taped onto the floor of the box (Ohl et al, 2001). Each animal was placed in a corner of the box under low illumination (50 Lux) conditions and was exposed to the apparatus during 5 min. Dependent variables included time spent in box and board (a board visit being recorded when the mouse moved all four paws into the board), the latency and total number of visits to the board, the number of holes explored and the time spent in hole exploration, grooming and rearing behaviours, and line crossings in the box.

Light/Dark Test

The light/dark test is based on the innate aversion of rodents for brightly lit large spaces and on the spontaneous exploratory behaviours of rodents to mild stressors, that is, novel environment and light (Crawley & Goodwin, 1980). Rodents present a natural conflict situation in which they show a tendency to explore in opposition to the initial tendency to avoid the unfamiliar (neophobia). Although the light/dark test was based on the model described by Crawley & Goodwin (1980), we have used it here with several structural modifications (Belzung and Le Pape, 1994; Bourin and Hascoët, 2003). The apparatus was a box consisting of a small (15 x 20 x 25 cm) PVC compartment with black walls and black floor dimly illuminated (50 Lux), connected by a 5 cm long tunnel to a large PVC compartment (30 x 20 x 25 cm) with white walls and floor, intensely lit (500 Lux). Mice were individually placed in the light compartment facing the tunnel at the beginning of the 5 min observation session.

Indeed, the anxiety-like behaviour measured in behavioural tests is sometimes confounded by changes in locomotor activity. For this reason, locomotor activity was measured by line crossings in each compartment. Dependent variables included time spent in the light and the dark compartment (a compartment visit being recorded when the mouse moved all four paws into the compartment), the latency to enter to the dark compartment, the movements per unit of time in each compartment, the transitions to each compartment, and the grooming, stretched attend and rearing behaviours in each compartment.

Elevated-plus maze Test

The elevated-plus maze (EPM) is based on exploration in a novel environment leading to the generation of an approach–avoidance conflict behaviour (Otto et al, 2001). The EPM consisted of a black plexiglas apparatus with four arms (29 cm long×5 cm wide) set in cross from a neutral central square (5 cm×5 cm). Two opposite arms were delimited by vertical walls (closed arms) and the two other arms had unprotected edges (open arms). The maze was elevated 40 cm above the ground and placed under indirect light (100 Lux). At the beginning of the 5-min observation session, each mouse was placed in the central zone, facing one of the open arms. The total numbers of visits to the closed arms and the open arms, and the cumulative time spent in open arms and closed arms were then observed on a monitor through a videocamera system connected to a videotracking analysis package [ViewPoint (Viewpoint S.A., France)]. An arm visit was recorded when the mouse moved all four paws into the arm.

The anxiety-like behaviour observed in the plus-maze was measured under the influence of specific anxiolytic drugs, such as benzodiazepines. The anxiolytic effectiveness of a drug is quantified by a reduction in the latency to enter the open arm and an increase in number of entries and total time spent into the open arm when compared to drug-free conditions.

2.2. Depression-like behaviour

Forced swimming test

The forced swimming test (FST) is a behavioural assay used to reproduce passive coping responses to stress that may model a relevant aspect of human depression in rodent species (Porsolt et al, 1977), introduced into a water tank devoid of exits. The initial behavioural reaction exhibited by the animals, lasting a few minutes, consists of vigorous attempts to escape from the situation. However, these active responses are soon abandoned and the animal starts to float in a state of rigid immobility that Porsolt defined as a 'despair' state and that is reduced by previous chronic but not acute treatment with antidepressants (Porsolt et al, 1977; Armario et al, 1988). In our experiments, mice were gently placed in individual glass cylinders (24 cm height, 11 cm diameter) containing 13.5 cm water at 25 ± 1ºC. This depth was sufficient to force the mice to swim or float since their hindlimbs did not touch the bottom of the cylinder. The water was renewed after each animal to avoid olfactory cues. Mice were exposed to the apparatus for 6 min during the test session, then removed from the cylinder, towel dried in a small cage, and returned to their home cages. The behaviour exhibited by each animal was videotaped using a remote-controlled video camera placed frontally to the apparatus. Thereafter, an observer unaware of the genotype or the environmental conditions exposure of each animal analyzed the videotape by the aid of a keyboard entry to the *Observer* software (Version 5.0, Noldus, The Netherlands). The behaviour in the FST was scored during the 6 min of the test based on behavioural categories described by Armario et al (1988). The duration and latency for the first occurrence of each of the following behavioural items was taken as a dependent variable: (a) immobility = time spent by the mouse floating in the water without struggling and making only those movements necessary to keep its head above the water; (b) swimming = the animal displays active swimming motions displacing the body around the cylinder, more than necessary to merely keep the head above the water; (c) struggling = the animal vigorously moves all four legs with body aligned vertically in the water, usually directed against the wall of the cylinder.

2.3. Fear-related behaviour

Fear Conditioning Test

Aversive emotional memories can be studied in the laboratory using classical fear conditioning, a behavioural task in which an initial neutral conditioned stimulus (CS) such as light or tone gains emotional properties after being paired with a noxious unconditioned stimulus (US) such as a footshock. Once the CS-US association has been made the occurrence of the CS initiates behavioural, autonomic and endocrine responses. The expression of these responses serves as a measure of the emotional memory created during the learning experience. Fear conditioning was conducted for both lines under basal conditions by the same experimenter in an isolated room and at the same time of the day. Male TgNTRK3 (n = 36) and wild type littermates (n = 45) were used. The fear conditioning apparatus consists of a transparent Plexiglas operant chamber (30 × 5 × 15 cm) placed inside a sound-attenuating box with a ventilating fan, which produced a white noise level of 60 dB. A transparent piece on the top of the box allowed a slight illumination inside the chamber (30 lux). The

grid floor was made of stainless steel rods (diameter 4 mm) spaced at regular intervals of 10 mm and through which scrambled electric foot shock could be derived. Between sessions, floor trays and shock bars were cleaned with soapy water and the chamber walls were wiped with a damp cloth. All tests were analyzed by the aid of the Startlefreezing program (Panlab SL, Spain). Behaviour was automatically recorded during CS presentation of each session and subsequently freezing, defined as the lack of movement except for breathing for at least 2 s, was analysed to give the percentage time freezing during exposition to CS.

For all the fear conditioning procedures, the day before fear conditioning (day 0), all mice were habituated to the chambers for 10 min. For fear conditioning (day 1), mice were placed again in the operant chamber, and after a 4 min baseline period, the animals were exposed to a CS-US pairing. The CS was an auditory clicker (X Hz, 80 dB, 30 s) and the US a mild electric footshock (0.2 mA, 2 s). Finally, 30 s after the shock, mice were returned to their home cages.

Contextual fear conditioning. Twenty-four hours later (day 2), fear conditioning was assessed by returning the mice to the conditioning chamber and measuring freezing behaviour. After two minutes of exposure, the tone starts for a period of 3 minutes during which freezing time is assessed. A week later, we performed a retest session with the same parameters of test session.

Non-contextual fear conditioning. Twenty-four hours later (day 2), fear conditioning was assessed by exposing the mice to a different chamber and measuring freezing behaviour. After two minutes of exposure, the tone starts for a period of 3 minutes during which freezing time is assessed. A week later, we performed a retest session with the same parameters of test session.

Memory reactivation and extinction training. A) The day after training (day 2), memory reactivation was introduced by a brief 2 min session in which the animals were exposed to a single presentation of the 60 s CS after 60 s baseline period. Memory reconsolidation was measured 3h [post-reactivation short-term memory (PR-STM)], 24 h [post-reactivation long-term memory (PR-LTM)], and 7 d (PR-LTM2)] after memory reactivation, using a 2 min session of 60 s CS after 60 s baseline period. B) Extinction involved 10 presentations of 60 s CS in a 20 min session (60 s interstimulus interval (ISI)), and 24 h later, extinction test was measured using a 2 min session of 60 s CS after 60 s baseline period.

2.4. Startle-response

Prepulse inhibition Test

The acoustic startle reflex paradigm is a behavioural paradigm readily translated across species, and is commonly considered as an operational measure of sensorimotor gating in animals as well as humans. The startle reflex is a response to a sudden, intense stimulus, and the magnitude of this response is reduced if a weak sub-threshold prepulse stimulus is presented shortly before the startle-eliciting pulse stimulus. This reduction in startle reactivity is refereed to as prepulse inhibition (PPI). The acoustic startle response (ASR) magnitude and latency are influenced by the stimulus intensity

(Pilz et al, 1987) the interstimulus interval (Davis, 1970), ongoing motor behaviour (Wecker and Ison, 1986), and is variable among individuals (Plappert et al, 1993). It is also influenced by genetic differences (Glowa and Carl, 1994), by the diurnal rhythm (Davis and Sollberger, 1971), by the sensory environment [background noise (Hoffman and Fleshler, 1963), illumination (Walker and Davis (1997)], prepulses (Reijmers and Peeters, 1994) and by the administration of different drugs (Davis, 1980). Startle response apparatus consists of a transparent Plexiglas operant chamber (30 × 5 × 15 cm) situated inside a sound-attenuating box with a ventilating fan, which produced a background white noise level of 60 dB. A transparent piece in the top of the box allowed slight illumination inside the chamber (30 lux). The grid floor was made of stainless steel rods (diameter 4 mm) spaced at regular intervals of 10 mm, and to which a non-restrictive cylindrical enclosure made of clear Plexiglas was attached. Between sessions, floor trays, the grid floor and the cylindrical enclosure were cleaned with soapy water. A high-frequency loud-speaker mounted directly above the animal enclosure inside the chamber produced both a continuous background noise of 60 dB and the various acoustic stimuli. Vibrations of the Plexiglas enclosure caused by the whole-body startle response of the animals were transduced into analogue signals by a piezoelectric unit attached to the platform. These signals were then digitalized, stored and analyzed by the aid of the "Startle" program (Version 5.0, Panlab SL, Spain). The average amplitude over the 40 ms was used to determine the stimulus reactivity. The threshold sensitivity of the *stabilimeter* was set up for each animal to ensure that startle response signals were inside the detection limit.

For the demonstration of PPI of the acoustic startle response (ASR), animals were presented with a series of discrete trials comprising three types of trials. These included pulse-alone trials, prepulse-plus-pulse trials, and "no-stimulus" trials, in which no stimulus other than the constant background noise was presented. A reduction of startle magnitude in prepulse-plus-pulse trials relative to that in pulse-alone trials constituted PPI. The pulse stimulus employed was 120 dB in intensity and 40 ms in duration. Prepulses of various intensities were employed: 70, 74, 78, 82, 86 and 90 dB, which corresponds to 10, 14, 18, 22, 26 and 30 dB above background. The duration of prepulse stimuli was 20 ms. The stimulus onset asynchrony (SOA) of the prepulse and pulse stimuli on prepulse-plus-pulse trials was 100 ms. The startle response was recorded for 65 ms (measuring the response every 1 ms) starting with the onset of the startle stimulus.

On the day before the habituation to startle response (day 0), all mice were habituated to the cylindrical enclosure for 1 min. For startle response habituation (day 1), mice were placed again in the cylindrical enclosure inside the operant chamber, and after a 5 min baseline period, the animals were exposed to 6 pulse-alone trials (120 dB, 100 ms, 15 s ISI) . Finally, 15 s after the last pulse, mice were returned to their home cages. Twenty-four hours later (day 2), prepulse inhibition was assessed by returning the mice to the operant chamber and measuring startle response within 48 trials comprising 8 blocks of trials each randomly distributed. The interval between successive trials was 15 s. The following formula was used to calculate the prepulse inhibition of a startle response: 100 – [(startle response on acoustic prepulse-plus-startle stimulus trials/startle response on startle alone trials) x 100]. Thus, a high percentage prepulse inhibition value indicates a good prepulse inhibition, i.e., the animal showed a reduced startle response when a prepulse stimulus was presented compared to the response when the startle stimulus was presented alone.

3. NEUROMORPHOLOGICAL CHARACTERIZATION

3.1 Immunohistochemistry

Animals were deeply anaesthetized with isofluorane and sacrificed. Then, mice were perfused through the left ventricle with 50 mL of phosphate buffered saline (PBS) 0.1M, pH = 7.4 (Sambrock et al, 1989), and 100 mL of cold-paraformaldehide (PFA) 4% in PBS by means of a peristaltic pump (5 mL/min). Brains were removed and postfixed overnight in the same fixative solution at 4ºC. 24 hours later, the brains were immerse in 30% sucrose cryoprotected solution overnight at 4ºC and they were frozen at -80ºC. 40-µm coronal frozen sections were distributed in two or three series of free-floating sections and stored in 0.1M PBS containing 30% ethylenglycol and 30% glycerol at -20ºC.

With the aid of a stereotaxic brain mouse atlas (Franklin and Paxinos, 1977), brain slices containing regions of interest were selected. Sections were washed twice with 0.1M PBS for 5 min, and treated with 0.1M PBS containing 10% methanol and 3% hydrogen peroxydase for 30 min. Slices were then washed twice with 0.1M PBS for 5 min and three times in 0.1M PBS containing 0.2% Triton X-100 (PBS-T) to increase tissue permeability and enhance antibody penetrance. Afterwards, the slices were incubated for 1 h with 0.1M PBS-T containing 0.25% gelatine (PBS-T-GEL) and 10% fetal bovine serum (FBS) to avoid unespecific antibody interactions. Primary antibody was diluted to the working dilution in 0.1M PBS-T-GEL and 5% FBS and slices were incubated overnight at 4ºC. On the next day, sections were washed three times in 0.1M PBS and incubated for 1 h in 0.1M PBS-T-GEL and 5% FBS with the biotynilated secondary antibody (1:250, Vector, UK). Sections were washed three times in 0.1M PBS-T and incubated for 1 h in avidin-biotin peroxidase complex (ABC Elite; Vector Laboratories, USA). After washing three times in 0.1M PBS-T and twice in 0.1M PBS, sections were incubated in 0.1M PBS containing 0.05% of 3,30-diaminobenzidine (DAB; Sigma, USA) and 0.1% hydrogen peroxide. Developer time ranged from 1 to 3 min and was specific for the antibody used. Finally, the reaction was stopped by three washes in 0.1M PBS, and slices were mounted in gelatinized slides and dried at room temperature for 24 h.

On the following day, slides were counterstained with cresil violet to facilitate the identification of neuronal nuclei and were dehydrated by an increasing graduation alcohol battery ending with xylene. Coverslips were mounted with Eukit (Electron Microscopy Science, USA).

3.2. Histochemical staining

Timm's Staining

The staining obtained using the Timm method, which is applied for the histochemical detection of zinc and heavy metals (Timm, 1958), clearly defines the borders and lamination of some areas of the cerebral cortex Timm-positive zones of the hippocampus of mammals, especially those occupied by mossy fiber (MF) endings.

Animals were deeply anaesthetized with isofluorane and sacrificed. They were then were perfused through the left ventricle with 50 mL of sodium sulfide and 100 mL of cold-glutaraldehyde solution by means of a peristaltic pump (5 mL/min). Thereafter, brains were removed and placed for 24 h in a postfixative solution of 3% glutaraldehyde and 20 % sucrose at 4ºC, after which the brains were frozen at -80ºC. When the dorsal hippocampus was identified during the process of cutting the brains horizontally in a cryostat, 40-µm horizontal frozen sections were distributed on gelatine-coated slides, with a small droplet of distilled water on the gelatinized slides.

Sampling started immediately below the most ventral extension of the septal pole of the fascia dentata, taking every second section. For morphometry, 5 sections were sampled pseudo-randomly, alternating between the right and left hippocampus. Methods used for visualization and measurements of the hippocampal terminal fields were similar to those described previously (e.g. Laghmouch et al, 1997). Morphometrical measurements were done blindly with respect to genotype. Areas of the MF terminal fields (CA4 (hilus), suprapyramidal MF (SPMF), and intra- and infrapyramidal MF (IIPMF); see Figure 2) were measured on an image analyzing system (Samba, Alcatel) and were expressed as a percentage of the whole regio inferior (hilus + CA3) to correct for possible slight variations in cutting plane or tissue shrinkage. This standarized method has been shown to yield reliable and replicable results (Schwegler and Lipp, 1983).

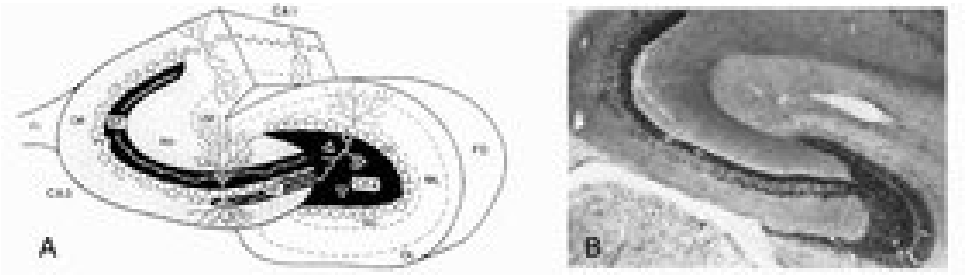


Figure 2. Diagram of a Timm-stained cross-section of the hippocampus.
A) Schema of hippocampal layes and MF projections at the midseptotemporal level. The hippocampal subregion hilus-CA3 (the area of morphometry) is indicated in black, stippled, and hatched areas. Black areas: suprapyramidal (SP), intra- and infrapyramidal (IIP) and hilar (CA4) MF terminal fields originating from the dentate gyrus. Stippled area: strata oriens (OR) and radiatum (RD). Hatched area: stratum lacunosum-moleculare (LM). CA1, subregion of the hippocampus without MFs; FI, fimbria hippocampi; FD, fascia dentata; OL and ML, outer and middle molecular layers of the fascia dentata; SG, supragranular layer; GC, granular cells.
B) Representative Timm-stained horizontal section of a mouse from C7BL/6 inbred strain.

3.3. *In situ* hybridization

Acute restrained mice were sacrificed 3 h after stress session with isoflurane overdose, brains were removed, and frozen in dry ice. Brains were sectioned at -20ºC in a cryostat microtome at 18 µm in the coronal plane through the level of the hypothalamic PVN and dorsal hippocampus. The sections were thaw-mounted on poly-l-lysine-coated slides, dried, and kept at -80ºC. In situ hybridization using ^{35}S-uridine triphosphate-labeled ribonucleotide probes for CRH and GR were

performed as described previously (Schmidt et al, 2002). The cDNA probes for CRH and GR contained the full-length coding regions of CRH (rat) and GR (mouse), respectively.

Briefly, for riboprobe *in situ* hybridization, sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine–HCl. Subsequently, the tissue was run into an alcohol train (70, 80, 90, 100 % diluted in distilled water). The antisense probes were transcribed from a linearized plasmid. Tissue sections (five brain sections per slide) were saturated with 100 µl of hybridization buffer (20 mM Tris–HCl (pH 7.4), 50% formamide, 300 mM NaCl, 1 mM EDTA (pH 8), 1X Denhardt's, 250 µg/ml yeast transfer RNA, 250 µl/ml total RNA, 10 mg/ml salmon sperm DNA, 10% dextran sulfate, 100 mM dithiothreitol (DTT), 0.1% SDS and 0.1% sodium thiosulfate) containing 1.5×10^{6} cpm ^{35}S-labeled riboprobe. Brain sections were coverslipped and incubated overnight at 55ºC. The following day, the sections were rinsed in 2XSSC (standard saline citrate), treated with RNAse A (10 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally sections were washed in 0.1XSSC for 30 min at 65ºC dehydrated through increasing concentrations of alcohol, exposed to Kodak Biomax MR film (Eastman Kodak, USA) and developed.

3.4. Stereology

For quantitative analysis of cell density, stereological methods were applied. An Olympus BX51 microscope was connected to a videocamera and with a motorized platine directed by a threedimensional automatic controller (microcator). The software CAST-GRID (Olympus, Germany) was used to ensure a random systematic sampling.

Estimation of the volume (Vref) of these regions was performed with the Cavalieri method, and the optical dissector method was used to estimate neuronal density (Nv). 10 dissector probes of 1739 µm2 (Sdis) with a thickness (Hdis) of 15 µm [V (dis) = Sdis x Hdis = 25890 µm3; guard zone = 5 µm to the surface of section] were analyzed per section, using a 40x objective to count neuronal nuclei in sampling probes. Estimation of total number of neurons was obtained according to the formula: N (neu) = Nv x V (ref), and the coefficient of error, CE = SEM/mean was calculated to evaluate the precision of the estimates. Sampling was optimized to produce a coefficient of error (CE) under the observed biological variability (West and Gundersen, 1990). CE, the estimated intra-animal coefficient of error, was calculated according to Gundersen and Jensen (1987). To estimate the volumetric shrinkage factor (SV), the thickness before and after processing was analyzed using the computer-driven z-axis of the microscope. This analysis revealed an average thickness shrinkage factor of about 0.86 that was similar in wild type and TgNTRK3 mice.

4. PHARMACOLOGICAL STUDIES

4.1. D-amphetamine and Dizocilpine (MK-801) challenge

The following drugs were used: D-amphetamine sulfate (5.0 mg/kg, Sigma, USA), and MK-801 (0.5 mg/kg, Sigma, USA). All drugs were dissolved in saline solution (0.9%) and administered intraperitoneally (ip), and the injection volume was 0.1 ml/kg.

Mice were tested in a baseline session to determine PPI and startle reactivity. Three days later, mice were assigned to receive a dose of D-amphetamine, MK-801 or vehicle (balanced for acoustic PPI, acoustic startle reactivity, startle chamber assignment, and treatment) and were tested in the PPI session. X TgNTRK3 and Y wild type littermates were treated with d-amphetamine (5 mg/kg), MK-801 (0.5 mg/kg) or saline 10 min before placing them into the PPI chamber.

5. HYPOTHALAMIC-PITUITARY-ADRENAL AXIS RESPONSE CHARACTERIZATION

5.1. Blood sampling

Blood sampling for the determination of neurohormonal levels in basal and stress conditions was performed by tail-nick procedure, wrapping the animal with a cloth and performing a 2 mm incision at the end of the tail artery. Massaging the tail of the animal allowed the collection of 100 μl of blood in an ice-cold EDTA-coated capillary tube (Sarstedst, Germany). All blood samples were kept on ice and later centrifuged (15 min, 1600 g at 4 °C). Plasma was transferred to clean, labelled 2 ml microcentrifuge tubes. All plasma samples were stored at -20°C until the determination of corticosterone by radioimmunoassay (RIA) as previously reported (Marquez et al, 2006).

5.2. Biochemical analysis

Corticosterone RIA used ^{125}I-carboximethyloxime-tyrosine-methyl ester (ICN-Biolink 2000, Spain), synthetic corticosterone (Sigma, Spain) as the standard, an antibody raised in rabbits against corticosterone-carboximethyloxime-BSA kindly provided by Dr. G. Makara (Inst. Exp. Med., Budapest, Hungary) and plasma corticosteroid-binding globulin was inactivated by low pH. Plasma glucose levels were measured by the glucose oxidase method using a commercial kit (Boehringer-Mannheim, Spain). All samples to be compared, from each experiment, were run in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was 4.9% for corticosterone and 4% for glucose.

5.3. Circadian rhythm

In non-stressful situations both CRH and AVP are secreted in the portal system in a circadian, pulsatory fashion, with a frequency of about two to three secreting episodes per hour (Engler et al, 1989). Under resting conditions, the amplitude of the CRH and AVP pulses increase in the early

morning hours, resulting finally in ACTH and cortisol secreting bursts in the general circulation (Horrocks et al, 1990). These diurnal variations are perturbed by changes in lighting, feeding schedules and activity and are disrupted by stress.

Male mice of either genotypes (14 TgNTRK3 and 10 wild type mice) were used for the measurement of corticosterone circadian rhythms (see below). Previous to blood extraction, animals were submitted to standarized handling to reduce the possible stressor effect of blood sampling procedure on corticosterone secretion. To measure corticosterone circadian rhythms, blood samples were obtained at 8:00 a.m. (at the beginning of diurnal phase) and 8:00 p.m. (at the beginning of nocturnal phase) (Moore and Eichler, 1976). A separate group of animals (12 TgNTRK3 and 14 wild type mice) was included to obtain basal corticosterone levels at 2:00 p.m. (diurnal phase midpoint) (Figure 1).

5.4. Acute stress procedures

We used novel environment and restraint as acute stressors in 14 TgNTRK3 and 10 wild type mice. Three days after measuring basal neurohormonal levels, mice were submitted to an open field during 20 min (novel environment). Four days later mice were immobilized in a metallic mesh during 30 min (restraint). Blood samples for neurohormonal and glucose determination, were obtained immediately after the exposure to either stressor (see below). One week later, animals were sacrificed using isofluorane and adrenal glands were dissected, weighed and processed to analyze adrenal/animal weight ratio, and the protein amount/adrenal weight ratio.

A different group of animals were submitted to acute immobilization (IMO) to the study of FOS immunoreactivity. Thirteen TgNTRK3 and 14 wild type mice were randomly distributed in stressed group receiving 30 min of immobilization (IMO) and control group [(acute IMO group n = 14 (7 TgNTRK3 and 7 wild type) and non disturbed control group n = 12 (6 TgNTRK3 and 6 wild type)]. Mice were immobilized by taping their limbs to the four metal arms radiating outwards of a customized immobilization board, where in addition, a central *velcro* band that hold down the shoulder of the mouse to the board was used to complete immobilization. This system allows a better immobilization of the paws and body, thus preventing possible lesions and allowing the obtention of more reproducible results. Acute IMO group was exposed to a single session of IMO stress for 30 min, which has been proved to produce maximal responses of the stress brain areas (Cullinan et al, 1995). In this experiment, cortiscosterone levels were not measured since this manipulation can affect Fos expression.

To investigate neuronal activity in response to external stimuli in rodents, the immediate early gene c-Fos is generally used as a marker. Neuronal c-Fos gene expression is cued by depolarization-induced Ca^{2+} influx (Morgan and Curran, 1991). By virtue of the fact c-fos mRNA and protein levels are generally near the level of detection in quiescent neurons, stimulus-induced c-Fos expression is widely used as a marker of neuronal activation.

5.4.1. c-Fos determination

After an appropiate stimulus, c-Fos expression occurs rapidly, usually within a few minutes, with a peak response within 30 min from the time of the initiation of stress. Fos protein is detectable by immunohistochemistry somewhat later, with maximal levels at 60-90 min after the stressor. Two hours after IMO exposure, animals were deeply anaesthetized with isoflurane, and perfused pericardially with PBS followed by 4% paraformaldehyde (PFA). Brains were extracted, postfixed overnight in 4% PFA, and cryoprotected in 30% sucrose. Cryostat sections (40-µm) were obtained and c-Fos labelled cells from the first two consecutive sections for each mouse were quantified by an investigator blinded to genotype using stereological methods as described (Sahún et al, 2006). The anatomical boundaries for the structures tested were defined based on the coordinates of Paxinos atlas (Paxinos and Franklin, 2001): cingulate cortex (Cg) (Bregma 2.58 to -0.82mm), prelimbic cortex (PrL) (Bregma 2.96 to 1.42mm), infralimbic cortex (IL) (Bregma 2.10 to 1.34mm), lateral septal (LS) nucleus [ventral (LSv) (Bregma 1.18 to -0.10mm) and dorsal (LSd) (Bregma 1.42 to -0.46mm)], bed nucleus of stria terminalis (BNST) [ventral (BSTv) and dorsal (BSTd) (Bregma 0.62 to -0.46mm)], central amygdaloid nucleus (CeA) (Bregma -0.58 to -1.94mm), paraventricular nucleus of the hypothalamus (PVN) (Bregma -0.58 to -1.22mm), hippocampus [CA1, CA2, CA3 and dentate gyrus (DG) (Bregma -0.94 to -3.88mm)], and LC (Bregma -5.34 to -5.80mm) were analyzed. In this experiment hormonal levels were not quantified to prevent any possible confounding effects.

5.5. Chronic stress procedures

5.5.1. Chronic immobilization stress (CIS) and social isolation

An independent group of animals was randomly assigned to social isolation [n = 32 (16 TgNTRK3 and 16 wild type] or group-housing [n = 46 (20 TgNTRK3 and 26 wild type)] conditions during 3 weeks. Then, each group was distributed into CIS or control subgroups. The animals were subjected to IMO stress (30 min/day) for 15 consecutive days and sacrificed 150 min after the last IMO (Govindarajan et al, 2006). On days 1, 3, 7 and 14, blood samples were taken at 0 and 90 min after the end of IMO (post-IMO), in order to study the dynamics of HPA hormone responses.

5.5.2. Chronic environmental stress (CES)

Changes in environment conditions, such as photointensity, photoperiod and spectral quality of the light are potential stressors affecting mouse behaviour and HPA responses (Stoskopf, 1983; Brainard, 1989). We used two different illumination conditions, high and low light intensity, during the light phase of the cycle. The high illuminated (stress) group was reared under high intensity light levels (800-1000 lux), and the low illuminated (non-stress) group received 50-100 lux in their home cages for 3 weeks. Stress/non-stress rearing conditions were maintained until animals were sacrificed.

We used two new independent groups of transgenic and wild type mice in order to avoid repeated testing influence on the CES effects. Each group was subjected to CES in the animal rearing room.

When the first group had been tested and sacrificed, the second group began the exposure to CES allowing reproducible CES effects.

The first group was exposed to forced swimming test (FST) 21 days after the initiation of CES [27 TgNTRK3 (14 stressed and 13 non-stressed) and 23 wild type mice (11 stressed and 12 non-stressed)]. Finally, 21 days after FST, a subgroup of mice were randomly assigned to acute restraint [n = 18 (10 TgNTRK3 and 8 wild type)] or non-disturbed control group [n = 13 (7 TgNTRK3 and 7 wild type)] to perform CRH and GR mRNA expression studies. The second group were exposed to the light/dark test 21 days after the initiation of CES [37 TgNTRK3 (18 stressed and 19 non-stressed) and 23 wild type mice (12 stressed and 11 non-stressed)].

6. GENE EXPRESSION PROFILING

6.1. Microarrays

RNA samples were derived from adult hippocampal tissue of 6 TgNTRK3 mice or 6 of their respective wild type littermates using TRIzol reagent and following the manufacturer's RNA isolation protocol (Invitrogen, #15596). One milliliter of TRIzol solution was added to each tube containing the frozen tissue block, and the tissue was homogenized by Polytron power homogenizer. After centrifugation, the RNA was precipitated from the aqueous layer, washed, and dissolved in Rnase-free water. Concentration was measured using Nanodrop spectrophotometer and RNA quality was assessed using the Bioanalyzer 2100 nanoelectrophoresis Lab-on-a–chip system (Agilent). All RNAs had RIN equal or above 8.3 and 28S/18S ratios above 1.35. The RNA samples were stored at -80ºC. 500 ng of total RNAs were amplified and labelled using the Low Input Linear Amplification Kit (Agilent 5184-3523) and hybridized following manufacturer's instructions.

Commercial in situ synthesized oligonucleotide microarrays (Agilent G4121A) were used containing 22393 spots with 60mer probes, 20869 of which represented as single or multiple copies a total of 20841 mouse transcripts. Two biological replicate experiments comparing a pool of two transgenic samples with a pool of two wild type samples were performed. Each pair of pools was hybridized twice with dye swapping to correct for dye bias effects, leading to a total of four microarray hybridization datasets.

Fluorescent images were obtained using an Agilent G2565BA scanner and quantified using GenePix 6.0 software (Axon, Molecular Devices) using the irregular feature finding option. Extracted raw data was filtered and normalized using MMarge (Lozano et al, unpublished), an in house web server implementation of the Limma package developed within the Bioconductor project in the R statistical programming environment (R Development Core Team, 2005). The two channels were balanced by lowess normalization using 0.3 as the span parameter with reduced weights in control and poor quality spots, followed by scaling across chips. An Empirical Bayes B statistic was computed taking into account dye swapping and the probes were ranked according to B value, although given the small number of replicates it was only taken as a ranking measure.

Differentially expressed genes were chosen using as cutoff criteria a B rank percentile above 95% and absolute FC above 1.19. Besides, we used the following quality filter: GenePix flag status found in 1 of the 4 hybridizations and normalized background subtracted intensitiy in at least one of the two channels above 250 in at least 1 of the 4 hybridizations. Data tables were visualized using the Array File Maker application (Breitkreutz et al, 2001).

For GO analysis Agilent identifiers were taken to infer functional overrepresentation using GOstat package in Bioconductor (Beissbarth and Speed, 2004).

6.2. Quantitative Real-Time PCR (qRT-PCR)

Validation of mRNA levels of selected genes detected in the microarray assay was performed using qRT-PCR with the LightCycler® 480 system. A previous step consisted in treating the RNA samples with DNase, in order to eminate DNA. cDNA was synthesized from 1 µg of total RNA with the use of the Omniscript® RT Kit (Qiagen GmbH, Hilden, Germany) in a 20 µl final volume, according to the manufacturer's instructions. Absence of genomic DNA contamination was determined by the amplification of a 126-bp PCR fragment and the non-amplification of a 235-bp fragment from cDNA samples with primers for *Gdx* (ubiquitin-like protein) transcript.

qRT-PCR was carried out in duplicates with 1 µl sample of cDNA solution, diluted 1/10, using the LightCycler® 480 SYBR Green I Master kit (Roche Diagnostics GmbH, Germany) in a LightCycler® 480 Real-Time PCR system (Roche Diagnostics GmbH, Germany) according to the manufacturer's recommendations, except that the final volume of the reaction was 20 µl. Reactions were performed in a 20 µl volume with from reverse transcription reaction 1 µl of one-tenth H_2O-diluted cDNA sample, 1.2 µl of each primer (Forward and reward; 0.3 µM/primer final concentration), 6.6 µl of RNase-free water and 10 µl of 2X SYBR Green I Master solution (Roche Diagnostics, Mannheim, Germany). The same aliquots of reverse-transcription reactions without added reverse transcriptase were used as negative controls. The cDNA solution was subjected to 45 cycles of PCR amplification by using specific primers from the selected genes. Gene expression was normalized using alpha-tubulin (*Tuba1*) as housekeeping gene. Oligonucleotide primers for selected target genes were chosen to span intron–exon boundaries and were designed with the PRIMER 3 program. Primers were synthesized by Sigma-Aldrich (Spain). Analysis was performed on a LightCycler 480 (Roche Diagnostics) under the conditions recommended by the supplier. Standard errors on all samples were generally ±2%.

qRT-PCR cycles were set at 10-min denaturation followed by 45 cycles of 95ºC for 10 s, 60ºC for 15 s, and 72ºC for 10 s and 95ºC. Dissociation curves were inspected for each pair of primers, and only dissociation peak must be present for each run of reaction before the results were considered to be valid. A 4-fold serial dilution of a reference sample was used for construction of standard curves with respect to each pair of primers. All samples were amplified in duplicate and the CT mean was obtained for further calculations. Duplicates with standard deviation (SD) ≥0.38 were excluded, and these samples were reamplified. CP values for each reaction were normalized to those for Tuba1 reactions included in each set of qRT-PCR runs and are expressed relative to values obtained with

RNAs from hippocampal cells. The ΔΔCt method (Relative Expression Software Tool (REST)) was used to quantify the relative amount of mRNA in each sample in comparison with the control samples. Next, 2−ΔΔCt of each gene was transformed into logarithmic scale (log2 ratio).

7. DATA COLLECTION AND ANALYSIS

c-Fos immunohistochemistry was analyzed with the help of CAST-GRID software package adapted to an OLYMPUS BX51 microscope (Olympus, Denmark). In situ autoradiographs were digitized, and relative expression was determined by computer-assisted optical densitometry (Scion Image, Scion Corporation). All behavioural tests were videotaped and analyzed by the aid of the Observer program (Version 5.0, Noldus). Parametric data are reported as the mean ± SEM. Analysis of the main effects of stress exposure and genotype, and interactions between these variables on behavioural and biochemical measures was performed with multivariate analysis of variance (MANOVA) followed by analysis of variance (ANOVA). The statistical analysis was performed using the SPSS for windows package. Significance levels were set at $p < .05$.

RESULTS

Lo que se debe aprender a hacer se aprende haciéndolo
[Aristóteles (384 BC – 322 BC)]

1. STRUCTURAL CHARACTERIZATION OF THE HIPPOCAMPUS AND AMYGDALA IN A MURINE MODEL OF *NTRK3* GENE OVEREXPRESSION (TGNTRK3)

1.1. TgNTRK3 mice showed functional and structural hippocampal alterations

1.1.1. Hippocampal stereological characterization of TgNTRK3 mice

The hippocampus is a key region in the limbic system, which plays a key role in short term memory, response to novelty and spatial navigation in humans and rodents (Figure 1A).

Brain sections of TgNTRK3 mice exhibited normal hippocampal cytoarchitectonics when compared with those from age-matched wild type littermates (Figure 1B). When the hippocampal formation was analyzed in detail, a preserved layering was observed in TgNTRK3 mice (Figure 1C).

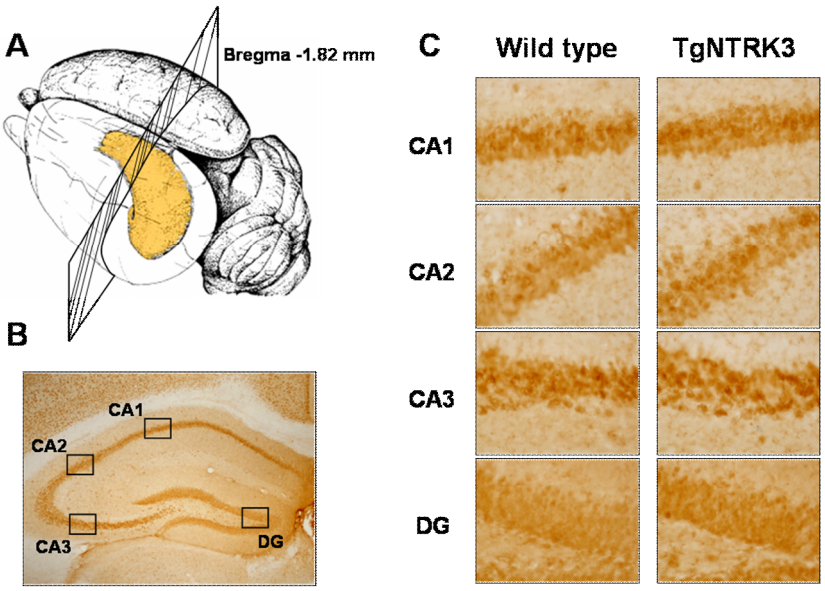


Figure 1. Stereological analysis in the hippocampus of TgNTRK3 mice.
A) Illustration of the hippocampus in the mouse brain. A plane is placed in the brain showing the location (Bregma -1.82 mm) of hippocampal immunohistochemistry presented in figure B.
B) NeuN immunohistochemistry showing the preserved layering in hippocampal subregions [CA1, CA2, CA3, and dentate gyrus (DG)] in TgNTRK3 mice. The squares represent the magnifications in figure C.
C) CA1, CA2, CA3 and DG hippocampal subregions magnification (400X) of a wild type and a TgNTRK3 mouse.

The analysis of the total volume of the hippocampus using the Cavalieri method showed no differences between genotypes. Nevertheless, in sections stained with the Nissl technique (0.1% cresyl violet), cell density and numbers in TgNTRK3 mice showed a tendency to an increase that reached its maximum statistical significance in the CA3 region. Figure 2A shows the mean density of NeuN-positive neurons in CA1 (20.4%; $t_{14} = 2.37$, $p < .05$), CA2 (21.2%; $t_{12} = 1.35$, $p < .01$), CA3 (32.2%; $t_{14} = 2.04$, $p < .001$), and dentate gyrus (DG) (23.8%; $t_{12} = 1.64$, $p < .01$) in TgNTRK3 mice.

To discern the possible differential effects of TrkC overexpression on glial and neuronal cells, we used specific stainings for both cell types (see methods). When glial, GFAP-positive cells were evaluated (Figure 2B), the mean density was significantly reduced in CA1 (-28.9%; t_{12} = .04, p < .05) and CA2 (-25.4%; t_{12} = 2.07, p < .05) of TgNTRK3 mice, with no differences in CA3 or DG (all p values < .05).

Conversely, when evaluating neuronal cell density, a significant increase was detected in all the hippocampal subregions in TgNTRK3 mice, thus suggesting that hippocampal-dependent cognitive processes could be affected in this mouse model.

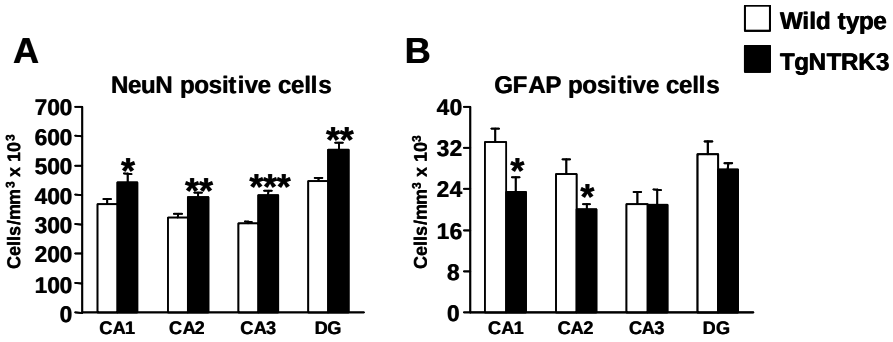


Figure 2. Stereological analysis of the hippocampus in TgNTRK3 mice.
A) A significant increase in NeuN-positive cell density in CA1, CA2, CA3, and dentate gyrus (DG) was observed in TgNTRK3 mice.
B) The density of GFAP-positive cells was reduced in CA1 and CA2 regions of TgNTRK3 mice.
Data are expressed as mean ± S.E.M. *P<.05 **P<.01 ***P<.001.

1.1.2. Hippocampal neurogenesis in TgNTRK3 mice

New neurons are continually born throughout adulthood in the subgranular zone of the DG. Although the function of adult hippocampal neurogenesis is not certain, there is some evidence that is important for learning and memory (Kempermann et al, 2004), and the regulation of stress (Malberg et al, 2000).

We have compared the effects of TrkC overexpression on the progenitor proliferation rate in the subgranular zone (SGZ) of the DG. The majority of BrdU-labelled cells were located within the SGZ of the DG where they appeared either singly or in clusters. Intensity of immunolabeling in individual cells was the same between genotypes. A similar basal-proliferation rate in TgNTRK3 (Figure 3; t_3 = -.27, p = .81) was found when compared with wild type mice.

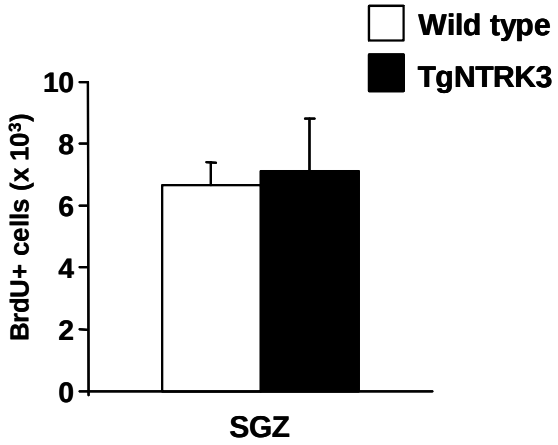


Figure 3. Stereological analysis of the subgranular zone (SGZ) of DG in TgNTRK3 mice. TgNTRK3 did not show differences in BrdU-positive cells in SGZ of DG vs wild type mice.
Data are expressed as mean ± S.E.M.

1.1.3. Hippocampal mossy fibers density in TgNTRK3 mice

In the hippocampus, granule cells of the DG send axonal projections, called mossy fibers (MF), to the CA3 region. As the DG is thought to contribute to the formation of new memories and to play a role in stress and depression, we were interested to measure the density of these projections.

The results obtained for the hippocampal MF density in both genotypes are presented in figure 4. Analysis of the mean area stained for hippocampal MF did not show differences between genotypes (Figure 4B). However, a more detailed analysis revealed that TgNTRK3 showed a slight increase in the percentage area for the hylus (Figure 4C; t_{13} = .03, p = .13), but normal percentage area for intra- and infrapyramidal (IIPMF) (t_{13} = 1.08, p = .96) or suprapyramidal (SPMF) (t_{13} = 1.96, p = .71) MF in comparison with wild type mice.

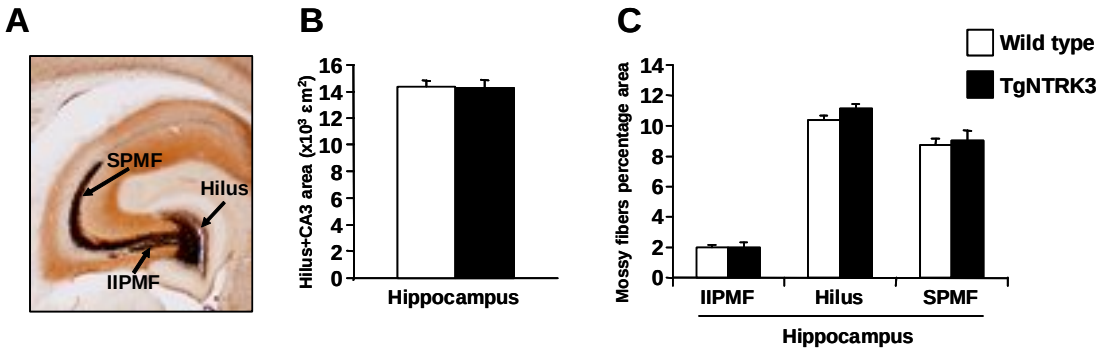


Figure 4. Hippocampal mossy fibers analysis in TgNTRK3 mice.
A) Representative Timm-stained horizontal section [Hilus, intra- and infrapyramidal (IIPMF) and suprapyramidal (SPMF) mossy fibers].
B) Analysis of the regio inferior (hilus+CA3) did not show differences between TgNTRK3 and wild type mice.
C) TgNTRK3 did not show differences in the percentage area of IIPMF or SPMF vs wild type mice; however, a slight increase in the hilus of DG percentage area was observed in TgNTRK3 mice.
Data are expressed as mean ± S.E.M.

1.1.4. Hippocampal gene expression profiles in TgNTRK3 mice

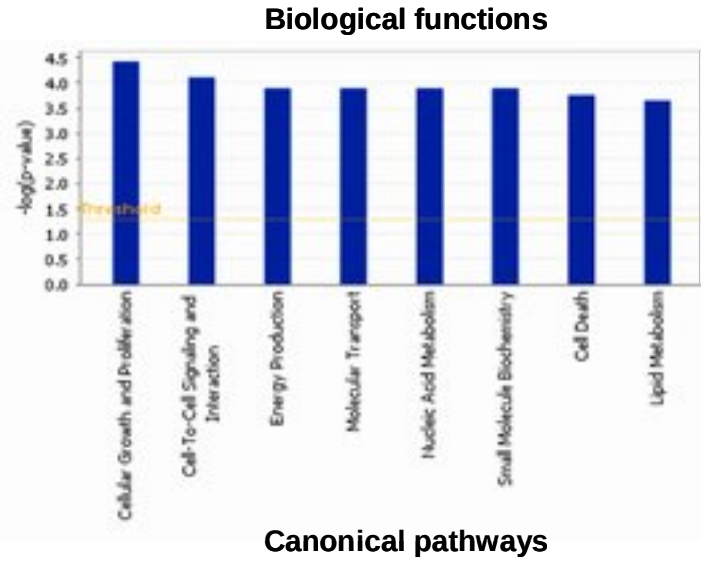
TrkC overexpression induce neuroplastic changes in brain pathways subserving emotion and cognition, such as hippocampus. Such changes may account for the information processing processes in TgNTRK3 mice. Here we compare gene expression profiles in mouse hippocampus from TgNTRK3 and wild type mice,. comprehensively to clarify the molecular mechanisms of hippocampal function through TrkC overexpression.

Only 69 candidates were shown to have at least an absolute 1.15 - fold expression change relative between the TgNTRK3 and wild type samples. To identify functional modules of gene expression and interacting partners that were relevant to the NTRK3 gene overexpression signature, we applied network-based analysis through the Ingenuity Pathways Knowledge Base (IPA) (Calvano et al, 2005). Fisher's exact tests were then performed to determine the likelihood that these genes of interest participated in a given function or pathway, relative to the total number of occurrences of these genes in all functional/pathway annotations stored in the IPA. These analyses produced 37 candidate genes that could be placed into 8 networks. The top three networks incorporated 32 of the candidate genes within a network of 105 molecules with significance *P*-values between 10E-18 and 10E-31 (Figure 5A). These networks were predicted to be involved in cellular growth and differentiation, and cell death through various signalling mechanisms (Figure 5B).

A

	Molecules in Network	Score	Focus Molecules	Top Functions
1	Akt, AKT2, CD128, CD160, Cyclin A, DCN, EDA2R, ERK2, FCGR2B, FRS3, HLA-C*, HLA-G, IMPA1, IRF2, LGALS7, LSR82, LYN, MAP3K7IP3, MFAP, NUP12, OTUD7B, PSMA, RBCK1, TAL1, TCAM2, TIPA, TNFRSF19, TRAF6, TRAF01, WISP1, WTAP, ZFAND5, ZMYND11, ZNF274, ZNF675	21	14	Cell Signaling, Cell Death, Molecular Transport
2	ALAD, APOA5, APOC1, APOC2, APOE, Ca2+, CABP1, CCR7, CD47, cholesterol, CSB1, CXCL9, CXCR3, DMP1, ERC1, Fcgr2, GH1, glutamic acid, HOXA13, ITPR, ITPR3, NPL3DA, PCMT1, RNA polymerase II, SLC17A6, SLC17A6, SLC9A8, SLC9A3R1, TRAP, TYL2, TNF, TRPC4, UBQLN1, UBQLN2, ZIC1	18	9	Cell Signaling, Molecular Transport, Vitamin and Mineral Metabolism
3	AKT2, amino acids, CCR7, CDKN2B, CHRM1, CTSS, CTTN, EEA1, FRAP1, GLRB, GLYCTK, GPHN, HRAS, IDE, IL9, ITPR3D, LTBD3, NEUNA2, MAP3K8, NEPT2 (includes EG:172725), NSF, NFκB, PDPK1, PDXK, PFN1, Pax5, RAB5B, Ras homolog, RHOA, RIL3, RPS6, RTN4, TG, TGFB1, THPO	18	9	Cancer, Cell Morphology, Cellular Growth and Proliferation

B



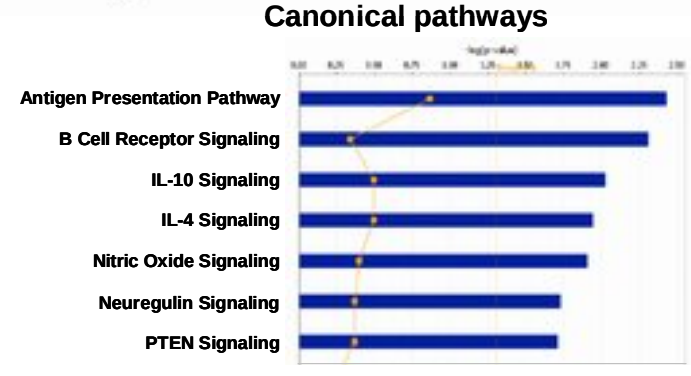


Figure 5. Ingenuity Pathway Analyses of differential gene expression in TgNTRK3 hippocampal region.
A) Interactome mapping predicts specific functional networks containing differentially expressed genes within the TgNTRK3 hippocampal region. The score reflects the statistical propability (P-value = 10E-18—10E-31) that the genes of interest participate in the specific pathway, relative to the total number of occurrences of these genes in all functional/pathway annotations stored in the IPA Knowledge Base. The number of focus molecules indicates the number of dysregulated genes within each network of 35 genes. The top function describes the predicted function of the network.
B) Graphical summary of the predicted biological functions and canonical pathways expected from the differentially expressed genes.

Of the three networks identified by IPA analyses, the first and third implicated Akt and NF-κB genes in functional pathways that are also incorporated multiple genes that were found to be dysregulated within the TgNTRK3 hippocampal gene expression profiles (Figure 6). Both NF-κB (Albensi and Mattson, 2000) and Akt (Horwood et al, 2006) have previously been implicated in processes of synaptic plasticity and memory.

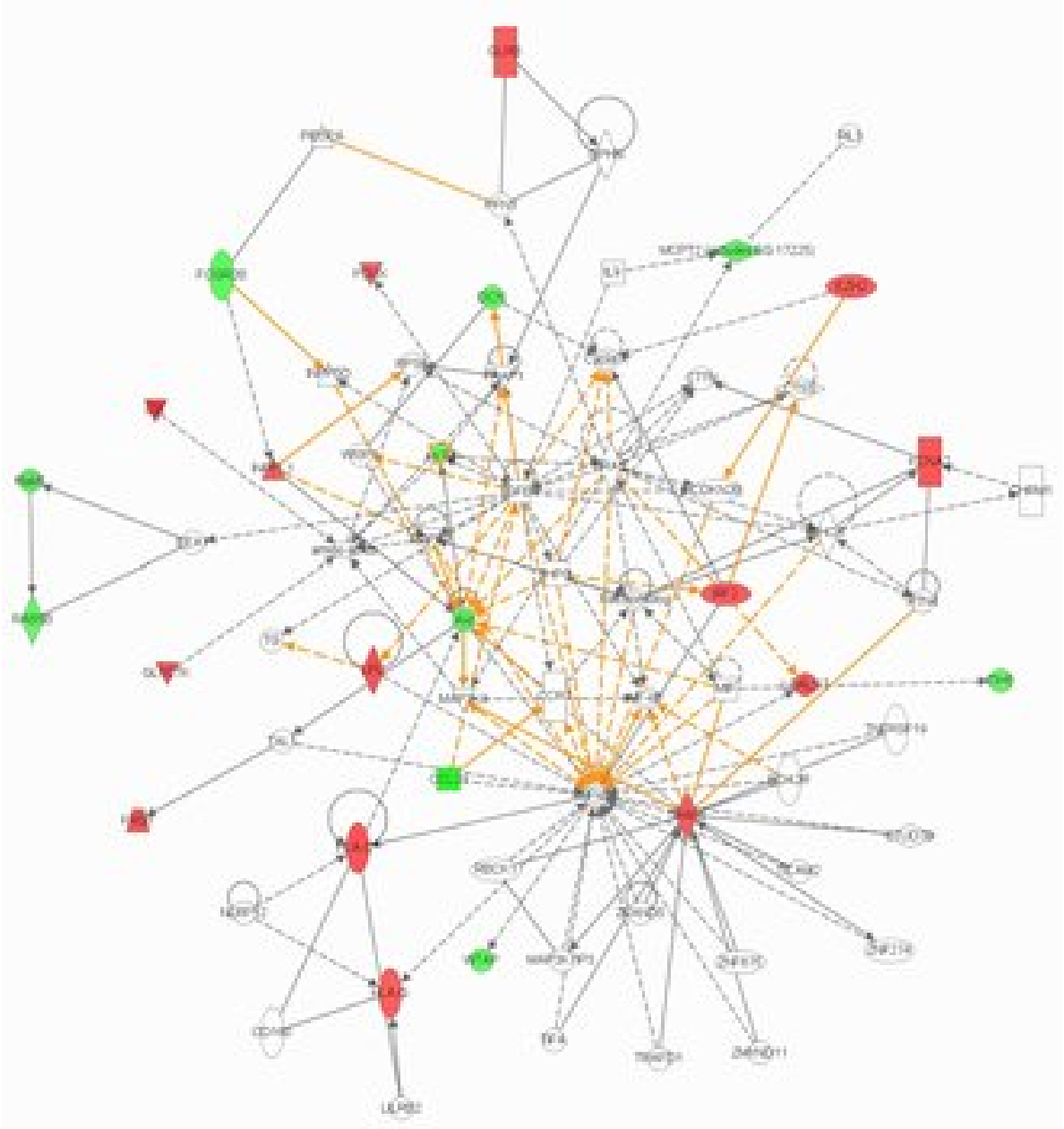


Figure 6. Schematic of network #1 and #3. Many of the differentially expressed genes in the TgNTRK3 hippocampal region form tight interrelationships within known pathways involved in cell signalling, cell death, molecular transport, cancer, cell morphology, and cellular growth and proliferation. Multiple dysregulated genes (red, upregulated; green, downregulated) form known interaction with each other suggesting a striking robustness of altered gene expression in TgNTRK3 hippocampal region. Hippocampal NTRK3 overexpression is predicted to alter Akt and NF-κB activity.

Validation of microarray results was performed using Lightcycler quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The differential expression of messenger RNA (mRNA) of 7 selected endogenous genes showed no differences in mRNA expression in hippocampus of TgNTRK3 mice (Table 1). Lipoprotein lipase (Lpl), CD47 antigen (CD47) and dynactin 3 (Dctn3) although differentially regulated in accordance with microarray data did not achieve statistical significance. However, Zinc finger protein of the cerebellum (Zic1), eukaryotic translation initiation factor 5B (Eif5b), pyridoxal (pyridoxine, vitamine B6) kinase (Pdxk) and inositol 1,4,5-triphosphate receptor 3 (Itpr3) gene expression were not different in TgNTRK3 with respect to wild type mice.

Gene	qRT-PCR relative exp.	p-value
Lpl	1.05	.66
Cd47	1.04	.76
Itpr3	-1.03	.70
Zic1	-1,05	.58
Eif5b	-1.08	.30
Pdxk	-1.08	.37
Dctn3	-1.10	.15

Table 1. Genes for qRT-PCR validation of microarray results in TgNTRK3 hippocampal region.

1.2. TgNTRK3 mice showed an amygdala hypertrophy

The amygdala plays a primary role in the processing and storage of memories associated with emotional events. In particular, research indicates that during fear conditioning, sensory and aversive stimuli converge to the BLA, and become associated. The BLA plays a central role in amygdaloid processing and modulation of emotional stimuli in the brain (LeDoux et al, 1990).

Brain sections of TgNTRK3 mice exhibited normal BLA cytoarchitectonics when compared with those from age-matched wild type littermates (Figure 7). Stereological analysis were performed in anterior part (BLAa, Bregma -0.70 to -1.34mm) and in posterior part (BLAp, Bregma -1.46 to -2.06mm) of the BLA.

The analysis of the total volume of the hippocampus using the Cavalieri method showed no differences between genotypes (BLAa: t_2 = -2.16, p = .16; BLAp: t_2 = -.61, p = .60; BLA Total t_2 = -1.29, p = .33). Nevertheless, in sections stained with the Nissl technique (0.1% cresyl violet),

amygdala cell density in TgNTRK3 mice showed a significant increase that reached its maximum statistical significance in the BLAp region. Figure 8 shows the mean density of cell density in BLAa (35.8%; t_2 = -3.99, p = .05), BLAp (46.5%; t_2 = -25.68, p < .01), and total BLA (40.2%; t_2 = -7.26, p < .05) in TgNTRK3 mice.

Evaluation of BLA revealed a significant increase in total cell density detected in TgNTRK3 mice, thus suggesting that amygdaloid-processes, such as emotional learning, could be affected in this mouse model.

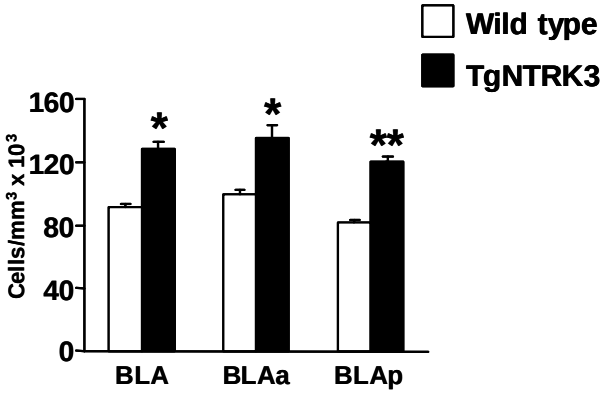


Figure 8. Stereological analysis of the basolateral amygdala (BLA). A significant increase in total cell density in BLAa, BLAp, and BLA was observed in TgNTRK3 mice. Data are expressed as mean ± S.E.M. *P<.05 **P<.01.

1.3. TgNTRK3 show increased consolidation and impaired extinction of fear memory

1.3.1. Contextual and non-contextual fear-conditioning in TgNTRK3 mice

We explored emotional memory formation and storage both dependent on hippocampal (contextual fear conditioning) and amygdala (non-contextual fear conditioning) LTP formation. In our experiments, both paradigms used the same training session involving the pairing of tone and footshock. In this training session the degrees of freezing response were similar in both genotypes thus discarding possible genotype differences in novelty-related responses or in the reactivity to either the sound or the shock (Figure 9A and 9B).

Freezing in the contextual conditioned response was significantly increased in both groups in the test session 24 h after training (Figure 9A), indicating that fear memory was acquired in both genotypes; however, TgNTRK3 showed substantially more freezing behavior (t_{10} = 10.47, p = .01) during the tone presentation than wild type mice. Seven days after the test session, mice were tested for conditioned fear to tone in the same chamber. When the tone was presented, TgNTRK3 exhibited higher freezing responses (t_{10} = .36, p < .001) than wild type mice.

In non-context fear conditioning, both groups showed an increase in freezing response in the test session compared to training (Figure 9B). As in the contextual paradigm, TgNTRK3 showed increased freezing response (t_9 = .76, p < .01) during the tone presentation in the non-contextual test. Long-

term fear memory measured 7 days after the non-context testing revealed increased fear memory in TgNTRK3 (t_9 = .36, p < .001).

TgNTRK3 mice showed an enhanced fear memory formation and storage in both contextual and non-contextual paradigms, that may indicate alterations in cognitive processes controlled by the amygdala and hippocampus.

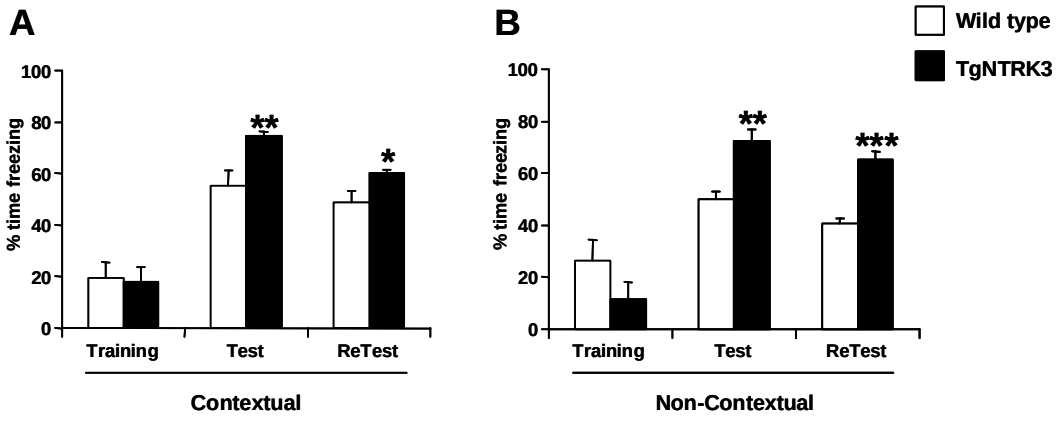


Figure 9. Effects of fear memory consolidation on conditioned freezing in TgNTRK3 mice.
A) TgNTRK3 showed higher freezing response in the test and retest sessions than wild type mice in both context, and **B)** non-context paradigms.
Data are expressed as mean ± S.E.M. *P<.05 **P<.01 ***P<.001.

1.3.2. Fear memory reactivation in TgNTRK3 mice

Memory reconsolidation is a distinct process that serves to maintain, strengthen or modify memories. Understanding the mechanisms of reconsolidation of normal mnemonic function could provide information about the dynamic aspects of psychiatric disorders characterized by strong and salient emotional memories. In rodents, reconsolidation is achieved by brief exposures to the neutral stimulus.

The effects of reconsolidation in associative memory on conditioned freezing in TgNTRK3 mice are shown in Figure 10. In order to examine long-term fear memory, contextual fear memory tests were conducted at 3 h (PR-STM), 24 h (PR-LTM), and 7 days (PR-LTM2) after reactivation. A repeated-measures ANOVA revealed a significant reduction of freezing response across trials in both genotypes ($F_{2,48}$ = 4.95, p < .05). TgNTRK3 did not exhibit differences in their freezing response across trials as compared to wild type mice. However, the reduction of freezing 7 days after last reconsolidation session was statistically significant in TgNTKR3 mice (t_{20} = .39, p < .05).

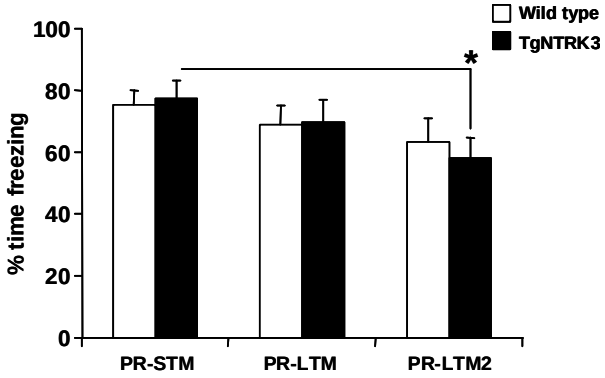


Figure 10. Effects of fear memory reactivation on conditioned freezing in TgNTRK3 mice. Freezing response was reduced across trials in TgNTRK3 (filled bars) and wild type (open bars) mice; however, TgNTRK3 showed a higher reduction of freezing 7 days after reactivation (PR-LTM2). Abbreviations: PR-STM (3 h after Reactivation), PR-

LTM (24 h after reactivation) and PR-LTM2 (7 days after reactivation). Data are expressed as mean ± S.E.M. *P<.05

Our results revealed that TgNTRK3 have similar freezing response than wild type mice in the retest sessions, suggesting that fear memory reconsolidation is not altered by *NTRK3* overexpression.

1.3.3. Extinction of fear memory in TgNTRK3 mice

A crucial role for the extinction process of fear memories has been hypothesized in maladaptative memory disorders, such as PD or PTSD. In rodents, the repeated exposure to the neutral stimulus without pairing it with the aversive stimulus, is a validated procedure to study the extinction process of fear memories.

A repeated-measures ANOVA analysis on extinction trial blocks revealed a significant reduction of the freezing response across trials in both genotypes (Figure 11A; $F_{9,270} = 9.01$, $p < .001$).

Delayed fear extinction response was observed in TgNTRK3 suggesting impairment in extinction of fear memories. Post hoc analysis revealed that, although freezing progressively decreased in both groups over extinction trial blocks, TgNTRK3 mice showed significantly higher freezing that wild type mice in the first, second, third and fourth extinction trial blocks (all p values < .05), with a non-significant trend for higher freezing in TgNTRK3 mice on the sixth trial block (p = .09) (see Figure XA).

Twenty-four hours after extinction, mice were given an additional extinction tone to test consolidation of extinction, and the percent time of freezing was assessed. Freezing levels in the extinction trial were 81% and 63% for TgNTRK3 and wild type mice, respectively (Figure 11B; $t_{19} = 1.46$, $p = .19$).

These results indicate that TgNTRK3 mice have an impaired extinction process, as revealed in high levels of freezing during a test 24 h after extinction training.

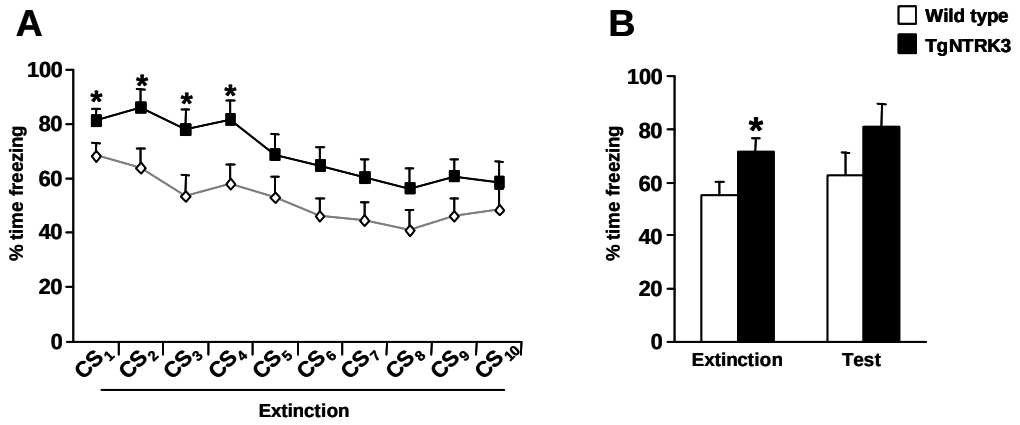


Figure 11. Effects of fear memory extinction on conditioned freezing in TgNTRK3 mice.
A) TgNTRK3 (filled bars) showed an impaired reduction in freezing response across extinction training vs wild type (open bars) mice.
B) The freezing response during the training session and 24 h later (test) was higher un TgNTRK3 than in wild type mice.
Data are expressed as mean ± S.E.M. *P<.05

1.4 TgNTRK3 mice showed sensorimotor information processing alterations

1.4.1. Startle response and prepulse inhibition (PPI) in TgNTRK3 mice

The aim of this study was to investigate the possible role of TrkC overexpression in PPI, a mesure of sensorimotor gating which is disrupted in PD and other mental illnesses (see Introduction). PPI consists in a reduced reaction of an organism to a strong stimulus (pulse), by introducing a previous weaker stimulus (prepulse). Deficits of PPI evidence the inability to filter out unnecessary information, which have been linked to abnormalities of sensorimotor gating.

When mice were exposed to the pulse, the mean startle amplitude levels did not show differences between genotypes (Figure 12A). This result indicates that startle response in TgNTRK3 mice is not modified. In addition, exposure to prepulses of different intensities revealed that TgNTRK3 have disruption of sensorimotor gating (Figure 12B; $F_{1,54} = 5.14$, $p < .05$, repeated-measures ANOVA), characterized by an enhanced PPI across prepulse intensities. Our results suggest that TgNTRK3 mice have a normal startle response but an altered sensorimotor information processing.

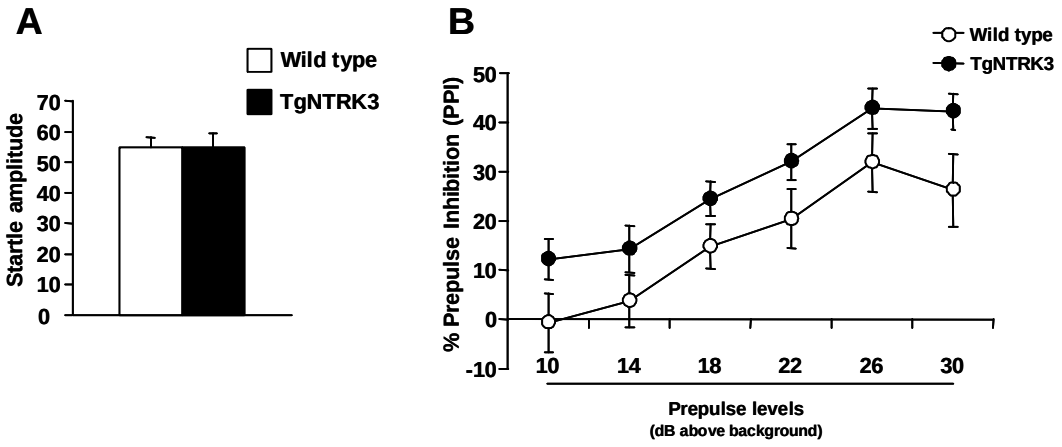


Figure 12. Effects of fear memory extinction on conditioned freezing in TgNTRK3 mice.
A) The mean startle amplitude (arbitrary units) in TgNTRK3 is similar than wild type mice.
B) TgNTRK3 showed higher PPI levels than wild type mice prepulse intensities.
Data are expressed as mean ± S.E.M. *P<.05

1.4.2. Pharmacological characterization of sensorimotor information processing

There was no significant effect of any of the treatments, MK-801 or d-amphetamine, on startle amplitude (Figure 13A); however, treatment with 5 mg/kg of d-amphetamine showed a non

significant higher decrease of startle response in TgNTRK3 mice (t_{14} = 1.64, p = .12) with respect to saline. These results suggest that TgNTRK3 mice exhibit higher sensibility to dopaminergic agonists in startle response.

In addition, although saline-treated wild type exhibited a slight PPI increase with respect to non-treated (basal) mice there was no effect of saline injection (Figure 13B; p > .05). Consequently, previous enhanced PPI in non-treated TgNTRK3 mice (Figure 12B) was not observed in saline group, which could be derived by the effect of injection treatment in PPI that may affect startle response

A repeated-measures ANOVA analysis on treatment with 5 mg/kg of d-amphetamine did not show disruption of PPI (Figure 13C; main effect of treatment, $F_{1,26}$ = .02, p = .89). Although this analysis revealed no genotype effect (p = .44), a statistical tendency was observed in genotype x treatment interaction (p = .09). Moreover, a post hoc analysis revealed that d-amphetamine-treated TgNTRK3 mice exhibited a higher reduction of PPI at lower prepulses [10 (-18%), 14 (-15%), 18 (-13%) and 22 (-10%) dB above background] levels than wild type. These results could indicate that reduced PPI by a dopaminergic agonist in TgNTRK3 mice is due to a higher increase of dopamine in neuronal terminals, which is in accordance with previous results of higher dopaminergic cellularity in catecholaminergic nuclei (Dierssen et al, 2006).

Treatment with 0.5 mg/kg of MK-801 markedly reduced PPI (Figure 13D; main effect of treatment, $F_{1,31}$ = 13.84, p = .001, repeated-measures ANOVA). In addition, the analysis revealed no effect of genotype (p = .35) and no genotype x treatment interaction (p = .94). A detailed analysis showed that treatment with MK-801 caused a reduction of PPI at higher prepulse levels (Treatment x prepulse interaction, $F_{5,155}$ = 2.172, p = .06). This prepulse-dependent effect of MK-801 was similar in both genotypes. Reduced PPI results in TgNTRK3 through NMDA receptors antagonist suggest similar function of glutamatergic circuit.

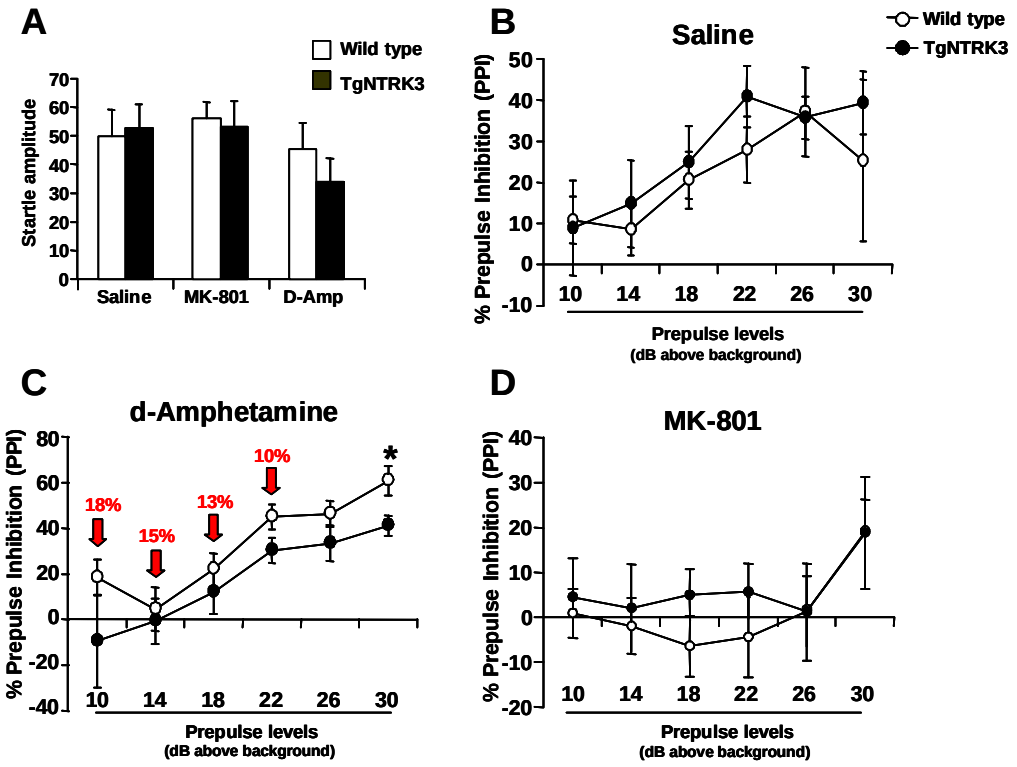


Figure 13. Effects of systemic drugs on startle response and PPI in TgNTRK3 mice.
A) Average startle amplitude in wild type and TgNTRK3 mice treated with saline, 5 mg/kg d-amphetamine or 0.5 mg/kg MK-801.
B) Saline-treated wild type showed similiar PPI levels than TgNTRK3 mice.
C) D-amphetamine-treated TgNTRK3 mice showed a higher reduction of PPI at low prepulse levels than wild type.
D) MK-801 treatment significantly decreased PPI similarly in both genotypes.
Acoustic prepulse intensities are above the 60 dB background noise. Data are expressed as mean ± S.E.M. *P<.05

These results indicate that TgNTRK3 mice present dysfunction of sensorimotor information processing in which an altered dopaminergic circuit could be involved.

2. STRESS HORMONAL AND BEHAVIOURAL ALTERATIONS IN TGNTRK3 MICE

2.1. TgNTRK3 mice showed a subtle hyperactivity of HPA axis in basal conditions

2.1.1. Adrenal weight in TgNTRK3 mice

The adrenal gland is an essential endocrine organ that is part of both the HPA axis and the sympatho-adrenomedullary system. Chronic stress exposure commonly increases adrenal weight, and is associated with increased maximal cortisol/corticosterone responses to ACTH. These chronic-stress-induced changes in adrenal growth and function may have implications for patients with stress-related disorders.

The analysis of the relative adrenal weight did not show significant differences in TgNTRK3 (t_{21} = 6.57, p = .66; Figure 14A) with respect to wild type mice, which indicates that in basal conditions, TgNTRK3 mice do not have hypertrophic alterations in the adrenal gland. Moreover, TgNTRK3 did not show significant differences in the ratio of the amount of protein per adrenal weight in comparison with wild type mice (t_{21} = .32, p = .57; figure 14B), that rule out a hyperplasia process in the adrenal glands of TgNTRK3 mice.

These results indicate a normal adrenal weight in TgNTRK3 mice and rule out a differential response of transgenic adrenal gland influencing corticosterone secretion levels.

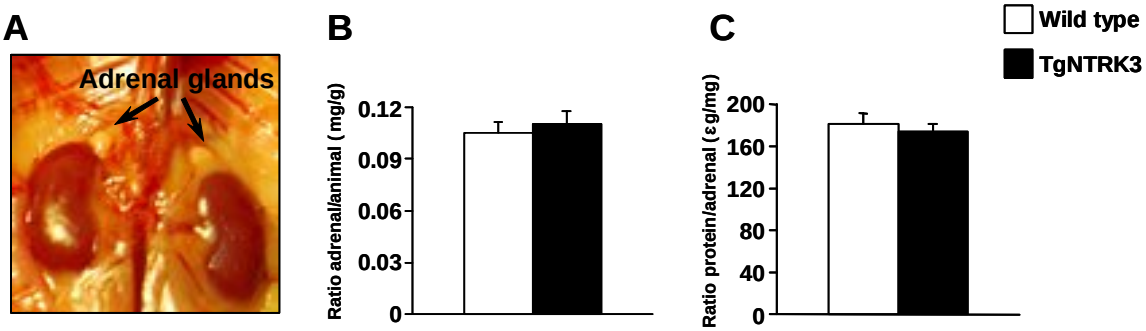


Figure 14. Adrenal weight analysis in TgNTRK3 mice.
A) Adrenal glands image.
B) The ratio adrenal/animal weight did not show differences in TgNTRK3 (filled bars) vs wild type (open bars) mice.
C) TgNTRK3 mice did not show differences in the ratio protein/adrenal weight.
Data are expressed as mean ± S.E.M.

2.1.2. Corticosterone and glucose circadian rhythm in TgNTRK3 mice

Cortisol production has an ACTH-dependent circadian rhythm with peak levels in the early morning and lower values at night in humans. In rodents, plasma corticosterone and glucose levels peak around the onset of the active period (dark phase) and decrease to lowest levels in the early morning (light phase). The factor controlling this rhythm is not completely defined and can be disrupted by a number of physical and psychological conditions.

The analysis of hormone levels (Figure 15) revealed no differences in corticosterone secretion between genotypes, either at early morning (8:00 a.m.; t_{22} = 1.34, p = .87) or at early night (8:00 p.m.; t_{22} = .13, p = .21). However, TgNTRK3 mice showed higher corticosterone levels than wild type mice in the middle of the light phase (2:00 pm; t_{24} = 2.69, p < .05). These results reveal an altered corticosterone circadian rhythm in TgNTRK3 mice have, which is more evident in the middle of the light phase, during the rest period.

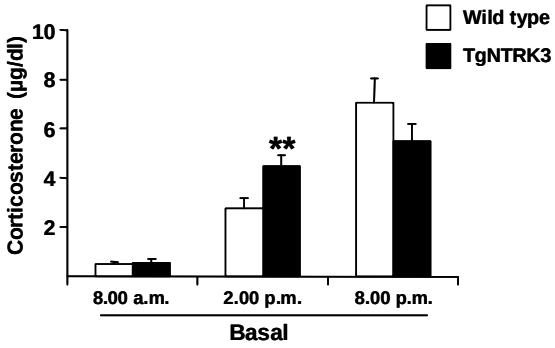


Figure 15. Circadian corticosterone radioimmunoanalysis in TgNTRK3 mice. Increased basal corticosterone levels in TgNTRK3 (filled bars) vs wild type (open bars) mice at midday.
Data are expressed as mean ± S.E.M. *P<.05 **P<.01.

Circadian glucose blood levels in TgNTRK3 mice were measured at early morning and early evening, and no differences were observed between genotypes [(8:00 a.m.; t_{21} = .95, p = .53) and (8:00 p.m.; t_{22} = .81, p = .90), respectively] in either sampling time. However, glucose could not measured in this experiment at 2:00 pm, to avoid hypovolemia. Glucose levels suggest a normal circadian oscillation in both genotypes, with a higher level at early evening.

2.1.3. Stress hormones characterization after clonidine administration in TgNTRK3 mice

Regulation of the HPA axis activity is exerted by different neurotransmitters, such as noradrenaline. As previously described (Dierssen et al, 2006), TgNTRK3 showed a higher noradrenergic cell density in LC, the main noradrenergic nucleus in the brain, along with altered basal neuronal activity. In order to analyze the possible influence of this altered noradrenergic system on

corticosterone secretion (see 2.1.2.), we administered clonidine, an α2 adrenergic agonist that reduces noradrenaline release.

A two-way ANOVA analysis in corticosterone levels revealed a statistically significant interaction effect of genotype x treatment (Figure 16; $F_{1,26} = 5.57$, $p < .05$). Moreover, a genotype ($F_{1,26} = 6.70$, $p < .05$) and treatment ($F_{1,26} = 12.74$, $p < .01$) effect were observed. As previously observed in basal conditions, saline-treated TgNTRK3 showed higher corticosterone levels ($t_9 = -2.24$, $p = .05$).than wild type mice. In addition, TgNTRK3 mice showed a consistent reduction of corticosterone levels through clonidine treatment ($t_{12} = 7.25$, $p < .001$), which reduced their increased corticosterone levels to similar levels of wild type mice.

The reduction of corticosterone levels achieved by acute treatment with clonidine in transgenic, but not in wild type mice reveals that noradrenaline is mainly affecting corticosterone circadian rhythms in TgNTRK3 mice.

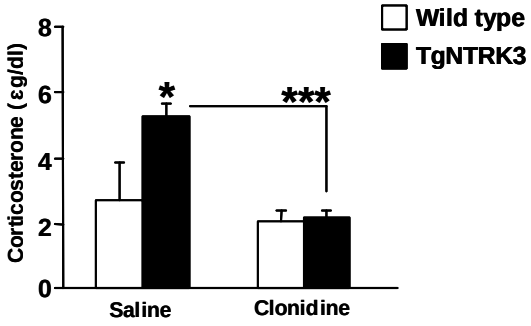


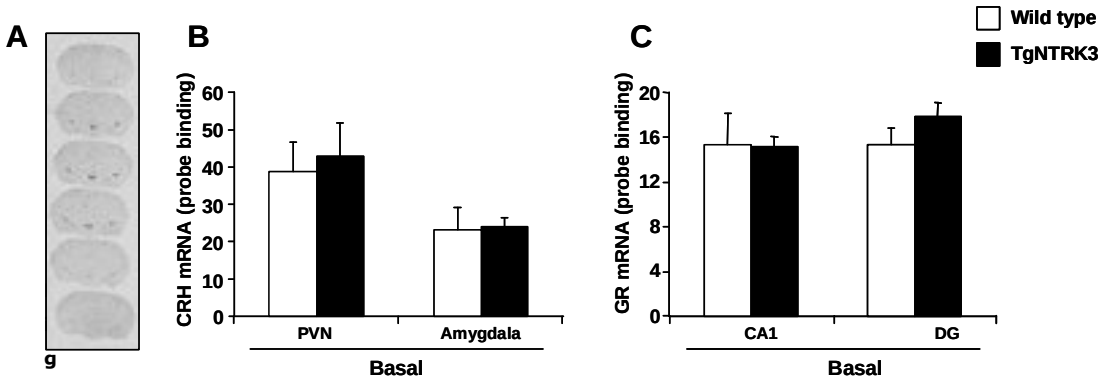
Figure 16. Corticosterone radioimmunoanalysis in TgNTRK3 mice after clonidine acute trearment. Higher corticosterone levels in saline-treated TgNTRK3 mice were significantly reduced to normal levels after acute treatment with clonidine.
Data are expressed as mean ± S.E.M. *P<.05 **P<.01 ***P<.001.

2.1.4. Corticotropin-releasing hormone and glucocorticoid receptor mRNA levels in TgNTRK3 mice

Given the important role of CRH and GR in stress-related disorders, studies of mRNA concentrations of CRH and GR were performed in basal conditions in TgNTRK3 mice by *in situ* hybridization.

CRH is produced by neuroendocrine cells in the PVN and by non-neuroendocrine cells in the amygdala. Analysis of the mRNA concentrations of CRH in the PVN or in the amygdala did not show genotype-dependent differences (Figure 17B; all p values > .05), thus indicating that TgNTRK3 mice do not present regional changes in CRH synthesis.

Another key brain region in HPA axis functionality and stress-related disorders is the hippocampus. In this brain region, GR are highly express and bind with high affinity to cortisol/corticosterone, regulating either directly or indirectly the HPA axis response. Analysis of the mRNA levels of GR in CA1 or DG hippocampal subregions did not show differences between genotype (Figure 17C; all p values > .05), thus suggesting that the inhibitory control of HPA axis is not altered.



g u re 17. *In situ* hybridization analysis in TgNTRK3 mice in basal conditions.

A) Film autoradiography slices illustrate the CRH binding probe in PVN and amygdala.

B) Normal CRH mRNA levels in PVN and amygdala in TgNTRK3 mice.

C) Normal GR mRNA levels in CA1 hippocampal subregion and dentate gyrus (DG).

Data are expressed as mean ± S.E.M.

2.2. TgNTRK3 mice showed reduced increase of CRH mRNA levels and c-FOS activation after acute stress in regions involved in HPA axis response

2.2.1. Stress homone analysis mice after acute stress in TgNTRK3

Acute stress is experienced in response to an immediate physical, emotional or psychological perceived threat. During an acute stress response, the autonomic nervous system is activated and the levels of cortisol/corticosterone, adrenalin and other hormones are increased.

After exposure to a mild stressor, such as novel environment, the increase in corticosterone levels was similar in both genotypes (Figure 18). However, after a moderately intense stressor, such as restraint in a metal mesh, the increase in corticosterone secretion observed in TgNTRK3 showed a subtle tendency to be higher than that of wild type mice (Figure 18; t_{22} = .58, p = .145). These results indicate that TgNTRK3 mice show a slightly more responsive HPA axis after a moderate, but not mild stressors.

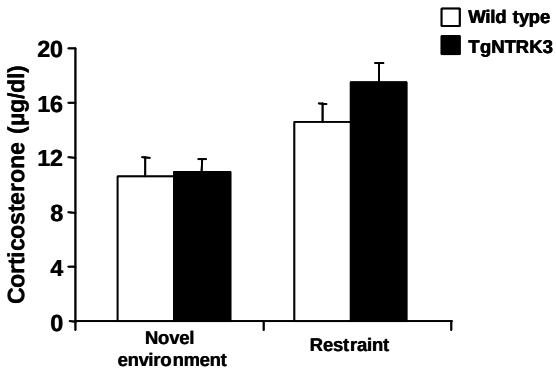


Figure 18. Corticosterone radioimmunoanalysis after acute stress in TgNTRK3 mice. Non-significant differences in corticosterone levels after novel environment and restraint exposure were observed between genotypes.

Data are expressed as mean ± S.E.M.

2.2.2. Corticotropin-releasing hormone and glucocorticoid receptor mRNA levels in TgNTRK3 mice after acute stress

Given the prominent role of the CRH system in regulating the stress response, the link between stress and psychopathology, and the potential role of CRH in psychopathology, it was important to determine how CRH system responds to acute stress exposure in TgNTRK3 mice.

As has been previously demonstrated, acute restraint stress increases CRH mRNA content in the amygdala and hypothalamic PVN (Kalin et al, 1994). In our experiments, wild type mice exhibited a significant trend to an increase of CRH mRNA levels in the PVN (Figure 19B; 164%; t_6 = .7, p = .06) after 30 min of restraint. However, TgNTRK3 mice showed no significant increase of CRH mRNA (122%; t_6 = 1.08, p = .5), with respect to basal conditions, in this region. Although the responses were not important in aither genotype, these results suggest that CRH synthesis in response to acute stress may be altered in TgNTRK3 mice. This alteration, could have an important role in hypothalamic response to chronic stress.

Another protein that plays a main role in regulating the stress response is the GR. Concretely, the hippocampal GR is implicated in negative feedback inhibition of the HPA axis (Jacobson and Sapolsky, 1991), and during the last decade it has received a prominent attention due to its putative role in stress-related disorders.

In our experiment GR mRNA expression was not highly affected by acute stress (Figure 19C) as was previously described by others (Herman and Spencer, 1998). *In situ* hybridization showed that GR mRNA expression in hippocampal subregions (CA1 and DG) was not significantly affected by acute restraint stress (all p values > .05) in either genotypes.

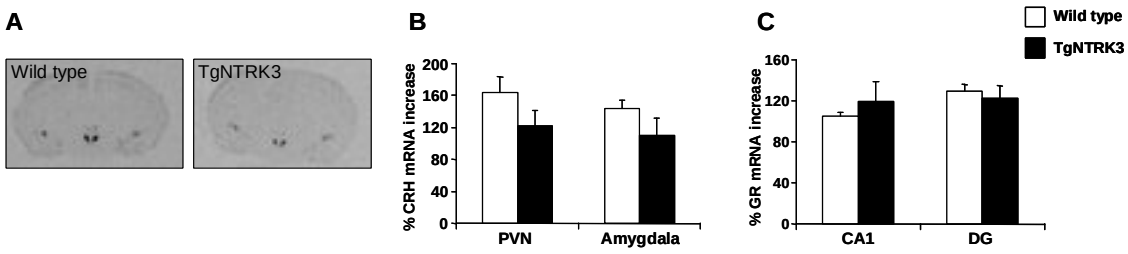


Figure 19. *In situ* hybridization analysis in TgNTRK3 mice after acute stress (restraint).
A) Film autoradiography slices illustrate the CRH binding probe example in PVN and amygdala of a wild type and TgNTRK3 mice exposed to 30 min of restraint in tube.
B) A nonsignificative lower increase was observed in the percentage of CRH mRNA increase in PVN and amygdala in TgNTRK3 (filled bars) vs wild type (open bars) mice.
C) Normal percentage of GR mRNA increase in CA1 and dentate gyrus (DG) hippocampal subregions in TgNTRK3 mice. Data are expressed as mean ± S.E.M.

2.2.3. c-Fos activation pattern after a single immobilization session in TgNTRK3 mice

Another important element in stress-related disorders pathophysiology is the failure to correctly activate or inhibit different brain regions after stressful stimuli. In our experiments, we used the immediate early gene c-Fos is generally as a marker of neuronal activity after stress.

We investigated the consequences of a single IMO on c-Fos expression in TgNTRK3 mice and wild type littermates. Immunohistochemical analysis of Fos expression, analyzed 120 min after the exposure to IMO showed a strong up-regulation of c-Fos immunoreactive cells in numerous brain areas implicated in stress response (Table 2), including different cortical areas, lateral septum (LS), BNST, thalamic and hypothalamic nuclei, and diverse brainstem nuclei. Mice not exposed to the IMO (basal condition) displayed lower c-Fos immunoreactivity in most of the stress brain areas investigated.

Table 2. Effect of an acute immobilization (30 min) on c-Fos expression in the TgNTRK3 mice

	WT_{basal}	TG_{basal}	WT_{IMO}	TG_{IMO}
Cortex				
Cg	1561.1 ± 432.8	970.4 ± 291.1	1416.5 ± 489.0	2154.2 ± 293.4 #
PrL	2256.9 ± 412.8	1990.1 ± 266.8	2193.8 ± 489.8	3847.2 ± 357.0 * ##
IL	2026.5 ± 308.1	2328.9 ± 195.5	4374.9 ± 956.1 #	4619.9 ± 854.1 #
Septal area				
LSv	6983.3 ± 554.6	6975.8 ± 951.9	8728.6 ± 1457.1	14553.9 ± 1526.2 * ##
LSd	1449.6 ± 432.3	1400.1 ± 193.3	1960.3 ± 326.2	2786.8 ± 369.6 ##
BST-amygdala				
CeA	1808.0 ± 461.5	1315.6 ± 375.8	5144.7 ± 490.8 ###	5542.9 ± 727.7 ###
BSTv	1713.9 ± 501.6	2126.7 ± 328.1	2757.9 ± 531.3	4082.5 ± 831.3
BSTd	1722.4 ± 346.2	2046.9 ± 299.1	3393.0 ± 587.4 #	3684.7 ± 484.4 #
Parvocellular PVN	2373.2 ± 981.3	2130.4 ± 255	24039.8 ± 4088 ###	15937.3 ± 3107.3 ##
Hippocampus				
CA1	5045.1 ± 1735.0	4335.4 ± 1100.3	2677.1 ± 693.8	4614.4 ± 843.0
CA2	8381.4 ± 2142.9	6984.8 ± 1232.3	3913.3 ± 768.6	6130.1 ± 1089.4
CA3	2769.2 ± 594.6	2805.4 ± 528.3	1779.4 ± 289.1	2918.6 ± 523.7
DG	2120.5 ± 233.5	3437.8 ± 260.8 **	1934.4 ± 320.6	2956.6 ± 408.4
Locus coeruleus	773.0 ± 199.1	449.9 ± 141.8	18391.3 ± 2608.3 ###	13151.6 ± 2235.6 ###

Table 2. c-Fos positive cells/mm3 after an acute stress exposure to 30 min of immobilization (IMO). Abbreviation: BSTd, bed nucleus of stria terminalis, dorsal groups; BSTv, bed nucleus of stria terminalis, ventral groups; CA1, field CA1; CA2, field CA2; CA3, field CA3; CeA, central nucleus of the amygdala; Cg: Cingulate cortex; DG, dentate gyrus; IL, infralimbic cortex; LSd, lateral septal nucleus, dorsal part; LSv, lateral septal nucleus, ventral part; PrL, prelimbic cortex; PVN, paraventricular nucleus of the hypothalamus.
Data are expressed as mean ± S.E.M. Differences between genotype: *P<.05 **P<.01; Differences between stress codition #P<.05 ##P<.01 ###P<.001.

A two-way ANOVA revealed significant effect of genotype and stress condition for LSv ($F_{2,22}$ = 5.412, p < .05) and PrL ($F_{2,22}$ = 5.778, p < .05), showing a higher increase in the density of c-Fos positive cells in TgNTRK3 stressed mice. In the basal group, ANOVAs showed higher Fos positive cells in TgNTRK3 dentate gyrus (DG) ($F_{1,10}$ = 14.16, p < .01) with no differences in other regions analyzed. Interestingly, in the IMO group, ANOVAs showed higher c-Fos response in the prelimbic cortex (PrL) ($F_{1,12}$ = 7.44, p < .05) and the lateral septal nucleus ventral (LSv) ($F_{1,12}$ = 7.62, p < .05) of TgNTRK3 mice (Figure 20A). Moreover, c-Fos response in stressed-transgenic mice showed a statistical tendency of increase of c-Fos positive cells in the CA1 ($F_{1,12}$ = 3.15, p = .1), CA3 ($F_{1,12}$ = 3.63, p < .1) and DG ($F_{1,13}$ = 3.88, p < .1) hippocampal subregions. Nevertheless, due to a great c-Fos response variability, TgNTRK3 showed a non-significant higher c-Fos positive cell density in cingulate cortex (Cg), lateral septal nucleus dorsal (LSd), and CA2 hippocampal subregion; and a non-significant lower density in parvocellular PVN (Figure 20B) and LC (Figure 20C).

C-Fos analysis revealed that TgNTRK3 mice may present with higher inhibitor inputs to hypothalamic pPVN which could reduce the HPA axis response to subsequent stressors.

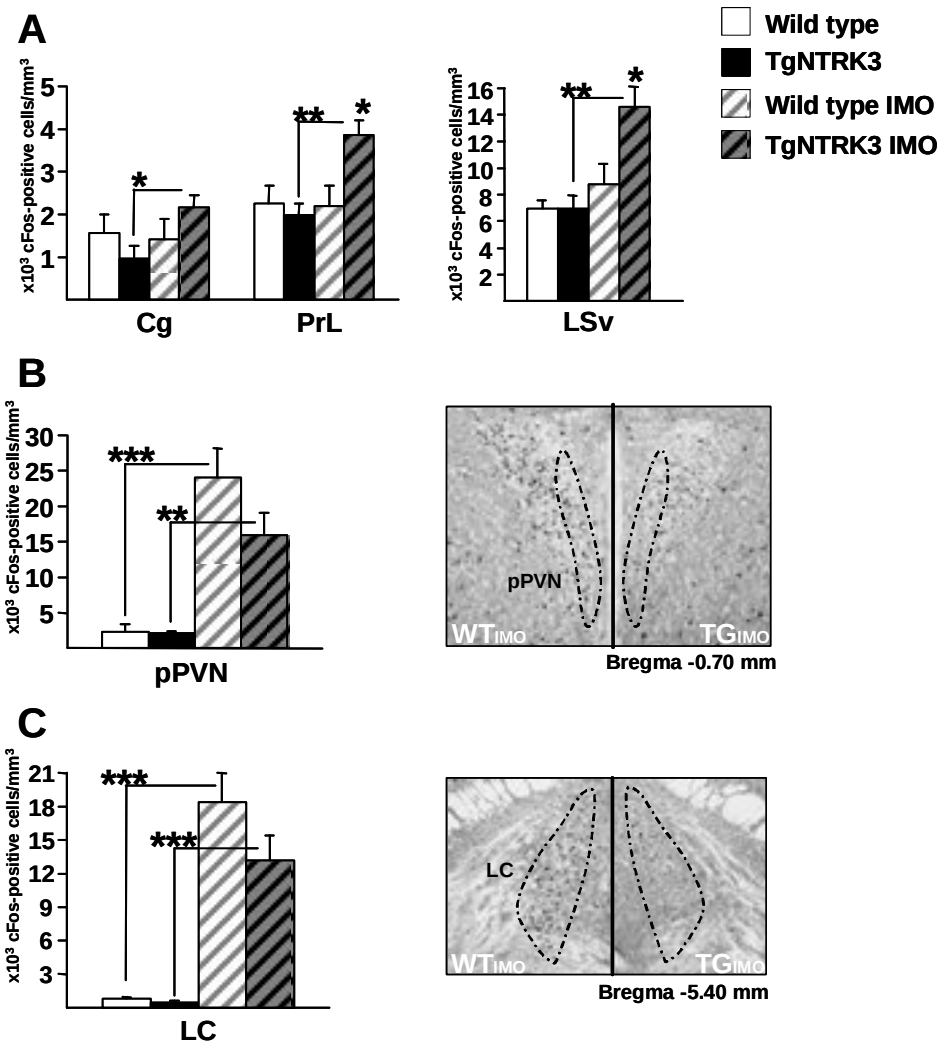


Figure 20. c-Fos immunoreactive cells in various brain regions of TgNTRK3 and wild type mice after an acute stress exposure to 30 min of immobilization (IMO).

A) Increased c-Fos positive cells in stressed TgNTRK3 vs wild type mice in Cg (cortex cingulated), PrL (prelimbic cortex) and LSv (lateral septal nucleus, ventral part).

B) Lower c-Fos positive cells in pPVN (parvicellular paraventricular nucleus of the hypothalamus) in stressed TgNTRK3 mice vs wild type.

C) Lower c-Fos positive cells in LC (locus coeruleus) in stressed TgNTRK3 mice vs wild type.

Data are expressed as mean ± S.E.M. *P<.05 **P<.01 ***P<.001.

2.3. TgNTRK3 mice showed an increase in anxiety-like behaviour after chronic environmental stress (CES)

We used two different illumination conditions, high and low light intensity, during the light phase of the cycle. The high illuminated (HL-stress) group was reared under high intensity light levels (800-1000 lux), and the low illuminated (LL-non-stress) group received 50-100 lux in their home cages for 3 weeks. Stress/non-stress rearing conditions were maintained until animals were sacrificed.

2.3.1. Corticosterone characterization of TgNTRK3 mice after CES

Chronic mild stress (CMS) models in rodents are used to study stress susceptibility in genetically modified mice. There are some reports that have found a decrease of corticosterone levels following CMS procedures (Murison and Hansen, 2001).

A two-way ANOVA analysis revealed that basal corticosterone levels after 3 weeks of CES did not show an interaction effect between genotype and light condition at the early morning (8.00 a.m.) or early night (8.00 p.m.) (Figure 21; all p values > .05). However, TgNTRK3 (LL+HL) showed a tendency to lower corticosterone levels at early morning ($F_{1,34}$ = 10.71, p = .1) than wild type mice.

A post hoc analysis showed that at early night, HL-animals have significant lower corticosterone levels (t_{47} = 5.42, p = .05) than LL-animals, with a higher effect in wild type mice (t_{21} = 6.48, p = .08). This reduction of corticosterone levels in the chronic stressed group would be in accordance with corticosterone data from other CMS

These results indicate that CES did not affect corticosterone circadian rhythms in TgNTRK3 mice.

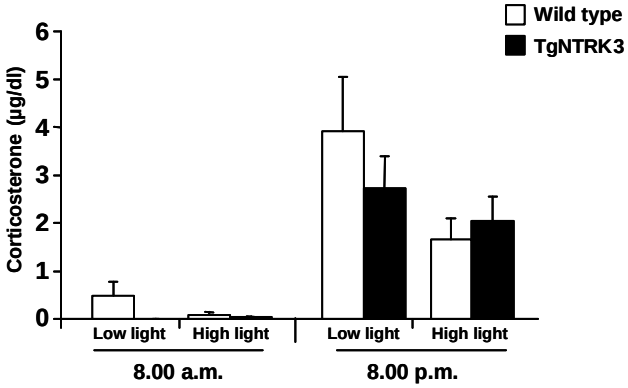


Figure 21. Circadian corticosterone radioimmunoanalysis in TgNTRK3 mice after CES. No observable effects of light condition in basal corticosterone levels were observed in TgNTRK3 mice.

Data are expressed as mean ± S.E.M.

2.3.2. CRH and GR mRNA levels in TgNTRK3 mice after CES

In order to study the effects of chronic stress in the synthesis of both CRH and GR mRNAs after a challenge, we restrained the animals and measured the percentage of increase of mRNA levels. As previously described (2.2.2.), *in situ* hybridization experiments did not show any significant difference in CRH (PVN and amygdala) or GR (CA1 and dentate gyrus hippocampal subregions) mRNA levels between genotypes under basal conditions.

It has been demonstrated that chronic stress increases CRH mRNA content in the PVN and reduces GR mRNA in the hippocampus (Herman et al, 1995). However, in our chronic stress procedures (chronic high light (HL) conditions), we did not observe a significant change in the expression of both mRNAs (data not shown; all p values > .05) in comparison to basal conditions (chronic low light (LL) conditions).

A two-way ANOVA analysis revealed a strong effect of light conditions in GR mRNA in DG after restraint, showing the HL-mice (wild type and transgenic groups) a lower increase in GR mRNA levels (Figure 22B; $F_{3,13} = 6.411$, $p < .05$). Such decrease in GR mRNA expression after chronic stress is in accordance with previous data (Makino et al, 1995). No interaction between the genotype and the light condition ($F_{3,13} < 1$) was observed in the increase of CRH or GR mRNA levels in any brain region studied.

Post hoc analysis showed that TgNTRK3, regardless of being exposed by LL or HL conditions, have a statistical tendency to lower increase of CRH mRNA levels in the PVN (Figure 22A; $t_{16} = .03$, $p = .01$) than wild type mice. No differences were observed in the increase of CRH mRNA levels in the amygdala or in GR mRNA in the CA1 hippocampal subregion (data not shown; all p values > .05).

As observed in basal conditions, our chronic stress procedure revealed that TgNTRK3 mice show a consistent lower increase of CRH mRNA levels.

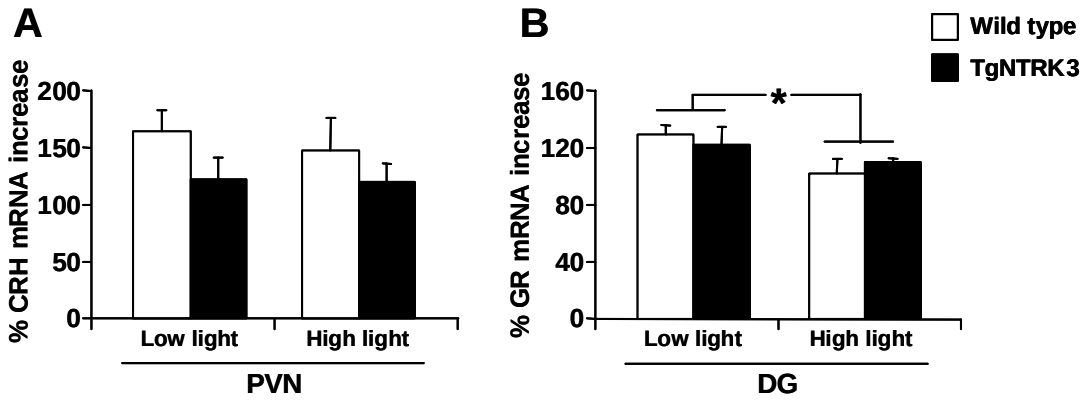


Figure 22. *In situ* hybridization analysis in TgNTRK3 mice after CES.

A) Tendency to a lower increase in CRH mRNA levels in PVN in TgNTRK3 (high and low light intensity groups) vs wild type mice.

B) Lower increase in GR mRNA levels in dentate gyrus (DG) in high light (wild type and transgenic mice) vs low light intensity groups.

Data are expressed as mean ± S.E.M. *P<.05.

2.3.3. Behavioural characterization of TgNTRK3 mice after CES

In the MHB, used to investigate exploration in rodents, a two-way ANOVA revealed an interaction between rearing condition (HL and LL) and genotype (TgNTRK3 and wild type) in grooming behavior, being the grooming time (Figure 23B; $F_{1,46} = 6.14$, $p < .05$), and the maximum duration of individual groomings (Figure 23C; $F_{1,46} = 5.71$, $p < .05$) decreased in HL-TgNTRK3 mice.

No differences were detected in anxiety-related behaviours between genotypes in mice reared in LL conditions, although LL-TgNTRK3 presented a tendency to a lower latency to hole exploration ($t_{21} = 9.70$, $p = .06$) than wild type mice. Moreover, LL-TgNTRK3 showed a lower latency to 1st board visit (Figure 23A; $t_{23} = 2.86$, $p < .01$) than HL-TgNTRK3 mice. These results can be interpreted as a higher impulsivity in LL-TgNTRK3 mice, since the exploration parameters (number and time of hole-pokes) were not modified by TrkC overexpression.

However, in HI rearing conditions, a significant lower percentage of time (Figure 23B; $t_{22} = 10.72$, $p < .01$) and a longer latency to initiate grooming behavior ($t_{22} = 1.99$ $p < .05$) were observed in TgNTRK3 with respect to wild type mice.

Due to the low aversive conditions of the MHB, HL-TgNTKR3 mice did not show evident increase of anxiety-like behavior. However, a reduction of specific patterns of grooming, which has been associated to the sensitization process after chronic stress (Flügge et al, 2001; Kramer et al, 1999), suggests that TgNTRK3 mice show different coping strategies in a novel environment after chronic stress.

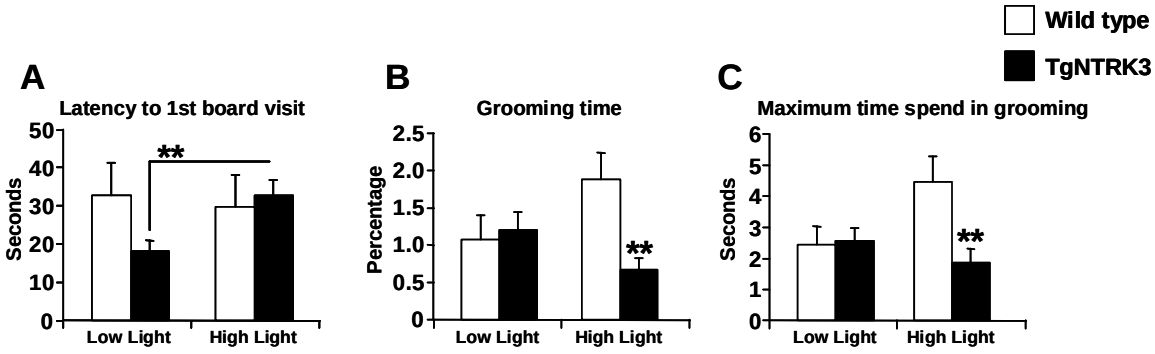


Figure 23. Modified Hole Board in TgNTRK3 mice after CES.

A) Tendency to a lower latency to 1st board visit in TgNTRK3 mice reared in low light and a significant reduction vs. TGNTRK3 reared in high light conditions.

B) Reduction in time spent in grooming in TgNTRK3 vs. wild type (open bars) stressed groups.

C) Reduction in maximum duration of individual groomings in TgNTRK3 vs wild type stressed groups.

Data are expressed as mean ± S.E.M. **P<0.01

In the *light-dark box*, which is based on the innate aversion of rodents for brightly lit large spaces, the analysis of the interaction effect of rearing light conditions and genotype (two-way ANOVA) showed a significant interaction in stretched attend time in the lit compartment ($F_{1,59}$ = 4.41, p < .05) with HL-TgNTRK3 showing increased stretched attend behavior. Moreover, a trend to significant interaction was observed in the time in the lit compartment (Figure 24B; $F_{1,59}$ = 3.40, p = .07), with reduced time in HL-TgNTRK3. In grooming time, HL-TgNTRK3 mice showed increased behavior in the dark ($F_{1,59}$ = 3.39, p = .07), and reduced in the lit compartment ($F_{1,59}$ = 3.72, p = .06). In movements per unity of time, HL-TgNTR3 mice showed reduced movements in the lit (Figure XD; $F_{1,59}$ = 2.93, p = .09) and in the dark compartment (Figure 24C; $F_{1,59}$ = 3.07, p = .09).

In LL conditions, post hoc analysis only showed a reduced grooming time in the dark compartment in TgNTRK3 mice (t_{28} = .06, p = .05) with respect to wild type. However, in HL conditions, TgNTRK3 showed a higher latency to initiate movement to the dark compartment (Figure 24A; t_{26} = 3.86, p < .05), lower transitions to the lit compartment (t_{26} = 10.24, p < .05) and a tendency to spend less time in the lit compartment (Figure 24B; t_{26} = .32, p = .07) than wild type mice.

Moreover, HL-TgNTRK3 showed less locomotor activity (movements/unity of time) both in the lit (Figure 24C;t_{26} = .25, p < .01) and in the dark compartment (Figure 24D; t_{26} = .22, p < .05] than HL-wild type mice.

Since time spent in the lit compartment is the most reliable parameter to assess anxiety-related behavior, and reduced locomotor activity could be attributable to higher levels of anxiety. These data reinforce the concept that TgNTRK3 is more sensitive to environmental stress, exhibiting different coping behaviors in this test.

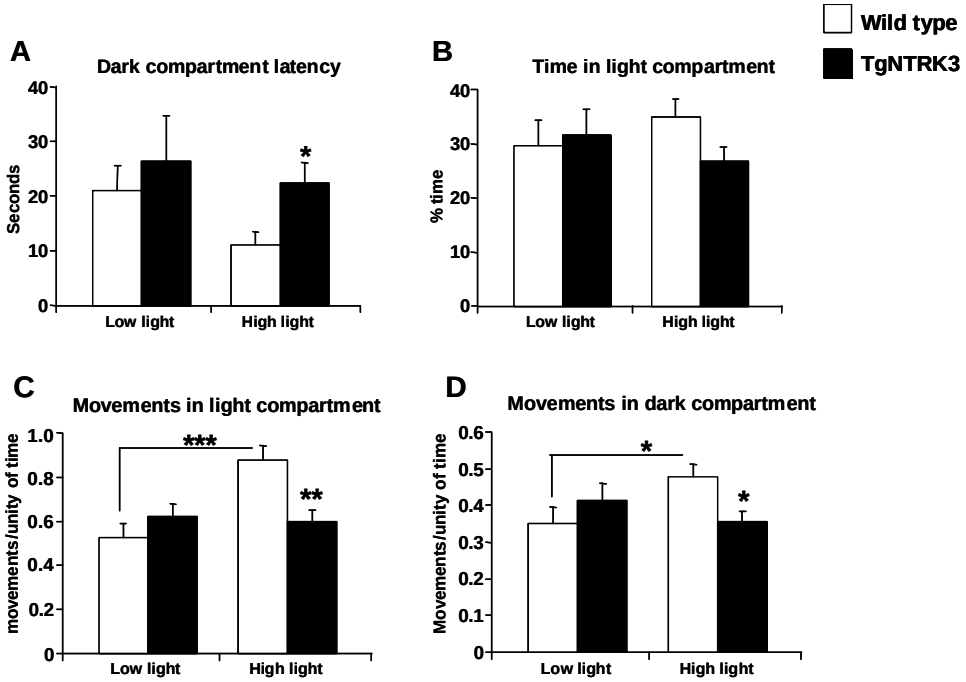


Figure 24. Light-dark box in TgNTRK3 mice after CES.

A) Increased freezing with longer latency to enter the dark compartment in stressed TgNTRK3 (filled bars) vs wild type (open bars) mice.

B) Reduced time in the light compartment in stressed TgNTRK3 vs wild type mice.

C) Reduction in exploratory behaviors (movements/unity of time) in the light compartment in stressed TgNTRK3 vs wild type mice and increase in exploration in stressed vs non-stressed wild type mice.

D) Reduction in exploratory behaviors in the dark compartment in TgNTRK3 vs wild type stressed mice and increase in wild type stressed with respect to non-stressed mice.

Data are expressed as mean ± S.E.M. *P<.05 **P<.01 ***P<.001.

In the *elevated-plus maze,* widely used to test anxiety in rodents, a two-way ANOVA revealed a trend to interaction effect of genotype and rearing light conditions in the latency to enter the open arms (Figure 25B; $F_{1,16} = 3.47$, $p = .09$), with longer open arm latency in HL-TgNTRK3. Moreover, a significant interaction effect was observed in time spent in center ($F_{1,16} = 4.80$, $p < .05$), in which a longer permanence was observed in HL-TgNTRK3 mice.

A detailed analysis showed that in the group reared in HL conditions, TgNTR3 showed significant higher time in center ($t_7 = 2.44$, $p < .05$), and a non significant lower ratio of open/total arms time (Figure 25A; $p = .19$) and higher stretched attend behaviour in protected arms (Figure 25C; $p = .31$) than wild type mice.

All these results in HL-TgNTRK3 correlate with the behaviour observed in the MHB and light-dark box, indicating an increased anxiety-like behaviour of TgNTRK3 mice after CES.

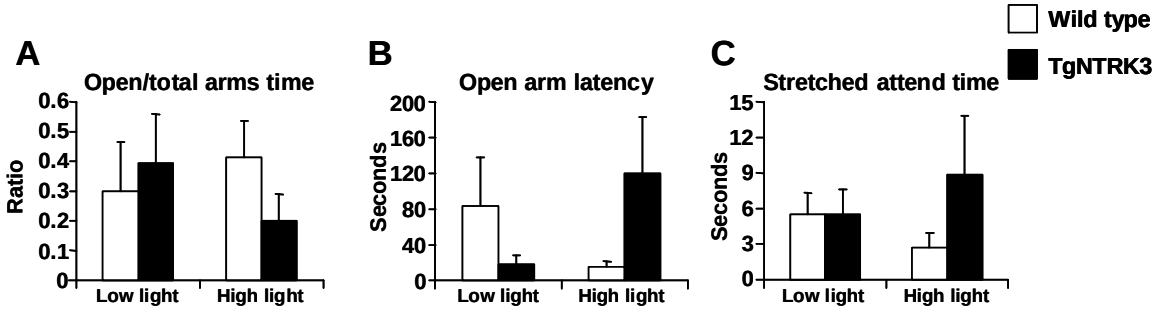


Figure 25. Elevated-plus maze in TgNTRK3 mice after CES.

A) Reduction in the open/total arms time ratio in TgNTRK3 mice reared in high light conditions.

B) Increased latency to enter the open in TgNTRK3 mice reared in high light conditions.

C) Increase in stretched attend time (protected arms) in TgNTRK3 mice reared in high light conditions.

Data are expressed as mean ± S.E.M.

In the FST, that predicts the efficacy of antidepressant treatments and the impact of stress exposure, the analysis of the effect of genotype and light condition showed an effect of genotype observed in the longer latency to struggling in TgNTRK3 mice (Figure 26A; $F_{1,49} = 4.00$, $p = .05$), and an effect of light condition observed in the shorter latency and higher duration of floating (Figure 26B; $F_{1,49} = 8.65$, $p < .01$ and $F_{1,49} = 5.52$, $p < .05$, respectively), and in the lower time of struggling in HL-mice ($F_{1,49} = 6.66$, $p < .05$). An interaction effect of genotype and light condition was evident in time of struggling ($F_{1,49} = 7.23$, $p = .01$), with lower struggling in HL- and higher in LL-NTRK3 mice.

A detailed analysis in LL-mice, revealed a significant increase in the percentage time of struggling in TgNTRK3 group (Figure 26A; t_{23} = 7.27, p = .05], with no differences in swimming or floating behavior. In the HL-mice, TgNTRK3 presented a significantly longer latency (Figure 26B; t_{23} = 193.16, p < .05) and reduced time of struggling (Figure 26A; t_{23} = .43, p = .079).

These results indicate that active behaviours, such as struggling, are affected in TgNTRK3 mice after CES, suggesting a different strategy to escape from a stress exposure.

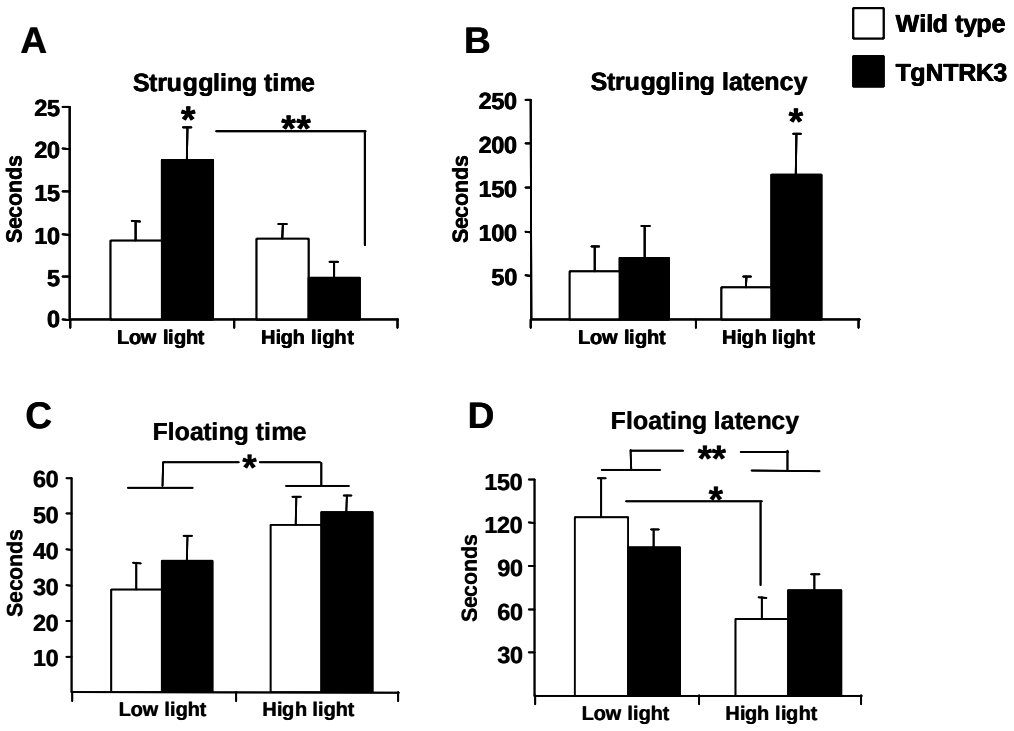


Figure 26. Forced swimming test in TgNTRK3 mice after CES.

A) Increase in time spent struggling in non-stressed TgNTRK3 vs wild type mice and reduction of this parameter after stress exposure.

B) Increased latency to struggle in TgNTRK3 vs wild type stressed mice.

C) Increase in time spent floating in stressed vs. non-stressed mice.

D) Lower latency to float in stressed vs. non-stressed mice.

Data are expressed as mean ± S.E.M. *P<.05 **P<.01.

2.3.4. Effects of a single immobilization session in anxiety-like behaviour in TgNTRK3 mice after CES

In the *elevated-plus maze,* a two-way ANOVA revealed a interaction effect of genotype and rearing light conditions in the latency to do stretched attend behavior in protected arms ($F_{1,17}$ = 5.49, p < .05), in which a shorter latency was observed in TgNTRK3 rearing in high light conditions, in comparison with wild type mice.

A detailed analysis showed that restrained animals showed similar anxiety-like behaviours, except for TgNTRK3 reared in high light conditions, which express the same anxiety-like behaviour than unrestrained TgNTRK3 mice (see 2.3.3.), as is reflected by a non-significant lower open/total arms

ratio (Figure 27A), longer latency to enter the open arms (Figure 27B) and a higher stretched attend behaviour in protected arms (Figure 27C) (all p values > .05) than wild type mice.

Our results suggest that a single exposure to restraint produced anxiety-like behaviour in TgNTRK3 mice previously exposed to CES.

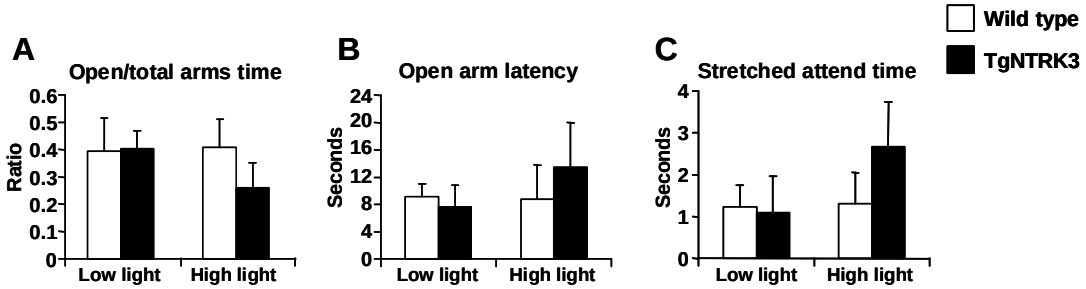


Figure 27. Elevated-plus maze in restrained TgNTRK3 mice after CES.

A) Reduction in the open/total arms time ratio in HL-TgNTRK3 mice.

B) Increased latency to open arm entry in HL-TgNTRK3 mice.

C) Increase in stretched attend time (protected arms) in HL-TgNTRK3 mice.

Data are expressed as mean ± S.E.M.

2.4. TgNtrk3 mice showed alterations in HPA axis response after chronic immobilization stress (CIS)

2.4.1. Stress hormones characterization of TgNTRK3 mice in a CIS and social isolation paradigm

Social isolation, that is a chronic psychosocial stressor, can contribute toward many emotional behavioral and physical disorders, such as PD. Moreover, CIS is a potent chronic stress model that induces anxiety behaviour and dendritic atrophy in amygdala (Vyas et al, 2003) and hippocampus (McEwen, 1999). As chronic stress alters limbic neuroarchitecture and function, our interest was to combine both chronic stressors in TgNTRK3 mice and analyze stress response.

A two-way ANOVA analysis of the effects of 3 weeks of social isolation in basal corticosterone levels revealed a statistically significant decrease of corticosterone levels caused by rearing in isolation (Figure 28A; $F_{1,77} = 7.33$, $p < .01$) and an interaction effect between genotype and rearing conditions ($F_{1,77} = 4.63$, $p < .05$). Post hoc analysis showed that the group most affected by social isolation was TgNTRK3 mice ($t_{34} = .01$, $P = .001$), which reduced their increased corticosterone levels to similar levels of wild type mice.

After chronic social isolation (3 weeks), a CIS procedure for 15 days was introduced, maintaining the rearing conditions in each group. A repeated-measures ANOVA revealed that corticosterone levels across trials were significantly increased in all the groups (Figure 28B; $F_{3,96} = 13.20$, $p < .001$), with no interaction between trial and genotype and/or rearing conditions ($F_{3,96} < 1$). This result is in accordance with other studies in which corticosterone levels increase across immobilization sessions.

Exposure to CIS did not show differences between genotypes or interactions between housed conditions and genotype in any immobilization session (Figure 28B). A detailed analysis revealed statistical differences in transgenic mice between housing conditions. Isolated TgNTRK3 mice showed significantly lower corticosterone levels in the 1st IMO session (t_{14} = .41, p < .05) and in the 2nd IMO session (t_{14} = .78, p < .05) than grouped TgNTRK3 mice.

Finally, a statistical tendency of interaction between housing condition and genotype was observed in corticosterone response recovery after the 15th IMO session (Figure 28C; $F_{1,35}$ = 2.879, p < .1). Post hoc analysis revealed that TgNTRK3 mice were highly affected by housing conditions, with the isolated group showing higher corticosterone levels (t_{14} = 5.92, p < .05) than transgenic mice housed in group. No differences were observed in wild type groups at this time sampling.

These results indicate that combination of social isolation and chronic immobilization altered TgNTRK3 stress response.

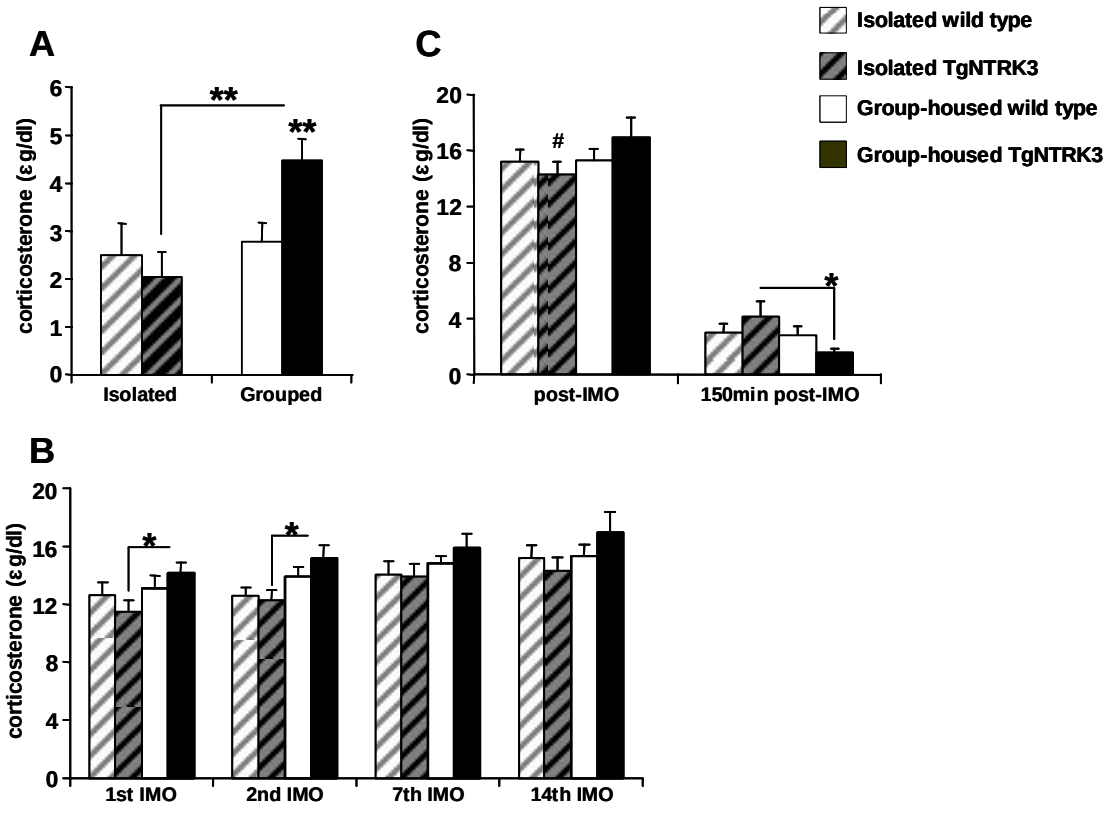


Figure 28. Corticosterone radioimmunoanalysis in TgNTRK3 mice after social isolation and CIS.

A) Social isolation for 3 weeks reduced the increased basal corticosterone levels in TgNTRK3.

B) Increased corticosterone levels in housed in group vs isolated TgNTRK3 mice in the 1st and 2nd IMO sessions.

C) Increased corticosterone levels in isolated vs. housed in group TgNTRK3 mice after 150 min of the 15th IMO.

Data are expressed as mean ± S.E.M. *P<.05 **P<.01.

DISCUSSION

-¿Alborotas y te enojas al discutir? Luego no tienes razón.

-Es que hablo con imbéciles.

-Pues entonces el imbécil eres tú al intentar persuadirles a gritos. El buen argumento, como el proyectil de las armas modernas, debe salir de la mente sin humo, sin fuego y con el menor ruido posible.

[Santiago Ramón y Cajal (1852-1934), en *Charlas de café*]

This thesis has been performed in the context of a project aimed at analyzing candidate genes for PD. In our laboratory, we have focused on neurotrophins as good candidate to explain part of the PD phenotypes by modifying specific brain structures, modulating cellular events, or affecting the development or the functionality of neurotransmitter systems affected in PD. As an experimental tool, we used a transgenic mouse model overexpressing *NTRK3* (TgNTRK3). In the present work we have centered on the study of altered emotional memories observed in PD patients (Ludewig et al, 2005), and on the role of stress in the aetiology and course of PD (Faravelli, 1985; Manfro et al, 1996).

We demonstrate in this work that overexpression of this gene is involved in altered cognitive processing by altering regions that process emotional stimuli, and is also involve in altered stress response to acute and chronic stress, which are associated to PD patients. Although we work with two transgenic lines with different *NTRK3* copy numbers, both lines showed a similar phenotype, which suggests that NTRK3 overexpression consequences are indistinguishable when the expression is higher than normal.

1. GENERAL CONSIDERATIONS

As a consequence of random insertion of *NTRK3* cassette in mouse genomic DNA, we obtained two founders with different copy numbers, which allows us to exclude insertional effects and moreover, to analyze the phenotypic consequences of genetic dosage variations. TrkC is a neurotrophic receptor which is involved in different signalling pathways, and its overexpression could produce gene expression changes of target genes or modulation of several signalling pathways. With this conclusions, it is difficult to predict and establish the relationship between genotype and phenotype, because we are not observing direct outcome from a single gene expression change, but secondary gene expression modifications that affects to transcriptome and proteome levels.

A second important consideration is referred to the genetic strain background used in this transgenic mouse model. We may take into consideration that TgNTRK3 phenotype could not be only affected by *NTRK3* overexpression, but by the genetic strain context. In this work, we have not used congenic strains, in which a homogenic genetic background is achieved by means of backcrossing; however, we have used mice from successive crossings, obtaining a reduced effect of genetic background, but an increased inter-individual differences. This means that to obtain the phenotype we need a higher number of animals per group; but it fact, human pathology never shows genetic strain's homogenicity and when a phenotype is observed might be more relevant.

2. RELEVANCE OF NTRK3 OVEREXPRESSION ON FEAR MEMORY PROCESSING

Analysis of TgNTRK3 mice revealed altered information processing to external stimuli associated to morphological and transcriptional changes in specific brain areas involved in this processment. Interestingly, cognitive disabilities were observed in TgNTRK3 mice evidenced by an altered emotional memory processing. In the discussion of this part, we analyze the cellular and behavioural changes

affecting cognitive process derived by NTRK3 overexpression, which could be involved in PD pathophysiology.

2.1. *NTRK3* gene overexpression affects regions involved in the processment of emotional stimuli

The first step to understand potential changes in the processing of external emotional stimuli is the analysis of brain regions that could influence behavioural responses, and in which we can extract conclusions about cognitive processment. These analysis allow us to interpret results from subsequent behavioural experiments. There are many regions that are involved in emotional information processing, however, different theoretical models have implicated brain structures localized in the temporal lobes, such as the amygdala and the hippocampus, in the neural circuitry underlying PD (Gorman et al, 2000).

For the analysis of both regions, we selected different tools to evaluate morphologically and transcriptome expression TgNTRK3 mice.

2.1.1. Hippocampal cellularity

Hippocampus is responsible for processing threatening or traumatic stimuli and plays a key role in the brain by helping to encode information into memories. The present study documents the presence of morphological modifications in the hippocampal formation of mice with overexpression of the neurotrophin TrkC receptor. NT-3, as well as the cognate receptor TrkC, have been localized to the hippocampus of normal adult rodents (Merlio et al, 1992; Zhou and Rush, 1994), and it has been suggested that TrkC promotes the survival of specific neuronal populations in the CNS, such as catecholaminergic neurons (Dierssen et al, 2006; Sahún et al, 2007).

Considering that neurotrophins control neuronal survival in a target-derived manner during the period of naturally occurring cell death in development, and have been implicated as regulators of neuronal differentiation and as modulations of synaptic efficacy (Thoenen, 1995), it is even conceivable that TrkC overexpression could have a physiological role in the survival of neuronal hippocampal cells in TgNTRK3 mice.

In this regard, hippocampal brain abnormalities results in PD patients have not been conclusive. For example, hippocampus of PD patients have not revealed differences (Vythilingam et al, 2000) or a slight decrease in volume (Uchida et al, 2003), and an abnormal hippocampal metabolism rate (De Cristofaro et al, 1993). in comparison with healthy subjects

Although TrkB/TrkC double mutants have shown non significant apoptosis in the Ammon's horn, where hippocampal pyramidal neuron cell bodies are located (Minichiello and Klein, 1996), we observed, through stereological studies, that TgNTRK3 mice presented an increase of neuronal cell density in all the hippocampal subregions. Studies that measured hippocampal cell density in humans

have revealed positive correlations between visual learning ability and the pyramidal cell density of hippocampal subregions (Tucker et al, 1996). These results in combination with previous hippocampal-LTP studies in TgNTRK3 mice (Sahún et al, 2007) suggest that transgenic mice present a hippocampal hyperactivation that would disrupt the process of memory for emotionally significant events. Any interference within this mechanism causes memories to be preserved as aversive experience.

In accordance with our results, haploinsufficieny of *NTRK3* gene caused neuron losses in the dentate gyrus of aged mice, probably due by its requirement in the support of dentate granule cells and their excitatory synaptic connections within the hippocampus (Von Bohlen und Halbach et al, 2003).

2.1.2. Hippocampal mossy fibers

Dentate gyrus granule cells form excitatory connections, so-called MF, with CA3 pyramidal neurons, a pathway that is implicated in certain forms of long-term potentiation (Zalutsky and Nicoll, 1990). Recently, selective and reversible inactivation of MF synapses in CA3 in mice consistently impaired leaning processes (Lassalle et al, 2000). The expression of TrkC in the dentate granule cells and in their postsynaptic targets, the CA3 pyramidal cells (Tessarollo et al, 1993), indicated that this neurotrophin receptor is involved in the formation of MF connections. In fact, TrkC is required for dendritic maturation and synaptogenesis, as shown in TrkC knock-out mice with reduced axonal arborization and synaptic density in the hippocampus (Martinez et al, 1998).

As an indirect measure of function of granule cells in TgNTRK3 mice, we estimated the amount of MF projecting from the DG to CA3 pyramidal neurons. We stained horitzontal sections with Timm's staining (Timm, 1958), which is a histochemical marker for hippocampal MF. The pattern of Timm's staining in TgNTRK3 MF was indistinguishable from that in wild type mice. We did not find significant differences in MF in hippocampal regions analyzed between genotypes, however, a slight increase in the percentage area of the hilus was observed in TgNTRK3 mice.

It could seem that this results are in contradiction with a higher cellularity in DG, however, homozygous mutant $TrkC^{-/-}$ showed no differences in density of hippocampal MF from that in wild type mice (Minichiello and Klein, 1996), but a clear reduction was observed in mice carrying mutant TrkB/TrkC alleles, giving another evidence of neurotrophic redundant function. Nevertheless, TrkC signalling is required for the correct formation and maturation of axonal trees and synaptic terminals of hippocampal MF (Otal et al, 2005). In this regard, we have not looking for structural changes, such as axonal branching or density of synaptic contacts in TgNTRK3 mice, but we hypothesize that TrkC overexpression may alter the maturation and formation of these hippocampal fibers.

2.1.3. Hippocampal gene expression profiling

NTRK3 overexpression in TgNTRK3 mice affects gene expression profile. As previously described, hippocampus plays a key role in learning and memory processes, and hippocampal gene expression

changes may affect some forms of information processing. We performed microarrays studies in hippocampal mRNA samples which revealed subtle gene expression changes in TgNTRK3 mice; however, identification of three gene networks evidenced an altered Akt and NF-κB cell signalling pathways in transgenic mice.

Concretely, NF-κB is widely used by cells as a regulator of genes that control cell proliferation and cell survival, and is involved in synaptic plasticity and memory (Albensi and Mattson, 2000). Active NF-κB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die. Part of NF-κB's importance in regulating cellular responses is that it belongs to the category of 'rapid-acting' primary transcription factors. Moreover, NF-κB plays a key role in regulating both the innate and the adaptative immune responses (Gilmore, 2006).

Akt or protein kinase B (PKB), which is also involved in synaptic plasticity and memory (Horwood et al, 2006), is an important molecule in mammalian cellular signalling and is involved in cellular survival pathways (Song et al, 2005). Stimulation of the PI3K-Akt kinase pathway following trophic factor receptor activation contributes to the survival of CNS neurons. Moreover, Akt binds an regulates many downstream effectors, e.g. NF-κB, thus resulting in transcription of pro-survival genes.

Previous results in TrkC$^{-/-}$ mutant mice, in contrast, addressed hippocampal gene expression changes toward synaptic vesicle and membrane proteins. TrkC$^{-/-}$ showed dramatical reductions for synapsin 1 (52–64% reduction), whereas the v-SNAREs synaptotagmin I and synaptobrevin 2 and the t-SNARE syntaxin 1 showed moderate decreases (19–45%) (Martínez et al, 1998). These results suggest that TrkC signaling leads to downregulation of both synaptic vesicle and synaptic membrane proteins responsible for synaptic vesicle docking and fusion, and neurotransmitter release at synaptic sites (Südhof, 1995; Hay and Scheller, 1997).

Taken together these observations support a role of TrkC in cell proliferation/survival and developmental maturation/function of synaptic structures, by regulating cell signalling pathways and presynaptic proteins (Wang et al, 1995; Takei et al, 1997), respectively. Because structural and biochemical changes in mice with modified expression of synaptic-associated proteins are linked to altered synaptic function and neurotransmitter release, it is tempting to speculate that CA^{2+}-dependent synaptic vesicle dynamics and neural transmission may be altered in TgNTRK3 mice.

2.1.4. Amygdala cellularity

The amygdala consists of several brain nuclei. The BLA) complex, including the basolateral and lateral nucleus, plays an important role in emotional memory (LeDoux, 2003). Although NT-3 mRNA is not expressed in the murine amygdala, TrkC mRNA expression is conserved in the amygdala of adult mice (Krause et al, 2008). However, the physiological significance of TrkC receptors in the adult amygdala is largely unknown. Haploinsufficiency of TrkC in mice did not alter neuronal densities within

the amygdala (Von Bohlen und Halbach et al, 2003). However, lateral, basolateral and basomedial nuclei of the amygdala revealed significant increases in densities of degenerated axonal fragments.

Substantial increases of neuron numbers were detected in BLA, which represents the largest of the amydala nuclei. These findings offer strong structural evidence for an involvement of the amygdala in a mouse model of PD. The remarkable degree of cell number increase in the BLA suggests that several subpopulations of neurons, possibly including projection neurons, may be affected in this mice model. The BLA is thought to play a major role in assigning emotional significance to external stimuli (LeDoux, 2003), and abnormalities in this nucleus is likely to contribute to the impairment of emotion processing encountered in TgNTRK3 mice, such as fear conditioning (see below).

In humans, in vivo structural imaging reports showed a smaller amygdalar volumes in PD patients (Massana et al, 2003), but significantly higher levels of glucose uptake in this region (Sakai et al, 2005) than healthy controls. Even though stereological analysis in TgNTRK3 did not show volume differences from than in wild type mice, increased cellularity provide new evidence for the existence of morphologic abnormalities in the amygdala by an altered expression of neurotrophic receptor TrkC. It is also important to point out that these findings imply that amygdala nuclei other than the BLA are intact in TgNTRK3 mice. It is possible, in fact likely, that more subtle cellular and neurochemical abnormalities occur in other amygdalar nuclei.

2.2. Altered fear memory consolidation and extinction in TgNTRK3 mice

Fear is an emotional response to threats and danger, and is a neural circuit that has been designed to keep the organism alive in dangerous situations.(LeDoux, 1994). In the case of anxiety disorders, such as PD, it is well understood that fear, escape, or avoidance behaviour and panic-like responses are ubiquitous throughout animal phylogeny (Gorman et al, 2000). However, the analogy of panic attacks to animal fear and avoidance responses is deficient.

Preclinical studies have shown that the amygdala plays a key role in fear circuitry, and that abnormalities in amygdala pathways can affect the acquisition and expression of fear conditioning. The amygdala receives direct sensory input from brainstem structures and the sensory thalamus, enabling a rapid response to potentially threatening stimuli, although it also receives afferents from cortical regions involved in the processing and evaluation of sensory information. The hippocampus also plays a role in fear conditioning, processing information about the environmental context in which the neutral stimulus is presented (Phillips and LeDoux, 1992). Potentially, a neurocognitive deficit in these cortical processing pathways could result in the misinterpretation of sensory information known to be the hallmark of PD, leading to an inappropriate activation of the 'fear network' via misguided excitatory input to the amygdala. In this animal model of PD, we observed amygdalar and hippocampal abnormalities, characterized by morphological and transcriptional alterations that could evidence a putative altered emotional brain processing in TgNTRK3 mice.

Using fear conditioning as a behavioural assay of the emotion humans experience as "fear", it has been possible, through studies of experimental animals, to map out in great detail just how the fear system of the brain works. As largerly explained in the introduction, fear conditioning paradigm, which involves the pairing of a neutral (conditioned) stimulus (CS) with an aversive (unconditioned) stimulus (US), was used in our transgenic model to measure the conditioned response (CR). As previously described, TrkC is widely expressed in specific brain regions involved in emotional learning processing, which suggests a physiologic implication of this neurotrophic receptor in fear memories.

Characterization of the conditioned response in TgNTRK3 mice after fear conditioning revealed an increase of fear memory consolidation and an impaired extinction process. In this work, we thoroughly examined the formation and storage of fear memories in TgNTRK3 mice and asserted in cognitive alterations observed in PD patients, such as consolidation and extinction.

2.3.1. Consolidation

Fear conditioning experiments in TgNTRK3 mice was aimed at further defining the role of NT-3/TrkC signalling pathway in memory consolidation of auditory fear conditioning. Fear memory consolidation is a process in which short-term memory (STM) is transformed, over time, into stable long-term memory (LTM) (Davis and Squire, 1984). The main role to consolidate fear memories into LTM is probably because they convey vital information about danger in the environment that might be important for survival. In this regard, recent studies have implicated neurotrophic factors signalling pathway in fear memory consolidation (Liu et al, 2004; Bekinschtein et al, 2008).

However, few evidences have determined the role of NT-3/TrkC system in fear memories. Previous studies suggest that conditional NT-3 mutant mice do not show impaired fear memory consolidation (Akbarian et al, 2001), suggesting that NT-3/TrkC signalling is not relevant in emotional memory; however, in this experiment, mice were repeatedly exposure to electrical footshocks, which could involve different learning process apart from acquisition.

In our fear conditioning paradigm, we used one-trial avoidance which has been widely used for the study of memory consolidation. Our findings indicated that TgNTRK3 mice show a higher freezing response after training to an associative presentation of tone and shock, both in contextual and non-contextual paradigms. These results strongly favour the view that TrkC-dependent process underlies memory consolidation and synaptic plasticity in the amygdala and build nicely on the findings of recent papers that have demonstrated the involvement of other neurotrophic factors in the amygdala in fear memory consolidation, including BDNF and TrkB (Rattiner et al, 2004).

The fact that TgNTRK3 maintain higher freezing response 7 d later of fear conditioning training suggest that previous consolidation has been strengthened, avoiding that 'forgetting' processes destroys the association between CS-US, in context and non-context paradigms. These results could indicate that in TgNTRK3 mice, emotional cognitive process induce changes in neuronal plasticity which are persistent in time.

NA system integrates and coordinates the response to fear stimuli. In previous studies in TgNTRK3 mice, a noradrenergic central function alteration has been observed (Dierssen et al, 2006), which could be affecting formation and storage of fear memory. The higher effectiveness of the NA synapsis in TgNTRK3 mice would diminish the emotional threshold of the individual's excitement and would alter the regulation of the fear and alarm system. However, amygdala and hippocampal alterations in this transgenic mouse suggest that other regions than LC, are also responsible for this abnormal behavioural response.

2.3.2. Reconsolidation

The idea that new memories undergo a time-dependent consolidation process after acquisition has received considerable experimental support. Recent studies have shown that established memories, once recalled, become labile and sensitive to disruption, requiring reconsolidation to become permanent.

In our experiments, retrieval is induced in a series of short reactivation sessions, which occurred at least 24 hours after training and consisted of presentation of the CS in the absence of the US. With this experimental procedure, we obtained information about the reactivation process that comprises short-term, long-term and very long-term reconsolidation. Our results suggest that TgNTRK3 mice have normal reconsolidation of fear memories, which is not contradictory with consolidation results, because several studies have defined both processes as nature independents.

Molecular studies have revealed an important role of NMDA receptors and beta-adrenergic receptors (Hurlemann et al, 2005) in reconsolidation. For example, propanolol, a beta-adrenergic receptor antagonist, has been consistently to block recognition and recall of emotional memories. This results suggest that alterations in NAergic system could contribute to affect reconsolidation. Although previous results revealed higher NAergic cellularity in TgNTRK3 mice, we did not observed obvious reconsolidation alterations; however, reconsolidation paradigm did not allow us to analyze freezing behaviour for more than a minute (sound) in each session, which we do not have information about longer conditioned responses.

2.3.3. Extinction

The persistence of fear memories is thought to be a major contributor to the morbidity associated with some psychiatric disorders, including PTSD, PD and social phobia (Cannistraro et al, 2003). Particularly, Studies in PD patients revealed a reduced extinction response in a fear conditioning procedure (Michael et al, 2007).

Biological mechanisms in fear extinction have not yet been fully characterized. However, for almost a century, fear extinction has been used as the base to behavioural treatment of phobias (Walker et al, 2002), and recently applied to the treatment of panic disorder using "flooding", "exposure" or desensitization" psychotherapeutic techniques. Exacerbated fear behaviors are accounted for, at least in part, by an excessive activity of fear circuits in the amygdala probably

influenced by an insufficient GABAergic inputs primarily form the prefrontal cortex that permits unwanted associations of fear memories to occur (Quirk and Gehlert, 2003). In addition, the role of endocannabinoid and catecholaminergic-mediated pathways has also been postulated (Maricano et al, 2002). The loss of afferent inhibitory tone has been evidenced in anxiety disorders, suggesting an exaggerated amygdaloid activity underlying the cognitive and peripheral symptoms of panic attacks (Quirk and Gehlert, 2003).

Animal research suggests that extinction is a form of acquired inhibition that suppresses a fear response. In other words, extinction is probably not simply an unlearning or forgetting but rather a new form of learning that changes the CS-US contingency in such a way that the CS no longer signals an aversive event and thereby inhibits the expression of the fear response (Rescorla, 1996). Laboratory studies implicate the amygdala during fear extinction. Another brain area that is apparently involved in extinction learning is the medial prefrontal cortex, probably mediating or regulating amygdala activity (Morgan and LeDoux, 1995; Phelps et al, 2004).

In extinction of fear memories, TgNTRK3 showed a reduced effect of CS-US disassociation. Taking into account our results in fear memory consolidation, it suggests that stronger consolidation process in TgNTRK3 together with reduced non-emotional 're-learning' process are the main cause of impaired fear memory extinction.

On the basis of previous work, modulation of dopaminergic and noradrenergic systems in mPFC is likely to modulate the rate of extinction (McCormick and Thompson, 1982; Morrow et al, 1999). As we described, TgNTRK3 mice show higher cellularity in dopaminergic and noradrenergic nuclei (Dierssen et al, 2006), which could be influencing in the altered extinction response observed in this model. Morevoer, recent studies revealed a crucial role of neurotrophic factors in fear memory extinction. TrkB in the amygdala has been suggested to be required for the consolidation of stable extinction memories (Chhatwal et al, 2006).

Given that research has established comparable findings in human and nonhuman animals, our results in TgNTRK3 mice are of special interest for future research. The translation of animal extinction research to human samples has received more attention lately due to the clinical relevance of the topic. That is extinction of conditioned fear can be viewed as a laboratory analog for the study of exposure treatment for anxiety disorders (Bouton et al, 2001). Knowledge of the conditions that facilitate or hamper extinction learning may help to sharpen exposure treatments in such a way that treatment outcome can be maximized in the short run (therapy efficacy) as well as in the long run (relapse prevention).

2.3. Impaired sensorimotor information processing in TgNTRK3 mice

Few studies have been done in the involvement of neurotrophic factors in sensorimotor information processing. The present data indicate that TgNTRK3 mice show enhanced PPI of acoustic startle. This is the first evidence that overexpression of a neurotrophin receptor leads to a

manifestation of altered PPI, features characteristic of some psychiatric disorders, such as schizophrenia.

The defensive nature of the startle response may be critical to understanding the biological relevance. Possible functions of the startle reflex are reduction of the latency for defensive flight (Pilz and Schnitzler, 1996) or protection during unexpected predator attack from behind (Yeomans and Frankland, 1995). Interestingly, TgNTRK3 mice exhibit enhanced PPI as measured by an attenuated startle response to a loud acoustic stimulus (pulse) when this is shortly preceded by a weak prepulse. PPI is a normal response in animals and humans and is though to reflect sensorimotor gating processes which filter extraneous sensory information. This phenomenon has been shown to be impaired in schizophrenics (Braff and Geyer, 1990), and reversal of PPI deficits has been found to be one of the most robust neurobehavioural models for predicting antipsychotic activity (Hunter et al, 2000). The enhanced PPI observed in TgNTRK3 mice in the absence of changes in startle amplitude in this study suggest that these animals may possess increased sensorimotor gatting mechanisms compared with their litter mate controls. In this regard, based on our previous study (Dierssen et al, 2006), we may have expected the TgNTRK3 mice to have shown a decrement in PPI rather than the observed enhancement, due to higher dopaminergic cells in the substantia nigra pars compacta (SNpc), and probably higher dopaminergic levels in output areas of SNpc, that could be affecting the PPI in our study.

In an effort to extend these findings, we examined the role of dopaminergic and glutamatergic systems on PPI in TgNTRK3 mice, by the aid of a dopaminergic agonist, amphetamine, and a NMDA receptor antagonist, MK-801. The effect of these drugs used in this study was similar as that described in other mouse lines (Varty et al, 2001).

PPI is significantly impaired in these mice after treatment with amphetamine, leading to the suggestion that this transgenic model may have alterations in dopaminergic brain areas, indicated by an increased sensitivity of TgNTRK3 mice to amphetamine. Thus, our data may be pointing at a modulatory influence in dopaminergic system as a consequence of TrkC overexpression. It should be noted, that the effect of amphetamine in the brain is not only on dopamine release, but on NA and serotonin release as well. However, in rats, previous studies have shown that the disruption of PPI caused by amphetamine release is critically dependent on dopamine release in the nucleus accumbens (Swerdlow et al, 1990). A reduced effect of amphetamine on behaviour could theoretically be caused by reduced amounts of dopamine released or by a down-regulation of postsynaptic dopamine receptors.

However, it may be that the increase of dopaminergic cell number in SNpc in these mice has led to a reduction of dopamine function in specific target areas. Although direct measurements of dopamine levels in these nuclei are needed for conclusive evidence, this notion may be supported by the present observation of disrupted PPI in TgNTRK3 mice, as acute treatment of dopamine agonists ip resulted in PPI deficit (Varty et al, 2001). Moreover, neurotrophin receptor function during development may determine behavioural abnormalities, such as dopaminergic hyper-responsiveness

and disruption of prepulse-inhibition, two features characteristic of schizophrenia (Rajakumar et al, 2004).

However, MK-801 reduced the startle reactivity in TgNTRK3 similar to effects observed in wild type mice. The reduced PPI in both genotypes is consistent with studies in mice (Varty et al, 2001) showing that NMDA antagonists reduce PPI. Although NT-3 has been reported to induce enhancement of glutamatergic synapses mediated by increasing both the efficacy of glutamate release from the presynaptic neuron and the neurotrophin-dependent postsynaptic enhancement of NMDA receptor responsiveness (Lessmann, 1998), MK-801 produced similar effects on PPI in TgNTRK3 and wild type mice, suggesting that NMDA receptor mechanisms were not critically involved in acoustic reactivity in TgNTRK3 mice. There also does not appear to be due to changes in hippocampal NMDA receptor levels, because our previous results indicated that overexpression of TrkC leads to significant changes in the level of hippocampal expression of NMDA receptors subunits, concretely to an increase of NR1 and NR2B protein levels in TgNTRK3 mice (Sahún et al, 2007). However, a more detailed analysis of NMDA receptor levels in other brain regions involved in the modulation of startle reactivity should be done in order to ascertain the implication of glutamatergic system in sensorimotor information processing in TgNTRK3 mice.

Recently, patients suffering from PD or posttraumatic stress disorder (PTSD) have been shown to have disruptions in PPI (Grillon et al, 1996, 1998; Ludewig et al, 2002, 2005). Concretely, PD patients showed abnormal early stages of sensory information processing in the absence of mediation, including deficits in PPI and habituation which could reflect a more generalized difficulty in suppressing or gating information, and leading to a cascade of downstream effects on cognition. In contrast to PD patients, TgNTRK3 mice showed an enhanced PPI response, however, and due to brain regions differences in the control of startle reactivity between different organisms, we hypothesize that in our transgenic mice, as in the case of PD patients, an altered sensorimotor information processing could lead to altered cognitive abilities.

3. IMPACT OF NTRK3 OVEREXPRESSION ON MOLECULAR, CELLULAR AND BEHAVIOURAL PROCESSES INVOLVED IN STRESS RESPONSE

The present study analyzed the effects of stress exposure in a transgenic mouse with overexpression of TrkC, the TgNTRK3 mice, validated as a PD model, focusing on the functionality of the HPA axis, the neuronal activation pattern and the behavioural changes under acute and chronic stress conditions. We hypothesized that TrkC may play a role in the susceptibility to stress showed in stress-related disorders, such as PD and MDD-RE, and also, whether comparable stress response signs could be observed in a mouse model of PD. Overexpression of TrkC resulted in alterations in HPA axis indicated by the alteration of circadian corticosterone rhythms and corticosterone release to acute and chronic stress which were accompanied by disproportionated activation of stress-related brain areas, and increased anxiety-like behaviour in transgenic mice.

3.1. Hypothalamic-pituitary-adrenal mechanisms in TgNTRK3 mice

3.1.1. Expression of CRH and GR in stress-related brain areas

It was shown that chronically elevated brain CRH levels produce marked changes in baseline and stimulus-evoked physiological, neurochemical, and behavioural responses, suggesting that chronic CRH hypersecretion is an important factor in the etiology of stress-related disorders (Linthorst et al, 1997). CRH is one of the major modulators of the HPA system (Engelmann et al, 2004). To test whether TgNTRK3 mice show an altered activity of brain CRH neurons, we studied the CRH mRNA levels.

Our results revealed that under basal conditions, TgNTRK3 mice present normal CRH mRNA levels in hypothalamus and amygmala. However, another A lower increase of CRH mRNA in PVN after exposure to an acute stressor (restraint) was observed in TgNTRK3 mice, an effect that may be explained by a higher increase in corticosterone secretion leading to an enhanced negative-feedback response in CRH synthesis. In fact, this is in agreement with the results obtained in PD patients in which there is a reduced response of ACTH to CRH (Schreiber et al, 1996) that could reflect a hypothalamic dysregulation resulting in an excess of endogenous CRH secretion.

Brain CRH neurons are involved not only in the control of HPA responses to stress, but they also play a major role in a wide variety of other stress-induced neuroendocrine, metabolic, autonomic, and behavioural responses (Owens and Nemeroff, 1991). Changes in CRH mRNA expression after a stressful stimuli suggest that in TgNTRK3 may present different coping-behavioural alterations

In addition, we studied putative changes in GR receptor expression in different hippocampal subregions. In accordance with previous studies, in which hippocampal GR mRNA expression is reduced after chronic stress (Kitraki et al, 1999), CES-stressed group showed a reduced GR mRNA increase after acute restraint exposure, and supported CES as a good model for chronic stress studies in rodents. Nevertheless, we need to ascertain the functionality of GR and other receptors involved in the HPA axis to get insight on the consequences of corticosterone hypersecretion observed after stressful events in TgNTRK3.

In conclusion, we demonstrated that transgenic mice overexpressing TrkC develop reduced activity of hypothalamic neurons to synthesize CRH after stress. Several studies have indicated that alterations in central CRH systems may lead to reciprocal changes in CRH receptors in the brain. For example, in rats, prolonged central administration of CRH decreased CRH receptor levels in the amygdala (Hauger et al, 1993). This data could suggest putative changes in CRH receptors in TgNTRK3 mice, however, a more detailed work should be address in this regard to obtain conclusive results.

3.1.2. Circadian adrenal response

The adrenal glands are responsible for regulating the stress response through the synthesis of glucocorticoids and catecholamines. One of the main function of glucocorticoids is to shut off the activation of the HPA axis triggered by stressors.

Under basal conditions, analysis of the absolute and relative adrenal weight did not show differences between genotypes, suggesting that glucocorticoids release in our acute stress experiments is not influenced by an adrenal mass effect.

However, we should be cautious in this regard in chronic stress paradigms. In fact, chronic stress causes adrenal gland alterations, such as adrenal enlargement, which could affect differently to TgNTRK3 mice. In our CIS and CES paradigms, we did not measure adrenal weight; however, adrenal gland differences between genotypes after chronic stress are few plausible due to little variation in corticosterone levels.

Studies in PD patients have been controversial but tend to show normal daytime (Cameron and Nesse, 1988; Holsboer, 1992) or slightly elevated baseline levels of plasma cortisol (Roy-Byrne et al 1986; Abelson and Curtis, 1996) and elevated nocturnal basal cortisol secretion (Abelson et al, 1996; Bandelow et al, 2000). In this way, hormone levels analysis in TgNTRK3 mice revealed higher corticosterone levels under basal conditions in the middle of the light phase, which correlates with nocturnal results in PD patiens.

3.1.3. Corticosterone hormone response to acute stress

Higher cortisol levels have been found in PD patients compared to healthy controls under conditions of minor environmental changes (Roth et al, 1992; Stones et al, 1999). However, TgNTRK3 mice did not show such differences in corticosterone levels when they were exposed to a mild stressor, such as novel environment. Although we did not observe corticosterone differences, HPA axis could be affected by behaviour displayed during exposure to the novel environment in TgNTRK3 mice. Moreover, we did not study corticosterone levels time after the termination of exposure to the stressor, which could give us valuable information of stress response to a mild stressor.

When we used restraint as stressor, a tendency to higher corticosterone levels in transgenic mice was observed. Restraint is a moderate-intensity stressful stimulus and has been described to activate consistently the stress response. We can not rule out a ceiling effect of adrenal secretion in mice exposed to restraint, which would reduce corticosterone differences. Moreover, we did not obtain blood samples time after the termination of restraint, and we can not rule out an altered corticosterone response in TgNTRK3 mice.

In addition, we could not measure ACTH response in TgNTRK3 mice due to technical problems. Stress causes ACTH release from anterior pituitary to the portal system, and is a reliable indicator of CRH and AVP activity.

3.2. Impact of NTRK3 overexpression in c-Fos activation of brain areas in TgNTRK3 after an acute stress

Stress exposure results in a rapid activation or inhibition of stress-related brain areas characterized by coordinated behavioural, autonomic, and endocrine responses, which are required for the maintenance of homeostasis.

By examining changes of c-Fos immunoreactivity in various brain regions upon exposure to immobilization, we also attempted to map central genotype-specific neuroendocrine pathways. We believe the existence of genotype-specific pathways and circuits is a clear step forward in the study of the abnormal stress response in TgNTRK3 mice.

It is well established that the HPA axis response is orchestrated by modulatory positive/negative influences of particular brain areas (Herman et al, 2003). Specifically, in PD, brain regions involved in perception of external and internal stimuli, hyperarousal and cognitive judgement may play a key role for HPA axis regulation. Fos immunohistochemistry studies were done to analyze the activation pattern of specific stress-related brain areas (Figure 1), because of their critical role in stress response (Herman et al, 2003).

In the mPFC, which plays a main role in the integration of cognitive-affective information and regulates the HPA axis response to emotional stress (Diorio et al, 1993), TgNTRK3 exhibited higher activation in prelimbic cortex subregion, which has been implied in inhibitory influence to the HPA axis (Radley et al, 2006), with respect to wild type mice. mPFC modulates PVN stress responses through modulation of brain regions that include the lateral septum (LS), bed nucleus of stria terminalis (BNST), thalamus, amygdala, hypothalamus and brainstem catecholamine cell groups (Hurley et al, 1991).

Another region that has been described to have a predominantly inhibitory role over the respose to stressful situations is the LSv (Seggie et al, 1974), that constitutes an interface between the hippocampus and the hypothalamus, and where TgNTRK3 mice showed higher c-Fos-positive cells in comparison with wild type mice. Intriguingly, hippocampus (Jacobson and Sapolsky, 1991) and central amygdala (Beaulieu et al 1986), which are key brain regions in the inhibition and excitation, respectively, of the HPA axis, showed similar activation in both genotypes. Moreover, another HPA-excitatory regions, such as infralimbic cortex (Radley et al, 2006) and ventral BNST (Choi et al, 2007), did not show differences in c-Fos response between genotypes. Remarkable was the reduction of neuronal activation occurred in stressed transgenic LC, a region that provide excitatory influence to the HPA axis and involved in the conduction of stress signals to forebrain areas (Ziegler et al, 1999).

As previously described by others, the stimulus-processing converges to the parvocellular subdivision of PVN (pPVN), which initiates the HPA response and release CRH and vasopressin to the anterior pituitary (Palkovits, 1992). The reduced activation of pPVN in stressed TgNTRK3 mice

correlates with the overactivation of inhibitory brain regions. These results illustrate that brain activation in response to acute stressful stimuli is disproportionated in TgNTRK3 mice and suggest an enhanced negative-feedback mechanisms probably to cope with an exaggerated stress response.

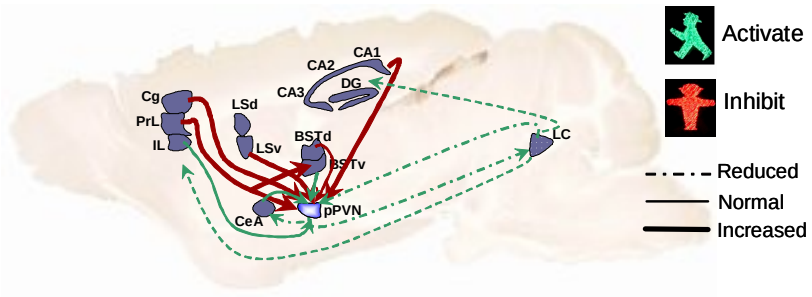


Figure 1. Neuronal efferent pathways to the hypothalamic paraventricular nucleus that are activated by immobilization stress in TgNTRK3 mice. Dashed, thin and think lines indicate reduced, normal and increased stimulation outflow, respectively. Green and red lines indicate activation and inhibition outflow, respectively. Abbreviations: BSTd, bed nucleus of stria terminalis, dorsal groups; BSTv, bed nucleus of stria terminalis, ventral groups; CA1, field CA1; CA2, field CA2; CA3, field CA3; CeA, central nucleus of the amygdala; Cg: Cingulate cortex; DG, dentate gyrus; IL, infralimbic cortex; LSd, lateral septal nucleus, dorsal part; LSv, lateral septal nucleus, ventral part; PrL, prelimbic cortex; PVN, paraventricular nucleus of the hypothalamus.

Nevertheless, as with other methods, detection of c-Fos immunoreactivity has some limitations. For example, an absence of *c-fos* induction does not necessarily indicate a lack of neuronal activity (Hoffman et al, 1993), and the appearance of *c-fos* mRNA is not necessarily followed by the production of Fos protein.

3.3. Corticosterone response after chronic immobilization stress (CIS)

The role of life experiences in the etiology of PD has been widely studied (DeLoof et al, 1989), and stress is thought to be prominent in the etiology and course of PD. The possible altered brain stress-processing in TgNTRK3 mice may influence adaptation/sensitization processes in chronic stress situations. Previous studies have shown that CIS in rats increased NT-3 mRNA levels in hippocampus (Smith et al, 1995c), suggesting that NT-3/TrkC system may play a role as a mechanism involved in adaptation/sensitization processes to stressors.

In this work, we have performed chronic stress experiments in TgNTRK3 mice to understand the biological implications of NT-3/TrkC system on chronic stress consequences. Additionally to chronic immobilization stress (CIS), we used social isolation due to its contribution toward many emotional, behavioral and physical disorders, including panic attacks, and determine animal-housed influences in the stress response (Levine, 2000).

House-conditions, that did not influence corticosterone secretion in wild type animals, elicited a differential HPA axis response in TgNTRK3 mice. Initially, social isolation reduced basal corticosterone secretion of transgenic to wild type mice levels, which could suggest group-housing stress effects in TgNTRK3 mice. When CIS was introduced, higher corticosterone release and an overreactivation of

HPA axis negative feedback was observed in transgenic group-housing, whereas lower corticosterone levels and hiporeactivation of HPA system negative feedback was present in isolated TgNTRK3 mice.

These results suggest that CIS alone or the combination of social isolation with CIS, generate specific changes in the HPA axis response, and could explain how the stress system in TgNTRK3 mice is affected. Moreover, these stressor-dependent differences in HPA response in TgNTRK3 mice could support the idea that PD patients show a HPA axis dysregulation, stressor- and/or comorbid disorder-dependent, which would produce controversial PD patient results.

3.4. Behavioural profile and coping strategies after chronic environmental stress (CES)

In this work, we have performed chronic stress experiments in TgNTRK3 mice to understand the biological implications of NT-3/TrkC system on behavioural chronic stress consequences.

Stress research in rodents has enlarged our knowledge about the pathophysiology of certain human stress-related disorders. The use of chronic stress models in rodents resembling chronic stress in humans has shown clear evidences that we are far away to have an accurate chronic stress procedure. For example, one of the most widely chronic stress model used is the chronic mild stress (CMS) model of depression, which consists to expose sequentially rats or mice, over a period of weeks, to a variety of mild stressors. CMS, instead of having high validity, has been criticized for being difficult to replicate. However, most of work realized with this stress model has shown clear behavioural changes in rodents that parallel symptoms of depression, such as decreased reactivity to rewards. It is very important to point out that there are a handful of reports of CMS causing significant effects in the opposite direction, termed as an 'anomalous' behavioural profile (Willner, 2005). Moreover, CMS is not a suitable model for anxiety-related behaviour evaluation due to its anxiolytic effect in rodent behaviour profile (D'Aquila et al, 1994).

Since PD patients are especially sensitive to minor environmental challenges (Roth et al, 1992), we selected a stress model based on environmental stressors, where light intensity conditions were modified to obtain a more reliable human model, the chronic environmental stress (CES). This model allows to stress mice without much interaction between researcher and mice.

3.4.1.Anxiety-like behaviour

As previously described, chronic stress models are commonly reported to reduce anxiety-like behaviours in rodents. In the MHB, which enables to detect alterations in a wide range of behaviours, e.g. anxiety-related behaviour, risk assessment or exploration, neither TgNTRK3 nor wild type mice showed anxiety-related behaviours either in stressed or in non-stressed conditions, mainly due to low aversive conditions in which the test was performed (50 Lux). However, self-grooming, an arousal-related behaviour (Ohl et al, 2001), was increased in wild type compared with TgNTRK3 mice stressed mice. Bolles (1970) proposed that stressed mice select the most appropriate behaviours (i.e. vigilance

vs. hygiene/body maintenance) to adapt to the stress situation. The increase in grooming behaviour is often related to the dearousal that appears following exposure to stressors, so that an increase in grooming behaviour may reflect a more efficient habituation process (Kalueff and Tuohimaa, 2004); in addition, TgNTRK3 stressed mice showed longer latency to start grooming, a behaviour that has been related to an impairment in the habituation process to a stressful situation (Spruijt et al, 1992), and suggest an increased apprehension towards a novel environment in TgNTRK3 stressed mice.

In the elevated-plus maze, which consists in higher aversive conditions to rodents than MHB, mice are exposed to highly illuminated and elevated open spaces. Under this ethological conditions, stressed TgNTRK3 mice showed higher anxiogenic responses, such as a reduced time in open arms or higher latency to enter to these arms. In accordance with the anxiolysis profile produced by CMS models, stressed wild type showed a reduced anxiety-like behaviour in comparison with non-stressed wild type mice. Then, here we have a relevant data that indicate, in our TgNTRK3 mice, different coping strategies to a chronic stress, which are observable by behavioural responses in aversive conditions. Nevertheless, the lower number of mice used in this experiment did that we were cautious in our affirmations.

In order to confirm and reproduce these results, we obtain a bigger naïve group of mice, and we exposed them to the same CES model. Then, this group went through a light-dark box test. As explained before, we observed a lower anxiety-related behaviour in stressed vs non-stressed wild type mice, reproducing the same effects of anxiolysis in a different behavioural test. Moreover, stressed TgNTRK3 mice showed higher anxiogenic behaviours, such as a tendency to spend less time in the light compartment and a similar activity-exploratory behaviour than the non-stressed TgNTRK3 mice, suggesting that CES, consistently, have induced an anxiogenic effect in transgenic mice.

All these behavioural data confirmed that TgNTRK3 mice are differentially affected by chronic stress, and that exposure to aversive conditions reveals a failure to adapt to chronic environmental changes, showed by an altered coping response. This results are in agreement with the current view that dysfunctions in neuronal plasticity are responsible for the development of stress-related disorders, such as depression and anxiety-related disorder, because TrkC overexpression or its developmental effects could be affecting the cognitive capabilities to cope with stress and give rise to maladaptative psychiatric disorders.

3.4.2. Depression-like behaviour

Given the growing 'neurotrophic hypothesis of depression' (Duman and Monteggia, 2006), which is based largely on observations that decreases in hippocampal BDNF levels are correlated with stress-induced depressive behaviours and that antidepressant treatment enhances the expression of BDNF, we used the FST, the most widely used tool for assessing passive coping strategies, after CES exposure.

In accordance with other chronic moderate stress studies in mice (Porsolt, 2000; Tannenbaum et al, 2002), stressed group showed an increase of passive behaviour (floating) with no differences

between genotypes, which suggest CES as a good chronic stress model in rodents. Since last years, active behaviours in the FST have reached higher relevance and the use of pharmacological agents have increased the ethological comprehension of these behaviours, such as swimming or struggling (Armario et al, 1988). In this regard, stressed TgNTRK3 mice showed a reduced duration of active behaviours, which have been closely related with stress susceptibility (Alcaro et al, 2002).

These results from CES may reflect again different coping strategies in TgNTRK3 mice, but only in escape behaviours. Previous results have shown that infusion of NT-3, or BDNF, into the dentate gyrus produced an antidepressant response in the FST paradigm, demonstrating a neurotrophic factor effect in passive behaviours (Shirayama et al, 2002). However, our results are not completely in contradiction to this data, because the efficacy of NT-3 could result from the ability of this factor to act on TrkB, as well as TrkC, receptors (Soppet et al, 1991). This TrkB-dependent effect of NT-3 hippocampal infusion is supported by the normal antidepressant response in NT3+/- mice (Saarelainen et al, 2003). Nevertheless, we suggest that TrkC receptor overexpression in TgNTRK3 mice may induce an abnormal active response which is affected by chronic stress. As depressive-related behaviours were not differentially affected in stressed transgenic mice, and we can not contribute with further data from other behavioural despair assays which measure depressive-related behaviours, such as tail-suspension test or learned helplessness test, we suggest that chronic stress affect TgNTRK3

CONCLUSIONS

In the first part of the work, the analysis of freezing response in fear conditioning revealed enhancement of fear memory formation in TgNTRK3 mice, that could be due to the alteration of the fear-related brain regions, such as amygdala and hippocampus. Moreover, transgenic mice showed impaired extinction of fear memories although transgenic mice presented a non-altered reconsolidation process. As well as this experiment the prepulse inhibition (PPI) was performed, in which startle reflex response is measured to a sudden, intense stimulus, and the magnitude of this response is reduced if a weak sub-threshold prepulse stimulus is presented shortly before the stimulus. Interestingly, PPI was enhanced in TgNTRK3 mice at different prepulse amplitudes (dB). For a better understanding of these results, we performed pharmacological studies with D-amphetamine (a dopamine agonist) and MK-801 (NMDA receptor antagonist) which showed alterations in dopaminergic and glutamatergic systems that could explain such PPI enhancement.

These results may reflect alterations of emotional memories' formation, storage and extinction in TgNTRK3 mice that underlie certain neuropsychiatric disorders, such as phobias and panic disorder, which are associated with lingering expressions of fear by a stimulus which has been linked with an aversive event. In addition, PPI that is an operational measure of sensorimotor gating and altered in several neuropsychiatric disorders, was enhanced in TgNTRK3 mice suggesting a role of this neurotrophin receptor in the inhibition of response to sensory and cognitive events.

In the second part of the work, the results obtained indicate a different response for both acute and chronic stress conditions in TgNTRK3 that could be due to the alteration of the stress-related systems in the brain. The TgNTRK3 mice showed 1/ an altered corticosterone circadian rhythm and corticosterone hypersecretion through moderate to intense stress stimuli could suggest alterations in TgNTRK3 HPA axis functionality; 2/ a disproportionated neuronal activation of brain regions involved in HPA axis activity regulation, with an increased inhibitory inputs to hypothalamic CRH neurons; and 3/ endocrine, behavioural, and CRH synthesis alterations in chronic stress studies that suggest an impaired ability to cope with stress. Homeostasis preservation requires continuous behavioural, autonomic, and endocrine adaptations to control disturbances caused by internal or external threats or stress. Therefore, our results suggest that TgNTRK3 mice could not achieved the right homeostasis control after repeated exposure to stressful stimuli, being unable to prevent or restore stress-induced homeostasis unbalance.

Our results show for the first time that exposure to different acute and chronic stressors produce a differential hormonal, gene expression, brain activation and behavioural responses in a mouse model proposed for PD, TgNTRK3. Previous work of our lab have shown that TgNTRK3 showed increased cellularity and a higher firing rate in LC, that may resemble the exaggerated stimulus-responsiveness and increased emotionality seen in patients with stress or anxiety disorders (Aston-Jones et al, 1994; Goddard and Charney, 1997), such as PD. The results obtained in this work suggest that *NTRK3* is an important candidate gene to explain the susceptibility to the onset of stress-related disorders, through an effect on neural

development and plasticity in specific brain regions related to the stress response and underlying sensitization to repeated stress. Thus, TgNTRK3 mice resemble the human situation in anxiety disorders, in which individuals at risk are predisposed to develop anxiety episodes after chronic stress. Further studies of the transgenic NTRK3 mice will enable a more complete understanding of the role of NT-3/TrkC system in the mechanisms of stress sensitization and susceptibility to stress.

In conclusion, we propose that an altered expression of TrkC could participate in the pathophysiology of panic disorder through affecting neurobiological systems involved in stress and fear responses, such as HPA axis and limbic system. Disturbation of these systems would cause innapropiate physiological and behavioral responses, and unleash to the psychiatric pathology.

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ANNEXE

1. ABREVIATURES

ACh	**A**cetyl**ch**oline
ACTH	**A**deno**c**ortico**t**ropin **h**ormone
ANH	**A**trial **n**atriuretic **h**ormone
AP-1	**A**ctivating **p**rotein-**1**
ASR	**A**coustic **s**tartle **r**esponse
AVP	**A**rginine-**v**asopressin **p**eptide
BDNF	**B**rain-**d**erived **n**eurotrophic **f**actor
BLA	**B**aso**l**ateral **a**mygdala
BNST	**B**ed **n**ucleus of **s**tria **t**erminalis
CB1	**C**annabinoid **r**eceptor **1**
CBG	**C**orticoid-**b**inding **g**lobulin
CE	**C**oeficient of **e**rror
CeA	**C**entral **a**mygdala
CES	**C**hronic **e**nvironmental **s**tress
Cg	**C**ingulate **c**ortex
CIS	**C**hronic **i**mmobilization **s**tress
CNS	**C**entral **n**ervous **s**ystem
CREB	**c**AMP **r**esponse **e**lement-**b**inding
CRH	**C**orticotropin-**r**eleasing **h**ormone
CS	**C**onditioned **s**timulus
CSF	**C**erebro**s**pinal **f**luid
CT	**C**omputed **t**omography
DAB	**D**i**a**mino**b**enzidine
DG	**D**entate **g**yrus
Dis	**Dis**ector
DRG	**D**orsal **r**oot **g**anglia
DST	**D**examethasone **s**uppression **t**est
DTT	**Dit**hio**t**hreitol
EDTA	**E**thylene **d**iamine **t**etraacetic **a**cid
EPM	**E**levated-**p**lus **m**aze
FBS	**F**etal **b**ovine **s**erum
FST	**F**orced **s**wimming **t**est
GABA	**G**amma-**a**mino**b**utyric **a**cid

GAD	**G**eneralized **a**nxiety **d**isorder
Glu	**G**lutamate
GR	**G**lucocorticoid **r**eceptor
GREs	**G**lucocorticoid-**r**esponse **e**lements
HCl	**Cl**or**h**ydric acid
Hdis	**H**eight of the **dis**ector
HPA	**H**ypothalamic-**p**ituitary-**a**drenal
ICV	**I**ntra**c**erebro**v**entricular
IEGs	**I**mmediate **e**arly **g**ene**s**
IIPMF	**I**nfra- & **i**ntra-**p**yramidal **m**ossy **f**ibers
IL	**I**nfra**l**imbic cortex
IL-1β	**I**nter**l**eukin-**1β**
IMO	**I**m**mo**bilization
Ip	**I**ntra**p**eritoneally
ISI	**I**nter**s**timulus **i**nterval
LC	**L**ocus **c**oeruleus
LS	**L**ateral **s**eptum
LTM	**L**ong-**t**erm **m**emory
LTP	**L**ong **t**erm **p**otentiation
LVGCCs	**L**-type **v**oltage-**g**ated **c**alcium **c**hannels
MAPK	**M**itogen-**a**ctivated **p**rotein **k**inase
m-CPP	**m**-**c**hloro**p**henyl**p**iperazine
MDD	**M**ajor depressive disorder
MeA	**Me**dial **a**mygdala
MF	**M**ossy **f**iber
mHB	**m**odified **H**ole-**B**oard
mPFC	**m**edial **p**refrontal **c**ortex
MRI	**M**agnetic **r**esonance **i**maging
NA	**N**or**a**drenaline
NF-κB	**N**uclear **f**actor-**κB**
NMDA	**N**-**m**ethyl-**D**-**a**spartate
NTRK3, TRC	**N**eurotrophine **T**irosine **K**inase **3**, **R**eceptor
Nv	**N**euronal density
OCD	**O**bsessive-**c**ompulsive **d**isorder
PAG	**P**eriaqueductal **g**ray
PBS	**P**hosphate **b**uffered **s**aline

PBS-T	**PBS** containing 0.2% **T**riton X-100
PBS-T-GEL	**PBS-T** containing 0.25% **g**elatine
PD	**P**anic **d**isorder
PFA	**P**ara**f**orm**a**ldehide
PFC	**P**refrontal **c**ortex
PLCγ	**P**hospho**l**ipase **Cγ**
PNS	**P**eripheral **n**ervous **s**ystem
POMC	**P**ro-**o**pio**m**elano**c**ortin
PPI	**P**re**p**ulse **i**nhibition
PrL	**P**relimbic **c**ortex
PR-LTM	**P**ost-**r**eactivation **l**ong-**t**erm **m**emory
PR-STM	**P**ost-**r**eactivation **s**hort-**t**erm **m**emory
PTSD	**P**ost-**t**raumatic **s**tress **d**isorder
PVN	**P**ara**v**entricular **n**ucleus of the hypothalamus
rCBF	**R**egional **c**erebral **b**lood **f**low
Sdis	**S**urface **dis**ector
SD	**S**tandard **d**eviation
SGZ	**S**ub**g**ranular **z**one
SNpc	**S**ubstantia **n**igra **p**ars **c**ompacta
SOA	**S**timulus **o**nset **a**synchrony
SPL	**S**ound **p**ressure **l**evel
SPMF	**S**upra-**p**yramidal **m**ossy **f**ibers
SSC	**S**tandard **s**aline **c**itrate
SV	**S**hrinkage factor
TH	**T**yrosine **h**ydroxylase
TrkB	**T**ropomyosin **r**eceptor **k**inase **B**
TrkC	**T**ropomyosin **r**eceptor **k**inase **C**
UFC	**U**rinary **f**ree **c**ortisol
US	**U**nconditioned **s**timulus
Vref	**V**olume of **r**eference
VTA	**V**entral **t**egmental **a**rea
α-MSH	**α**-**m**elanocyte-**s**timulating **h**ormone
µCi	**µC**ur**i**er

2. ARTICLES DERIVED FROM THIS THESIS

Part of the results obtained in this thesis were published in scientific papers:

Sahún I, Delgado-García JM, **Amador-Arjona A**, Giralt A, Alberch J, Dierssen M, Gruart A. Dissociation between CA3-CA1 synaptic plasticity and associative learning in TgNTRK3 transgenic mice. The Journal of Neuroscience 2007 Feb; 27(9): 2253-2260.

Dierssen M, Gratacòs M, Sahún I, Martín M, Gallego X, **Amador-Arjona A**, Martínez de Lagrán M, Murtra P, Marti E, Pujana MA, Ferrer I, Dalfo E, Martínez-Cué C, Flórez J, Torres-Peraza JF, Alberch J, Maldonado R, Fillat C, Estivill X. Transgenic mice overexpressing the full-length neurotrophin receptor TrkC exhibit increased catecholaminergic neuron density in specific brain areas and increased anxiety-like behavior and panic reaction. Neurobiol Dis 2006 Nov; 24(2): 403-18.

Moreover, scientific revisions were written during these years:

Gratacòs M, Sahún I, Gallego X, **Amador-Arjona A**, Estivill X, Dierssen M. Candidate Genes for Panic Disorder: Insight from Human and Mouse Genetic Studies. Genes Brain Behav 2007 Jun; 6 (s1): 2-23.

The main results of this thesis will give rise to other scientific papers:

Amador-Arjona A, Belda X, Gagliano H, Delgado R, Erhardt A, Keck ME, Armario A, Dierssen M. The neurotrophin-3 receptor TrkC plays a critical role in stress susceptibility and adaptation/sensitization to chronic stress. Biol Psychiatry (Submitted).

Amador-Arjona A, Sahún I, Sotelo J, Crusio W, Dierssen M. Overexpression of the full-length neurotrophin receptor TrkC changes hippocampal differentiation phenotype and impairs extinction of fear memories (In preparation).

Collaborations in other scientific papers:

Gallego X, Murtra P, Zamalloa T, Canals J, Pineda J, **Amador-Arjona A**, Maldonado R, Dierssen M. Dysregulation of the opiod system in the locus ceruleus of a mouse model of panic disorder. Genes Brain Behav (Submitted).

Gallego X, **Amador-Arjona A**, Robles N, Armengol L, Gratacòs M, Sabriá J, Estivill X, Dierssen M. Altered anxiety-related phenotype in BAC transgenic mice overexpressing the α3, α5, and β4 subunits of the nicotinic receptors in brain (In preparation).

In the following pages, there are enclosed scientific papers published that contain results from this thesis.

Dierssen M, Gratacòs M, Sahún I, Martín M, Gallego X, Amador-Arjona A, Martínez de Lagrán M, Murtra P, Martí E, Pujana MA, Ferrer I, Dalfó E, Martínez-Cué C, Flórez J, Torres-Peraza JF, Alberch J, Maldonado R, Fillat C, Estivill X.
Transgenic mice overexpressing the full-length neurotrophin receptor TrkC exhibit increased catecholaminergic neuron density in specific brain areas and increased anxiety-like behavior and panic reaction.
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Sahún I, Delgado-García JM, Amador-Arjona A, Giralt A, Alberch J, Dierssen M, Gruart A.
Dissociation between CA3-CA1 synaptic plasticity and associative learning in TgNTRK3 transgenic mice.
J Neurosci. 2007 Feb 28;27(9):2253-60.