Sapienza University of Rome Doctoral school of Neuroscience

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The Repeated cross fostering protocol as a mouse model of panic disorder: suggestions for new treatments from behavioral and molecular characterization

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INTRODUCTION

CHAPTER I. Panic Disorder: definition, epidemiology, etiology, treatments, clinical research and PD theories

1) Definition and epidemiology of Panic Disorder

Panic disorder (PD) is a heterogeneous psychiatric syndrome that affects 3-5 % of the population. The DSM-V includes panic disorder in the anxiety disorders (DSM-V 2013). Recurrent panic attacks (PAs) are the hallmark feature of diagnosis panic disorder. Individuals with this disorder experience recurrent panic attacks and are persistently concerned or worried about having more panic attacks or change his/her behavior in maladaptive ways because of the PAs. Panic attacks are abrupt surges of intense fear or intense discomfort, that reach a peak within ∼10 minutes; can occur in calm or in anxious state

and are accompanied by physical (incapacitating periods of acute-onset respiratory, cardiovascular, gastrointestinal, autonomic) and/or cognitive symptoms. To diagnose panic disorder in addition to PAs four or more of following symptoms occur:

1. Palpitations, pounding heart, or accelerated heart rate;
2. Sweating;
3. Trembling or shaking;
4. Sensations of shortness of breath or smothering;
5. Feelings of choking;
6. Chest pain or discomfort;
7. Nausea or abdominal distress;
8. Feeling dizzy, unsteady, light-headed, or faint;
9. Chills or heat sensations;
10. Paresthesia (numbness or tingling sensations);
11. De-realization (feelings of unreality) or depersonalization (being detached from oneself);
12. Fear of losing control or “going crazy”;
13. Fear of dying.

Another criterion to diagnose PD is that at least one of the attacks has been followed by 1 month (or more) of one or both of the following:

1. Persistent concern or worry about additional panic attacks or their consequences
2. A significant maladaptive change in behavior related to the attacks

The recurrent PAs are categorized in the DSM-V as being either unexpected (also called spontaneous) (uPA), or expected (ePA). The uPAs occur in the absence of a clear

external trigger whereas ePAs occur where an external cue (e.g., situation where uPAs have occurred, or when confronted with a generally feared phobic situation or stimulus) is associated with the induction of the PA (Shulman et al. 1994). Collectively, recurrent PAs can lead to agoraphobia, which is a conditioned avoidance response that occurs when people with PD begin to fear situations that are associated with PA or where escape might be difficult or help might not be available (e.g., planes, elevators etc.) if a PA were to occur.

Already in 1993 Briggs and colleagues identified two subtypes of PD based on the presence or absence of prominent respiratory symptoms (Briggs, Stretch, and Brandon 1993). Studies demonstrated that the respiratory subtype patients feel a stronger suffocation and have more panic attacks than the non-respiratory subtype patients during the carbon dioxide challenge tests (Biber and Alkin 1999; Valenca et al. 2002; Abrams, Rassovsky, and Kushner 2006). In addition in this group there is a higher family history of panic disorder, less comorbidity with depression, a longer duration of panic disorder, lower scores on the scale of neuroticism and, in general, higher scores on scales of severity for panic disorder. These subjects are particularly sensitive to methods of artificial induction of panic. From the respiratory point of view there is a greater sensitivity to the panicogenic effects of CO_2 (Freire et al. 2008).

Epidemiology. Anxiety disorders are a heterogeneous classification that has a lifetime prevalence of about 20% in the general population. Panic Disorder represents one of the most severe anxiety disorders and current estimates are that about 7–10% of the population experience occasional PAs and the prevalence of PD in the general population is ∼2–5% (Goodwin et al. 2005; Kessler et al. 2006).

Lifetime prevalence estimates are 22.7% for isolated panic attacks only, 0.8% for PA with agoraphobia without PD (PA-AG), 3.7% for PD without AG (PD-only), and 1.1% for PD with AG (PD-AG). Persistence, number of lifetime attacks, and number of years with attacks all increase monotonically across these four subgroups (Kessler et al. 2006).

The age of the onset for panic disorder varies considerably with the median age which ranks among 20-24 years in United States population. A small number of cases begin in childhood, and onset after age 45 years is unusual but can occur. The rates of panic disorder show a gradual increase during adolescence, particularly in women, and possibly following the onset of puberty, and peak during adulthood (DSM-V 2013). Women are more frequently affected than men, at a rate of approximately 2:1. The gender differentiation occurs in adolescence and is already observable before age 14 years (Kessler et al. 2006; Sheikh, Leskin, and Klein 2002; DSM-V 2013). Although panic disorder is very rare in childhood, first occurrence of "fearful spells" is often dated

retrospectively back to childhood. As in adults, panic disorder in adolescents tends to have a chronic course and is frequently comorbid with other anxiety disorders (in particular with agoraphobia), depressive, and bipolar disorders and possibly mild alcohol use disorder. A subset of individuals with panic disorder develops a substance-related disorder, which for some represents an attempt to treat their anxiety with alcohol or medications. Comorbidity with other anxiety disorders and illness anxiety disorder is also common especially in individuals with more severe agoraphobia (Social phobia it has been referred in 15%-30% of PD individuals; the obsessive-compulsive disorder in 8%-10% of them and generalize anxiety disorder in 25% of them). The separation anxiety disorder (SAD) during childhood resulted associated with panic disorder (DSM-V 2013).

2) Etiology of Panic Disorder

Although the etiology of PD is largely unknown, several studies demonstrated that there is a strong heritability in first degree relatives (∼11%) and monozygotic twins (30–40%) [see meta-analysis and reviews by (J M Hettema, Neale, and Kendler 2001; Schumacher et al. 2011)]. This heritability was already been referred in first studies in which panic disorder was called anxiety neurosis (COHEN et al. 1951).

Additional data have shown a higher risk of panic disorder in adult first-degree relatives when the age of onset was less than 20 years (Goldstein et al. 1997). However, the major basis of genetic contribution to anxiety disorders is provided by the higher concordance rates for monozygotic twins compared with dizygotic twins (Marco Battaglia et al. 2009; Bellodi et al. 1998; G Perna et al. 1997; Torgersen 1983). The use of the two biggest databases of twins' information, the "Virginia Adult Twin Study of Psychiatric and Substance Use Disorder" (VATSPSUD) and the "Vietnam Era Twin" (VAT), permitted to observe a variance of panic disorder heritability due to a genetic factor for 30%-40%, being the rest of the variance because of individual-specific environment, with an estimated heritability of 44% (K S Kendler, Gardner, and Prescott 2001; G Perna et al. 1997).

Many genetic studies have tried to identify linkage or association to clarify molecular basis of genetic factors in panic disorder [for a review see (Gratacòs et al. 2007)]. Linkage studies permit to indicate approximatively a chromosome region of one gene or genes associated with a defined phenotype. On the other hand in the associative studies the association between a specific DNA sequence and the disease is analyzed in a sample of subjects.

Total genome scans, in the case of panic disorder, have yielded some interesting chromosomal regions, including 7p15 (Crowe et al. 2001; Logue et al. 2003; Knowles et al.

1998), 13q32 (Hamilton et al. 2003; Weissman et al. 2000) 1q32, 11p15 (Gelernter et al. 2001) and 9q31 (Thorgeirsson et al. 2003). Recently, one study found that one region on chromosome 4q31-q34 shows strong evidence of linkage (Kaabi et al. 2006). Also, in a recent study, evidence for linkage reached genome-wide significance in one region on chromosome 15q (near GABA-A receptor subunit genes) and was suggestive at loci on 2p, 2q and 9p (A. J. Fyer et al. 2006) chromosomes.

In the review of Gratacòs is reported a table presenting a list of several genes probably involved in genesis of panic disorder (Gratacòs et al. 2007). Most of all are genes for neurotransmitters, receptors or enzymes involved in neurotransmitters' catabolism or synthesis, and have been considered basing on pharmacological or clinical evidences (Furukawa, Watanabe, and Churchill 2007; Watanabe, Churchill, and Furukawa 2009). Among the drugs with clear panicolytic properties, selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, sertraline or paroxetine, or venalfaxine, a selective serotonin-norepinephrine reuptake inhibitor, have been extensively used. Among the panicogenic agents, drugs increasing the synaptic availability of noradrenaline, such as yohimbine or caffeine, or acting on the adenosine or CCKergic systems are used as provoking agents in diagnostic explorations. This is in line with clinical investigations that have shown abnormal NAergic, serotoninergic or GABAergic systems regulation in

patients with panic disorder and during panic attacks (Balaban and Thayer; Bremner et al. 1996; Goddard, Brouette, et al. 2001). Thus, many genetic studies have been directed to explore the elements of the serotonergic, NAergic, GABAergic or CCKergic systems.

However more recently, Maron and colleagues conducted a meta-analysis of the use of linkage and candidate genes in association studies, which founded over 1000 polymorphisms and 350 candidate genes, for their association with PD (E Maron, Hettema, and Shlik 2010).

Although there are several promising, replicable candidate genes, most studies produced inconsistent results.

Therefore, even though there is a strong genetic predisposition for PD in monozygotic twins and first degree relatives, the specific genes associated with PD and recurrent PAs may be more heterogeneous than the symptoms associated with PAs, and there is most likely multiple gene polymorphisms that may contribute small but cumulative risks for the symptoms and presentation of PD.

Although the importance of genetic factors in the etiology of panic disorder, the non-complete concordance between monozygotic twins and the studies about families have clearly indicated that the genetics is not the only factor able to completely determine the susceptibility to develop panic disorder. Thus, a growing

number of studies investigated how early-life adversities add their effects to, or interact with (figure 1), genetic risk factors to affect behavior (Rutter, Moffitt, and Caspi). Extensive research has been devoted to the identification of elements that may act as risk factors and/or precipitants of internalizing conditions, and to characterize the clinical precursors of anxiety and depressive disorders (Kenneth S Kendler, Kuhn, and Prescott 2004; Kenneth S Kendler et al. 2003; Faravelli et al. 2007; G. A. Fava et al. 1981). Different types of adverse events seem to affect the individual susceptibility to develop anxiety disorders – including panic disorder (PD). Heterogeneous adverse events, such as physical illnesses, changes in social activities, loss of – or threatened separation from – a loved one, appear to play a role in affecting the individual susceptibility to panic attacks (Manfro et al. 1996; Horesh et al. 1997).

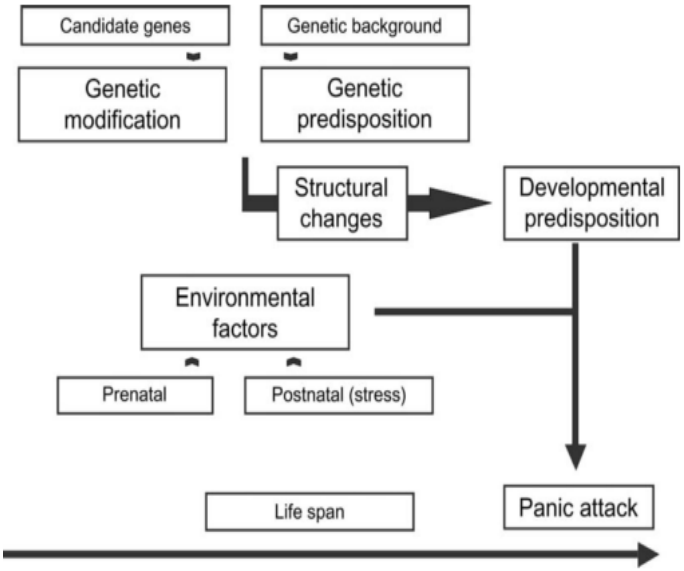


Figure 1: Pathogenic factors in panic disorder. Panic disorder is distinct from other forms of anxiety disorders, such as GAD, mainly based on pharmacological dissection and more recently on twin studies that have shown a, at least partially, independent structure of genetic and environmental risk factors. ***Gratacòs et al 2007***

Regarding the early physical adverse event, in 1997 Bouwer and Stein have proposed the hypothesis of the traumatic suffocation events, basing on the CO_2 ability to provoke a panic attack. In their studies the frequency of traumatic suffocation was significantly higher among the panic disorder patients (19.3%) than among the comparison subjects (6.7%). Within the panic disorder group, patients with a history of traumatic suffocation

were significantly more likely to exhibit predominantly respiratory symptoms and nocturnal panic attacks being part of respiratory subtype of PD (Briggs et al. 1993). By contrast patients without such a history of traumatic suffocation were significantly more likely to have predominantly cardiovascular symptoms, occulovestibular symptoms, and agoraphobia (Bouwer and Stein 1997).

Another series of studies investigated the impact of several early traumatic events such as loss of parents, separation by them or abuse events, on the development of mental illness including panic disorder. These studies pulled out much evidence that these types of early adverse events make it more likely the onset of panic disorder (Breier, Charney, and Heninger 1986; Fierman et al. 1993; Noyes et al. 1993; Servant and Parquet 1994; Friedman et al. 2002; Ogliari et al. 2010; Branchi and Cirulli 2014). There are many researches that indicate a relationship between the separation anxiety disorder and panic disorder and will be described in detail in the chapter concerning the theories about panic disorder (chapter 2).

Another informative clue to PD etiology is the age of onset, which has a mean age range at diagnosis from 22 to 23 years in US population (DSM-V 2013), but the incidence of PAs and PD show a gradual increase during adolescence (Reed and Wittchen 1998) that coincides with sex hormone surges and sexual maturation that begins at ∼10–12 and ends at ∼15–17 years of age [see

review (Kessler et al. 2010)]. This developmental stage is accompanied by critical cortical growth and remodeling which begins in pre-adolescence and continues to develop until early adult- hood when PAs and PD typically get diagnosed. Of particular relevance to anxiety, fear, and panic states, there is also evidence that this is a critical period for development of connectivity of the prefrontal cortex with the amygdala and brain stem centers (Gee et al. 2012; Gee, Humphreys, et al. 2013; Gee, Gabard-Durnam, et al. 2013), all structures that are critical for developing fear and panic, and heavily implicated in anxiety disorders such as PTSD and PD. This connectivity with the prefrontal cortex appears to be critical for extinction of fear memories and preventing over-generalization of threatening cues (Kheirbek et al. 2012). Another striking feature of PD is that, compared to men, women show earlier age of onset and is twice as males to develop PD (Reed and Wittchen 1998; Sheikh, Leskin, and Klein 2002). The initiation of fluctuating sex steroid hormones over the menstrual cycle in women [see review (Nillni, Toufexis, and Rohan 2011)] could be an important factor that contributes to the higher rates of PA and PD in women, but other factors such as early life stress or higher incidence of trauma such as sexual abuse or domestic violence in women could also account for this vulnerability.

3) Treatments for Panic Disorder

A number of neurochemical hypotheses are also proposed for the etiology of panic disorder, primarily based on the pharmacological therapies that work in treating PAs and PD. For example, symptoms associated with PAs in PD, and laboratory-induced PAs can be rapidly treated with benzodiazepines (Charney and Heninger 1985; Tesar and Rosenbaum 1986; Ballenger et al. 1988) which effectively enhance inhibitory GABAergic tone. Panic attacks associated with PD can also be treated with slower-acting pharmacological therapies that enhance monoaminergic (e.g., serotonin, norepinephrine, epinephrine, dopamine, and histamine) activity globally [using tricyclic antidepressants (Rifkin et al. 1981) or monoamine oxidase inhibitors (MAOI) (Kelly, Mitchell-Heggs, and Sherman 1971)] or by specifically targeting serotonergic or noradrenergic systems with reuptake inhibitors [see review (Cloos and Ferreira 2009)].

Most evidence suggests that there is reduced inhibitory GABAergic tone in patients with Panic Disorder: for example PD patients have reduced $GABA_A$ receptor binding in frontal cortex (Nikolaus et al. 2010), or deficits in central GABA concentration (Goddard, Mason, et al. 2001). In addition the GAD1 gene that codifies for the enzyme responsible for GABA synthesis has been shown to be associated with PD (John M

Hettema et al. 2005). For these reasons benzodiazepines which enhance GABA activity are effective at treating panic symptoms (Nutt et al. 2002; Borwin Bandelow et al. 2008; Baldwin 2005; Cloos and Ferreira 2009) and represent a fast-acting panicolytic treatment; however, routine usage makes these drugs less effective due to desensitization, and there are many side effects and safety concerns such as sedation.

First evidence for involvement of serotonin and noradrenergic involvement in anxiety and PD pathology was due to the effectiveness of tricyclic antidepressants (TCA), such as imipramine and clonipramine, for managing symptoms in these disorders [see meta-analyses (Bakker, van Balkom, and Spinhoven 2002; Giampaolo Perna, Guerriero, and Caldirola 2011)]. Although TCAs have pharmacological actions at many receptors, they primarily act as serotonin and norepinephrine reuptake inhibitors at the serotonin and norepinephrine transporters (5-HTT, and NET, respectively) with low affinity for dopamine transporters, which increases synaptic concentration of the neurotransmitters to enhance neurotransmission. Other lines of evidence came from pharmacological inhibition of monoamine catabolism using monoamine oxidase inhibitors (MAOIs) such as phenelzine for the treatment of PAs and PD, but this is considered a third- or fourth-line approach since it requires a tyramine-restricted diet, and can produce serious side effects such as hypertensive crisis. Selective serotonin reuptake

inhibitors (SSRIs) and norepinephrine reuptake inhibitors (SNRIs) are also effective treatments for PAs and PD, and the safety and efficacy of these compounds will be discussed in the subsequent sections. It is important to note that unlike benzodiazepines, these are not fast-acting panicolytic compounds. In some cases, TCAs (and also SSRIs and NRIs) increase anxiety initially, and begin to show anxiolytic and panicolytic properties after 2–3 weeks of daily treatments. Thus, the mechanisms by which these compounds are panicolytic are through compensatory changes that occur with repeated use, and a therapeutic option is to initially co-administer a low dose of a benzodiazepine with SSRIs to PD patients, which has been shown to result in a 41% response rate, compared to 4% response rate for placebo + SSRI group in the first week of treatment (Goddard, Brouette, et al. 2001). Currently SSRIs and NRIs represent the first-line treatment for PAs and PD due to their similar efficacy in treating PAs (M. H. Pollack et al. 2007; M. Pollack et al. 2007), with some evidence that SSRIs are more tolerable and safe. There are several FDA-approved SSRIs for treating PAs, including fluoxetine, paroxetine, and sertraline, and NRIs such as venlaflaxine. In regards to efficacy, TCAs are arguably as effective as SSRIs and NRIs, but they are considered a second-line approach for treating PAs and PD due to side effects and tolerability (Johnson, Federici, and Shekhar 2014).

CHAPTER II: Panic Disorder Theories: clinical research

1) PD Theory based on Anxiety Sensitivity

The concept of "*anxiety sensitivity (AS)"* refers to the fear of anxiety-related sensations, which arises from beliefs that these sensations have harmful somatic, psychological or social consequences, which can last over the anxiety episode (Reiss 1986). To measure this anxiety sensitivity, Reiss and colleagues (1986), have developed an "anxiety sensitivity index (ASI)" based on a 16 items questionnaire. It has been observed that ASI has a normal distribution in the population and can be considered a vulnerability factor which enhances the probability to develop an anxiety disorder.

AS has been associated with PD (Foot and Koszycki 2004; White et al. 2006; Naragon-Gainey 2010), and the level of AS is greater among individuals with anxiety disorders in general (i.e., PD, social phobia, specific phobia, generalized anxiety disorder, obsessive-compulsive disorder, post-traumatic stress disorder, and agoraphobia without panic) as compared with nonclinical controls (Olatunji and Wolitzky-Taylor 2009). However, prospective studies have shown that AS specifically predicts the onset of panic (Benítez et al. 2009) and that PD significantly differ from other anxiety disorders patients in AS levels, suggesting unique features of AS in

PD (Olatunji and Wolitzky-Taylor 2009). Further evidence of the correlation between anxiety sensitivity and panic disorder come from one study on neural activity in response to emotional stimuli in the corticolimbic network in a sample of patients affected by PD (Poletti et al. 2015). The main result of this study is a correlation between AS and brain activity in core structures involved in emotion processing in panic disorder [such as the amygdala, insula, cingulate and prefrontal cortex, which interact to identify the emotional significance of the stimuli and to generate and regulate affective states (Phillips et al. 2003b; Phillips et al. 2003a)]. Functional magnetic resonance demonstrated that higher levels of AS in PD patients, are associated to greater activations in anterior cingulate cortex (ACC) and insula in response to emotional faces (Poletti et al. 2015). This finding is consistent with the literature emphasizing the role of the insula and ACC in the processing of threat-related stimuli other than in the regulation of affective states and in the definition of the emotional significance of the stimuli (Phillips et al. 2003b; Phillips et al. 2003a). In addition, the insula and the ACC, together with midbrain periaqueductal gray matter, have been suggested to be involved in the pathophysiology of panic disorder (Graeff and Del-Ben 2008).

However these studies show some limitations. Indeed they do not clarify the cause of anxiety sensitivity and its role in the etiology of panic attacks and all studies

refer to single panic attack and no to the panic disorder at all.

2) PD Theory of Catastrophic Misinterpretation

One of the most important cognitive theories of panic disorder has been proposed by Clarks in 1986. Within this model, panic attacks are said to result from the catastrophic misinterpretation of certain bodily sensations. The sensations which are misinterpreted are mainly those involved in normal anxiety responses (e.g. palpitations, breathlessness, dizziness etc.) but also include some other sensations. The catastrophic misinterpretation involves perceiving these sensations as much more dangerous as they really are (e.g. perceiving palpitations as evidence of an impending heart attack or losing control or an imminent faint). These catastrophic thoughts produce anxiety and consequently increase the intensity of bodily sensations leading to a vicious circle that falls in a panic attack. The constant attention to the somatic sensations leads to a chronic vigilance and increased sensitivity to the normal physical sensations (Clark 1986).

Often this theory and the theory of anxiety sensitivity are considered together but actually there are some differences between them. The most peculiar is that in the theory of AS individuals who suffer of panic disorder are completely conscious of the causes of their sensations (do not misinterpreted them as in Clark's

theory) but nevertheless they are frightened because they believe that these sensations can be physically or mentally harmful.

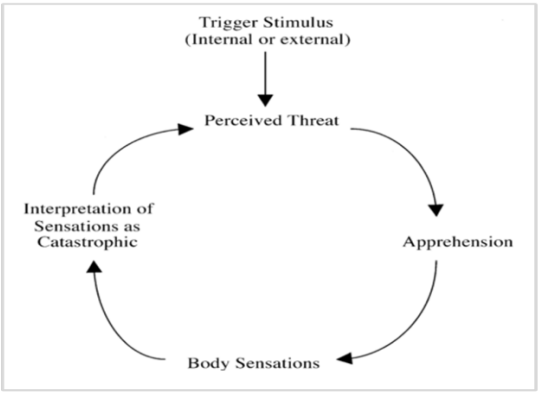
Many studies have been conducted to verify Clark's theory (Khawaja and Oei 1998; Austin and Richards 2001). First set of studies have demonstrated that inducing subjects to interpret bodily symptoms in catastrophic manner, it is possible to raise the level of vigilance and , in susceptible individuals , trigger panic attacks (Ehlers et al. 1988; Margraf et al. 1987)

Another set of research evaluated whether PD individuals had a higher attention towards neuro-vegetative bodily sensations in comparison with healthy people. For example PD patients interpreted catastrophically ambiguous information related to internal sensations (McNally, Riemann, and Kim 1990) and they overestimated their heartbeats (Ehlers et al. 1995).

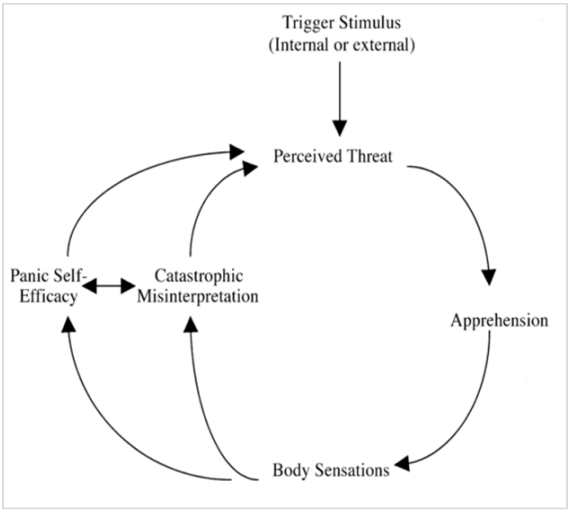
Other studies demonstrated that cognitive factors can influence the way in which PD patients interpret physiological reactions experienced after CO_2 or sodium lactate administration, which are able to induce panic attacks. Indeed in several studies the possibility to interrupt the agents' administration or the presence of a trusted person during these tests is able to inhibit the surge of panic attacks (Abelson et al. 2001; Salkovskis, Clark, and Hackmann 1991; Rachman, Levitt, and Lopatka 1987).

Casey integrated the Clarks' cognitive model with the theory of self-efficacy (figure 2) to explain the evidence that cognitive factors can decrease the likelihood of experiencing a panic attack as described in previous studies (Casey, Oei, and Newcombe 2004; Bandura et al. 1987).

According to supporters of cognitive models an increase of self-efficacy would be one of the mechanisms underlying the success of cognitive behavioral therapies.



Cognitive model of panic attacks (Clark, 1986)



Integrative Cognitive Model of panic attacks (Casey 2004)

Figure 2. Representation of Clark's and Casey's models of PD

Clark's theory has stimulated much research behind the new psychological therapy (cognitive behavioral therapy) effective in the treatment of PD but raised much criticism (Roth, Wilhelm, and Pettit 2005). Indeed the circularity of this model makes difficult to distinguish in time the causes and the consequences of panic attack and doesn't explain the reason why PD patients associate unpleasant cognitive symptoms with potential threats (Windmann; Roth, Wilhelm, and Pettit 2005).

3) PD Theories of Conditioning

Conditioning theory has a long and distinguished tradition in helping to understand the etiology of anxiety disorders and it was one of the first types of theory applied to the cause of PD (Bouton, Mineka, and Barlow 2001). Generally, conditioning theories suggest that when stimuli, events, or situations (conditioned stimuli [CSs]) are paired with a panic attack (and all of its associated physiological sensations), the learning that may occur can allow the CSs to trigger panic and anxiety when they are encountered again. This sort of theory has taken a number of different forms when applied to PD. Early conditioning theories focused on the role of conditioning in the onset of agoraphobia or situational panic attacks (i.e., conditioning to external or exteroceptive cues). However, perhaps the best known version of conditioning theory applied to PD originated in an important article by Goldstein and Chambless (1978)

that described a process they termed "fear of fear." In their work Goldstein and Chambless reintroduced the notion of interoceptive conditioning, in which low-level somatic sensations of anxiety or arousal effectively became CSs associated with higher levels of anxiety or arousal. Thus, they posited that early somatic components of the anxiety response can come to elicit significant bursts of anxiety or panic. These were also expected to generalize to other stimuli (Goldstein, A. J., & Chambless 1978). Thus, the focus of conditioning theory changed from exteroceptive conditioning in explaining agoraphobia and situational panics to interoceptive conditioning in explaining the cause of more "spontaneous" or apparently uncued panic attacks. Thus, interoceptive cues linked with the onset of an event can be associated with later aspects of the event. Collectively, this work is important in showing that an intero-interoceptive relation (RAZRAN 1961) forms with each drug administration such that animals learn to respond to an early event in anticipation of a later event. In an analogous fashion, early physiological changes during a panic attack may become signals for more intense and aversive physiological arousal (e.g., a panic attack, or intense fear) and thus elicit a panic attack (CR) on their own (Barlow 2002).

For example, a slight rise in heart rate accompanying the beginning stages of a panic attack may become a conditioned stimulus (CS) signaling a larger rise in heart rate characteristic of the later stages of a panicogenic

response including other associated sensations (e.g., tachycardia, heart pounding, chest tightness, breathlessness). Such learned relations then alter the function of formerly benign bodily events such that they become significant fear-evoking events in their own right. Under the right conditions and in the context of relevant vulnerabilities (S Mineka and Zinbarg 1996) such learning may contribute to the development of hypervigilance, anxious apprehension, avoidance, and even panic disorder (Bouton, Mineka, and Barlow 2001; Barlow 2002; Finlay and Forsyth 2009).

Recently Grillon and colleagues developed another theory based on conditioning (Grillon et al. 2007; Grillon 2002). This theory starts from the evidence that individuals with panic disorder perceive panic attacks as unpredictable and because predictability is fundamental to Pavlovian conditioning, failure to predict panic attacks could be due to a basic deficit in conditioning. Results of their studies suggest that individuals with panic disorder suffer from a deficit in declarative associative learning. Such a deficit points to impaired hippocampal function that may disrupt cognitive processing of internal and external cues predictive of a panic attack (Grillon et al. 2007; Grillon 2002). Further researches are necessary to define whether this deficit has a causal role in etiopathogenesis of panic disorder or is only a trait-marker.

4) PD Theories basing on Separation Anxiety

Donald Klein was the first to suggest that the separation anxiety disorder (SAD) during childhood could be a precursor of panic disorder in the adulthood (Gittelman R and Klein 1984; Klein 1964). Mattis and Ollendick have proposed a theory in which the separation anxiety or an intense discomfort, during a separation from attachment figures, could be an important way of PD development in children and adolescents (Mattis and Ollendick 1997). They speculated that repeated experiences of separation can scare children and grow in intensity until became panic attacks. Thus children with SAD, who live with great suffering the separation from caregivers, have a high risk to develop PD when experience numerous or prolonged events of separation. This last affirmation has been contested by Doerfler who find no correlations between the number or duration of separation experience and the risk to the develop PD (Doerfler, Toscano, and Connor 2008).

Most researches confirmed the strictly correlation between SAD and PD leading to suppose common mechanisms of development (Battaglia et al. 1995; Bandelow et al. 2001; Aschenbrand et al. 2003; Doerfler, Toscano, and Connor 2008; Roberson-Nay et al. 2012). In this purpose a twin study conducted by Battaglia and colleagues (2009) demonstrated that shared genetic determinants appear to be the major underlying cause of the developmental continuity of childhood separation anxiety disorder into adult panic disorder and the

association of both disorders with heightened sensitivity to CO_2. Inasmuch as childhood parental loss is a truly environmental risk factor, it can account for a significant additional proportion of the covariation of these 3 developmentally related phenotypes (Battaglia et al. 2009).

5) PD Theory of Hyperventilation

Ley's (1982) hyperventilation theory of panic fear is the first respiratory theory about the etiology of panic disorder. This theory supposes that the panic attack consists of a synergistic interaction between hyperventilation and fear, the nature of which is a positively accelerating loop: with excessive expiration of CO2, moderate over-breathing produces relatively mild symptoms (e.g. slight dizziness) which can be tolerated for prolonged periods. If, however, respiration rate increases somewhat, the symptoms of hyperventilatory hypocapnea increase in both number and intensity very rapidly to the point where tolerance gives way to alarm and fear. Details of the reports of agoraphobics who suffered panic attacks indicate clearly that the symptoms of hyperventilatory hypocapnea preceded the experience of fear (Ley 1988; Ley 1985).

This theory is much debated, indeed, while some clinical evidence seems to support it, many studies seem to refute it entirely. In first case the symptomatology of hyperventilation syndrome show many common features

with PD, such as dyspnea, sense of suffocation, dizziness and anxiety (Gardner 1996). On the other hand several studies in which individuals were been instructed to hyperventilate obtained controversial results. Indeed these studies did not demonstrate that the hyperventilation lead to trigger panic attacks in PD patients (Garssen, Buikhuisen, and van Dyck 1996; Wilhelm, Gerlach, and Roth; Gorman et al. 1988; Nardi et al. 2004).

These results demonstrated that, although low level of pCO_2 is common in some panic attacks and maybe in basal conditions in PD patients, much panic attacks are triggered by mechanisms different from hyperventilation.

Thus these findings have led some researchers to consider falsified the theory of Lay (Roth, Wilhelm, and Pettit 2005).

6) PD Theory of false alarm suffocation

A few years later the Ley's theory, Klein proposed the theory of *"false alarm suffocation"* for the etiology of PD (Klein 1993). This theory suggests a physiological misinterpretation by the control center of an advanced and sophisticated suffocation alarm system. This produces sudden respiratory distress followed swiftly by a brief hyperventilation, panic, and the urge to flee. Carbon dioxide hypersensitivity is seen as due to the deranged suffocation alarm monitor.

Between Ley's and Klein's theories there is a significant difference concerning the role of the hyperventilation: in Ley's theory the hyperventilation is the cause of panic attacks whereas in the Klein's theory is a compensatory response to a false alarm of suffocation (Roth, Wilhelm, and Pettit 2005).

Klein's theory is the result of an intense clinical research on CO_2 inhalation in PD patients. Although already in 1951 Cohen and colleagues (COHEN et al. 1951) described the panicogenic properties of CO_2, only in the 80's some researchers developed experimental protocols that involved the CO_2 inhalation. For example in a study of Van den Hout the inhalation of air mixture compose by 35% CO_2 and 65% O_2 was able to trigger in healthy subjects a short but intense respiratory response accompanied by neurovegetative symptoms similar to those reported during a panic attack (Van den Hout and Griez 1984). However in PD subjects the similar procedure was able to induce a transient increment of anxiety similar to that experienced during a panic attack (M. R. Fyer et al. 1987; Griez et al. 1987; G Perna et al. 1994; Nardi et al. 2000). The CO_2-hypersensitivity observed in PD patients is not present in subjects suffering of generalized-anxiety disorder, phobia, obsessive compulsive disorder or mood disorder (G Perna et al. 1999; Verburg, Griez, and Meijer 1994; G Perna et al. 1995).

Gorman and colleagues developed a different protocol during which twenty minutes inhalation of 5% CO2 enriched air mixture provoked an intense panic attack and demonstrated that this procedure was more panicogenic than the voluntary hyperventilation (Gorman et al. 1984).

Over the years many variation of these protocols have been developed, with different concentrations or time of administration but independently from the protocol the CO_2 inhalation is able to trigger panic attacks more in PD patients than in healthy subjects [for a review including the different protocols see (Rassovsky and Kushner 2003)].

Several studies demonstrated also an abnormal respiratory response to CO_2 in PD patients including a higher increment of breathing frequency, of tidal volume and minute ventilation after CO_2 inhalation (Sardinha et al. 2009; Maddock & Carter 1991; Wilhelm et al.2001).

Overall clinical studies demonstrated that PD patients also show high variability in several respiratory parameters, also in basal conditions, suggesting a possible malfunctioning in breathing control system supporting Klein's theory (Abelson et al. 2001; Schwartz et al. 1996). In particular, according to this theory substances that stimulate the breathing highlight alterations already present in the basic condition in PD subjects.

In addition the chronic administration of antipanic drugs (such as SSRI or TCA) in PD patients was able to

decrease CO_2 reactivity (Bertani et al. 1997; Pols et al. 1996; Giampaolo Perna et al. 2002). These findings suggested that the CO_2 hyper-reactivity can be a central trait for PD and a useful model to study this disorder.

The theory of false alarm suffocation stimulated scientific activity and studies highlighting the relationship between CO_2 hypersensitivity, respiratory disorders in PD patients and SAD (as above mentioned in paragraph 4.4). Additional evidence in support of Klein's theory is the alteration in neurotransmission systems involved in breathing, in PD subjects, that will be describe in next section.

6.1) Neurobiological basis and substrates of Panic disorder

The first neurobiological theory of panic disorder has been proposed by Gorman and colleagues in 1989 and then revised in 2000 (Gorman et al. 1989; Gorman et al. 2000). These authors suggested that PD comes from an abnormal sensitivity of fear conditioning networks. These complexes have been extensively studied by LeDoux and Davis, and involved prefrontal cortex, insula, thalamus, amygdala and its projection toward the brainstem.

Clinical and preclinical studies demonstrated the importance of amygdala in fear perception and panic response as well. In humans amygdala stimulation elicits responses similar to anxious responses whereas bilateral lesions of this structure decrease anxiety and fear (Adolphs et al. 1994; Adolphs et al. 1995). Neuroimaging

studies demonstrated that the amygdala is active during observation of scared but no happy faces (Morris et al. 1996). According to Gorman's model the sensory input of the conditioned stimulus crosses the anterior thalamus, reaches the lateral nucleus of amygdala until arrives in the central nucleus of amygdala. This latter nucleus represents the control center of information that coordinates autonomic and behavioral responses (figure3).

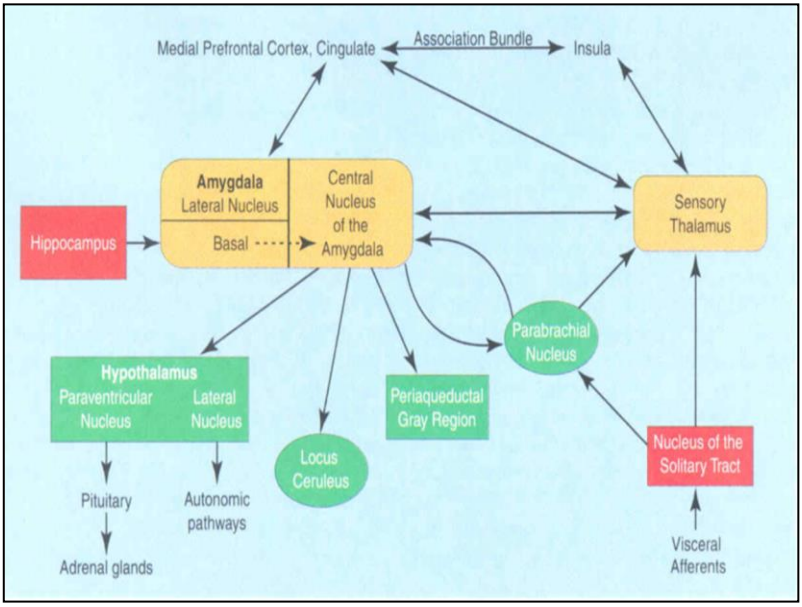


Figure 3. Neuroanatomical pathways of viscero-sensory information in the brain (Gorman et al 2000)

Projections from the central nucleus of amygdala reach several areas: the parabrachial nucleus that produces an increment in breathing; the lateral nucleus of hypothalamus that activates sympathetic nervous system causing autonomic arousal; the locus coeruleus that contributes to the heartbeat increment and to the fear response; the periaqueductal gray substance responsible of defensive behaviors. In addition the hippocampus maintains contextual information. Moreover the amygdala receives information also from cortical regions involved in processing and evaluation of sensory information. According to the Gorman's theory, panic disorder depends on deficits in some of these structures (figure 4) (Gorman et al. 2000; Ohta et al. 2008; Tanii et al. 2009; Eduard Maron et al. 2004).

Further evidence support the role of amygdala and brainstem structures in panic disorder demonstrating a linkage between the CO_2 hypersensitivity and acid sensing ion channels (ASIC) which are activated by acidosis (following CO_2 inhalation) and are localized in several structures including amygdala and brainstem. ASICs are also linked to PD (Smoller et al. 2014; Ziemann et al. 2009; Maren 2009).

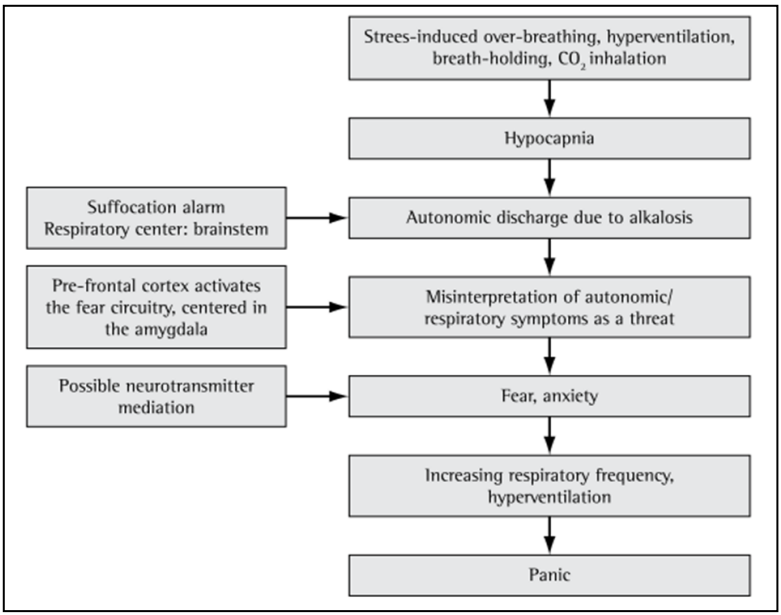


Figure 4. Panic attack mechanism (Sardinha et al. 2009)

Gorman and colleagues proposed a putative circuit that might be involved in PD and a theory for the etiology of this disorder (figure 5). According to this model, PD subjects have genetic vulnerability to the disorder. Early adverse events and attachment binding alterations can produce hypersensitivity in the network which mediates fear conditioning response, through a gene environment interaction.

These conditioning mechanisms could be also involved in avoidance behaviors typical in panic disorder.

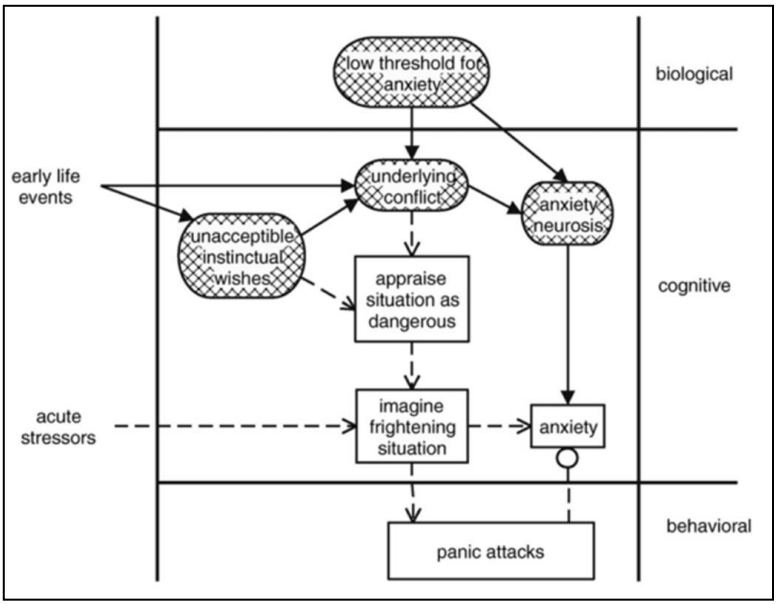


Figure 5. Causal modeling of panic disorder theory (L. Fava and Morton 2009).

CHAPTER III. Panic Disorder (PD): pre-clinical research

Due to the impossibility to interview animals and ask them about feelings and sensations, animal models of panic are usually based on exposure to dangerous context, trying to discriminate between animal responses to real or potential threat, between fear and anxiety, between panic and generalized anxiety disorder. The reaction to diverse drugs, whose differential effectiveness has already been measured in human patients, is needed to validate the animal model.

The etho-experimental approach has laid the foundation for the preclinical study of emotions and emotional disorders, even if this reasoning does not allow exploring the molecular mechanisms underlying anxiety disorders, and new therapeutic strategies. This approach is based on the empiric observation of defensive behaviors showed by animals and according to this examination in 1988 Blanchard and Blanchard have provided a behavioral, functional and pharmacological distinction between anxiety and fear (D. C. Blanchard and Blanchard 1988). The discrimination between these behaviors has a strong relevance for the pre-clinical approach in the study of emotional disorders such as panic disorder or generalized anxiety disorder (GAD).

The function of fear is to prompt the animal to move away from a real and imminent danger, such as when a rat is in proximity to a cat. Behaviors showed by the animal differ depending on the presence or not of an escape way. In the presence of a way out the animal will move away, whereas other defensive strategies such as immobility and attack will be shown by the subject whether there is no possibility to elude such situation (D. C. Blanchard and Blanchard 1988). Both these defensive strategies are decreased by antipanic drugs such as SSRI and TCA but not by drugs used in anxiety disorder (GAD) (D. C. Blanchard, Griebel, and Blanchard 2001; Poltronieri, Zangrossi, and de Barros Viana 2003).

The function of anxiety responses is to prepare the individual to detect and deal with threats. Anxiety facilitates reaching the individual's goals, by adopting a more careful approach when potential dangers are detected, such as for example the presence of predator. In this case anxiety induces behaviors of risk assessment and defensive quiescence (D. C. Blanchard and Blanchard 1988; McNaughton and Corr 2004). These behavioral strategies are instead decreased by treatments for GAD but not by them used in PD (D. C. Blanchard, Griebel, and Blanchard 2001).

The etho-pharmacological approach has laid the basis for studying emotions such as fear and anxiety in animals. In addition pharmacological studies support the theory which sustain that PD is due to alteration in fear

circuits and not in anxiety ones (McNaughton and Corr 2004).

Animal models of PD are based on the analysis of defensive behavior in response to different kind of stimulus. According to the stimulus used to elicit defensive behavior tests are classified in: 1) tests based on predator exposure; 2) tests based on conditioning 3) tests based on structures' stimulation and on administration of substances (such as sodium lactate and CO_2). These tests will be described in the following paragraphs.

1) *Animal Test for studying PD*

1.1) Test based on predator exposure

Rodent models of PD are based on the analysis of defensive behaviors. Cognitive symptoms during a panic attack, for example fear of dying, are suggested to be considered homologous to those attributable to rat when exposed to a cat (McNaughton and Corr 2004).

Blanchard and colleagues developed a paradigm in which rats are exposed to predator (cat) and this paradigm permits to study behaviors related to fear. If there is no escape way, the rat shows freezing behavior (immobility) for the majority of time of predator exposure. This paradigm has also good pharmacological validity indeed antipanic drugs, such as imipramine,

decrease the avoidance behavior during threatening stimulus exposure (R. J. Blanchard et al. 1997).

A similar test, namely “rat exposure test (RAT)”, has been developed for the mouse. In this case a mouse is exposed to the presence of a predator (an awake rat) separated by a wire grid (figure 6) (Yang et al. 2004). During this test several behavioral parameters are measured: time spent by the mouse in each section of the apparatus, time in contact with the grid and defensive behaviors such as stretch attend posture or freezing or burying (tunnel closing with the bedding). Some of these behaviors, such as latency to flight, have been associated with fear response.

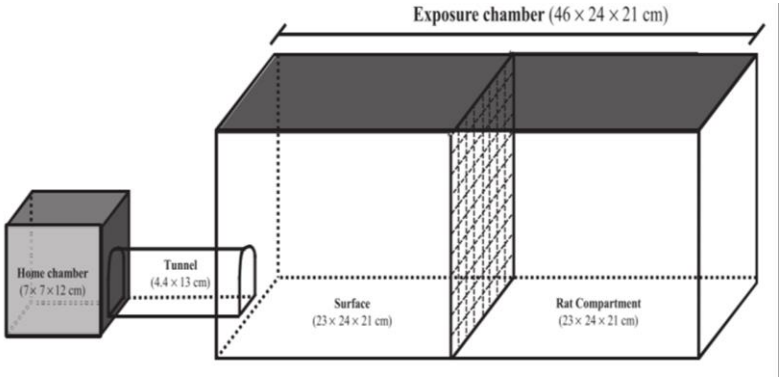


Figure 6. Schematic side view of rat exposure test (RAT) (Campos et al. 2013)

This test has been pharmacologically validated for the study of anxiety disorders: administration of anxiety-inducing drugs induced an enhancement in avoiding

behaviors, whereas the administration of anxiolytics, such as benzodiazepine, leads to reduction of these behaviors (Carvalho-Netto et al. 2007; Litvin et al. 2007).
In addition, it has been recently demonstrated that the defensive response in this test is also sensitive to drugs known either to attenuate (alprazolam and chronic fluoxetine) or induce (caffeine) panic attack in humans, suggesting the RET as a useful test to assess the effects of panicolytic and panicogenic drugs, as well (Campos et al. 2013).

A similar, more complex behavioral test eliciting defensive behaviors in rodents is the “mouse defense test battery (MDTB)”.
The MDTB consists of five tests associated either with potential threat (contextual defense) or with the actual presence of an approaching threat (i.e. a rat). After a period of habituation in the apparatus, a rat is approached to the subject at various speeds. Defensive behaviors showed by the mouse, in the presence of the approaching rat, as flight, avoidance, freezing and attack are considered fear indices. Once the rat is removed, risk assessment behaviors are shown by the mouse and these behaviors have been considered anxiety indices (Griebel, Blanchard, and Blanchard 1996).
This test has been pharmacologically validated both with anti-panic and anti-anxiety drugs. Anti-panic drugs (fluoxetine, SSRI, imipramine) potentiated in acute the flight response, with chronic treatment decreasing the intensity of these reactions, as in clinical observations. In

addition panicogenic substances (e.g. yombina) potentiated the flight response (Griebel et al. 1995; Griebel, Perrault, and Sanger 1997; Blier and Ward 2003; Eduard Maron and Shlik 2006; R. J. Blanchard et al. 1997).

Overall, the paradigms described are based on the exposure of the animal to a predatory threat. The quantitative and qualitative analysis of avoidance behaviors provides a measure of fear. Pharmacological studies have shown good validity of these models for the study of panic disorder, using drugs already known for their effects in the clinical practice.

1.2) Test based conditioning

Models based on conditioning are among the first developed for the study of panic (Bouton, Mineka, and Barlow 2001). Fear conditioning paradigms have been widely used in preclinical research for the study of networks involved in fear response. Panic disorder is considered a disease related to fear as described in the first chapter (D. C. Blanchard, Griebel, and Blanchard 2001; McNaughton and Corr 2004). For this reason experimental research in preclinical field can suggest possible networks altered in PD patients.

In fear conditioning experiments a neutral stimulus (CS) or context is associated with an aversive unconditioned stimulus (US), for example a foot-shock. The behavior showed by the animal, for example

freezing, is used as conditioning index (Fanselow and Bolles 1979; Young and Fanselow 1992).

A fear-potentiated startle response (FPS) is also used as fear conditioning test and this response depends on classical pavlovian learning. The amplitude of the startle response elicited by a stimulus (for example a loud noise) is measured concurrently or less than a CS previously coupled to an aversive stimulus (e.g. foot-shock). A measure of fear is obtained subtracting the amplitude of the two startle responses (Grillon 2002).
The advantage of this test is the possibility to measure fear levels at a specific time point.

Although pre-clinical studies demonstrated the existence of conditioning mechanisms involved in anxiety disorder including panic disorder (as described in first chapter) there are not yet enough clinical research that confirm their importance in the etiology of panic disorder so it is necessary more research in this field.

1.3) Test based on administration of human panicogenic substances

There are some substances which have panicogenic properties such as sodium lactate, CO_2 and doxapram hydrochloride and clinical and pre-clinical research have widely used them for studying PD.

Doxapram hydrochloride is a respiratory stimulant with panicogenic effects (Abelson et al.; Abelson et al. 1996). Preclinical evidence demonstrated that the

doxapram mechanism of action depends on the direct stimulation of chemoreceptors localized on carotid bodies and brainstem. Doxapram is able to induce panicogenic effects as high levels of anxiety, panic attacks, increment of respiratory frequency (Abelson et al. 1996; Y. J. Lee et al. 1993).

Sullivan and colleagues demonstrated that the administration of doxapram is able to induce both anxiety and panic measurable in different animal tests: contextual, cue fear conditioning, open field and social interaction tests. They also demonstrated that its effect depends on the activation of central nucleus of amygdala. Clinical evidence suggested an higher reactivity to this substance in PD patients in comparison with healthy subjects suggesting the use of doxapram to validate animal models of panic disorder (Sullivan et al. 2003).

A different substance used in preclinical PD research is the sodium lactate. Clinical research demonstrated that sodium lactate infusion induces hyperventilation, enhancement of heartbeat rate and blood pressure and cognitive symptoms similar those of panic attack. In addition PD patients are more susceptible to the substance in comparison with healthy subjects or individual who suffer of different psychiatric disorders (Gorman et al. 1986; Liebowitz et al. 1985). In 2000 Sajdyk and colleagues developed a paradigm using sodium lactate. Physiological response to the substance was detected through a catheter in freely moving rats

while an arousal behavioral index was detected during the analysis of social interaction test. Authors observed that rats responded to a lactate infusion with significant increases in heart rate, blood pressure and experimental anxiety. They also demonstrated the role of basolateral nucleus of amygdala in this phenomenon; indeed rats which were primed with chronic subthreshold GABA receptor blockade in the basolateral nucleus developed a sensitivity to sodium lactate, similar to human panic disorder patients (T. J. Sajdyk and Shekhar 2000; T. Sajdyk et al. 2008). These results are in agreement with the evidence which suggests a role of basolateral amygdala and fear networks in panic disorder. However the Sajdyk's paradigm needs of pharmacological validation.

As extensively described in 4.6 section, CO_2 is able to induce panic attack in PD patients. Inhalation of CO_2 demonstrates several interesting characteristics as a model to induce panic in the laboratory. Not only is the inhalation of CO_2 an efficient means of provoking panic and anxiety in PD and healthy individuals but it is also a relatively easy and non-invasive procedure (Rassovsky and Kushner 2003). In addition CO_2 sensitivity is a common trait in all animals and can represent an useful endophenotype to measure and investigate panic disorder molecular mechanism, using a real translational approach (as described above in this work) (Marco Battaglia and Ogliari 2005; T. J. Sajdyk and Shekhar 2000).

There is few preclinical research in this field (D'Amato et al. 2011) and further investigation on the potentiality of this endophenotype to measure panic in animals, as well as in humans is presented in here.

EXPERIMENTAL SECTION

AIMS

In this section I will describe the aims of the different experiments performed during my PhD.

EXPERIMENT 1

The first aim was to validate the Repeated cross fostering (RCF) protocol in mice as a useful manipulation procedure affecting individual emotionality. This method differs significantly from the classical maternal separation (Handling) usually applied in rodents in order to evaluate the effects of an early adverse environment. I assesed the short and long-term effects of these early manipulations, comparing the Handling and the RCF protocols in outbred mice. Several behavioral, molecular and physiological parameters (mother-pups interaction; stress response; emotionality; CO_2 panic-related response; gluco- and mineral-corticoid receptors mRNA expression; etc.) have been considered.

EXPERIMENT 2

The aim of the second experiment was to analyze possible molecular mechanisms underlying the panic-related CO_2 hypersensitivity showed by RCF animals (Experiment 2a). Moreover, on the basis of the molecular

suggestions founded in this first part, I evaluated different pharmacological treatments (chloridiazepoxide, chlorogenic acid and amiloride) able to recover the normal respiratory response to hypercapnia (Experiment 2b).

EXPERIMENT 3

The aim of the third experiment was to verify the cognitive capability of RCF animals trough learning tests (such as active avoidance test and novel recognition test) and investigate the capability of 6% CO_2 exposure to condition animals' behaviors, in RCF and Control subjects. Indeed, humans with PD show behavioral conditioning to panic attacks and develop PA also in absence of unconditioned stimulus.

EXPERIMENT 4

The aim of the fourth experiment was to investigate whether the CO_2 hypersensitivity showed by RCF animals was a transgenerational transmissible trait.

Experiment 1. Short- and long-term behavioral effects of two different manipulations of the early environment: comparison between Handling and Repeated Cross Fostering

INTRODUCTION

The developmental programming hypothesis suggests that the early environment, whether by nutritional, hormonal or behavioral processes, can give rise to persistent modifications of the adult phenotype. In particular, when facing a challenging environment, epigenetic modifications may occur that modify the behavioral, physiological, hormonal and neurobiological profile of the developing individual, to optimize its future coping strategies (Bock et al. 2014). Several studies in rodents have investigated the effects of a challenging environment, experimentally altering the external or internal pup's milieu, and various postnatal manipulations, differing for severity, time and duration schedules have been applied to developing animals. In the majority of studies (see also Moles et al. 2004; Moles et al. 2008) pups were directly stressed exposing them to low temperature, poor mothering, saline injection,

unfamiliar odors and others (Oddi, Luchetti, and D'Amato 2015). The most common manipulation applied to developing rodents consisted in exposing young animals to daily sessions of separation from the mother during the first 1–2 weeks of life (Pryce & Feldon, 2003). Maternal separation is adversative and pups search for the mother by emitting calls and by seeking olfactory and thermal cues of her presence. This indicates the establishment of an attachment bond between the infant and the mother in the first 2 weeks of life, with signs of distress (e.g., ultrasonic vocalizations (USVs)) following maternal separation that are already detectable in the first few postnatal days (PNDs). Rather than repeated separations, unpredictability of the early environment may represent a stressful condition for pups. Repeated cross-fostering (RCF) has been used in mice as a postnatal manipulation to model human early environmental instability, a risk factor for internalizing disorders (including separation anxiety disorder-SAD-, panic disorder-PD-and CO_2 hypersensitivity, (K S Kendler et al. 1992; Forman and Davies 2003; Marco Battaglia et al. 2009)). Even though animal models are not expected to reproduce clinical disorders exactly, a translational model of PD should allow to differentiate panic attack (PA) from fear, on the basis of respiratory symptoms (over-reaction to hypercapnia) and lack of increments in stress hormones (Schenberg et al. 2014). Cross-fostering is a routine procedure used in many laboratories that consists in giving pups to a lactating female different

from the biological mother, usually within 24–48 h from birth (Oddi, Luchetti, and D'Amato 2015). RCF consists in repeating the same procedure every day for the first 4 days of life. Changes in maternal (olfactory, gustatory, tactile, thermal, etc.) cues connected with the RCF procedure may disrupt the associative learning process that is necessary for establishment of the attachment bond in the developing infant (Landers and Sullivan 2012).

The temporary separation from the mother, or the absence/malfunctioning of the attachment bond (RCF protocol) may act on different molecular system and differently affect the development of emotionality and vulnerability to specific psychopathologies. In this experiment, we evaluated the short- and long-term behavioral effects of two different manipulations of the early environment. In one case pups experienced short separations (Handling) from the mother, which interferes with continuity of the bond; in the other case, pups experienced the Repeated Cross-Fostering procedure, which is aimed at interfering with bond formation. The effects of maternal separation in rodents, mice especially, yield little agreement among laboratories and strains (see for example Millstein and Holmes, 2007).

To help resolving these issues, I analyzed the specificities of the RCF vs. Handling protocols effects on behavioral readouts and on the panic-related respiratory responses to carbon dioxide (CO_2) among outbred strains in the

same laboratory. Different response to these manipulations would support the relative selectivity of behavioral and molecular mechanisms involved in response to different types of adversities.

MATERIALS AND METHODS

Animals

NMRI outbred mice (Harlan, Italy) were used in all experiments. Mice were mated when they were 12 weeks old. Mating protocol consisted in housing 2 females with 1 male in transparent high temperature polysufone cages (26.7 × 20.7 × 14.0 cm) with water and food available ad libitum. Room temperature (21 ± 1 ◦C) and a 12:12 h light dark cycle (lights on at 07.00 p.m.) were kept constant. After 15 days, males were removed and pregnant females were isolated, left in clean cages, and inspected twice a day for live pups. All animal used procedures were in strict accordance with standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609/EEC) and the Italian legislation on animal experimentation (DecretoL.vo 116/92).

Experimental Manipulations

On PND1 litters were culled to 8 pups (4 males and 4 females) and assigned to handling (H) or repeated cross-fostering (RCF) procedure

Repeated cross-fostering

On postnatal day 1 (PND1), after having spent the day of birth (PND0) with the biological mother, litters were culled to 8 pups (4 males and 4 females) and assigned to experimental Repeated Cross Fostering (RCF) or control (CT) treatment. Differently from the ‘‘classical’’ cross-fostering procedures (Bartolomucci et al. 2004), RCF pups changed caregiver every 24 h: 4 times in the PND1-PND4 time interval by following a rotation scheme, each dam shifted to 4 different litters and each litter was shifted to 4 different dams (see also Figure 1). The daily procedure consisted of first removing the mother from the cage, then removing its entire litter, and immediately introducing this litter into the home cage of a different dam whose pups had just been removed. The RCF pups were then semi covered with the home cage bedding of the adoptive mother, which was then reintroduced in the cage and left with this litter for the next 24 h. The entire procedure lasted about 30 s and took place every day between 10.30 and 11.00 a.m. This was repeated daily, four times until reaching the fourth adoptive mother, with which pups were left until weaning (PND0:

biological mother, PND1-PND4: adoptive mother 1–4, PND4-PND28: fourth adoptive mother- Figure 1). Adoptive dams were lactating females with pups of the same age as fostered litters. Control litters (CT) were picked up daily and reintroduced in their home cage, covered with home cage bedding and had their biological mothers returned within 30 s; this procedure took place from PND1 to PND4 in order to control the possible effect of manipulation necessarily required by RCF procedure.

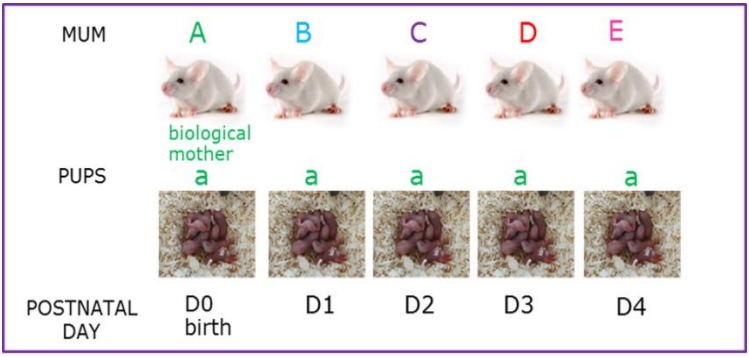


Figure 1. Schematic representation of RCF procedure. Pups "a" born from mum "A" spend the first day (D0) with their mum. Then they change caregiver for four consecutive days spending D1 with mum "B", day 2 with dam "C", day D3 with mum "D" and finally from D4 to weaning with adoptive mum "E". Also pups “b, c, d, e” receive the same treatment.

A total of two experimental groups resulted from the early manipulation: RCF and their controls (RCF and CT). Animals were weaned when 28 days old, and then separated by sex and left in cage with littermates. A total number of 10 RCF and 10 CT litters were used for all experiments described in this thesis.

Handling

According to the well validated paradigm called ‘‘handling’ ’(Pryce et al. 2005) pups were briefly handled and separated from the dam for 15 min daily. This procedure took place from PND1 to PND14 between 9:30 and 11:00 am. Controls litters (N-H), once completed the culling procedure, were left undisturbed for the first 2 weeks of life. A total of two experimental groups resulted from the early manipulation: Handled (H) and their controls (N-H). Animals were weaned when 28 days old, and then separated by sex and left in cage with littermates. A total number of 10 H and 10 N-H litters were used for all experiments described in this thesis.

Short and Long-Term Effects of Repeated cross-fostering manipulation

The effects of H and RCF on offspring were compared according to eight different physiological, molecular, and behavioral parameters collected during development and

adulthood. Body weight (1) was measured in infancy (PND8) and adulthood (PND90). Maternal behavior (2) was observed during the first week of life to exclude the action of poor nurturing on offspring's responses. USVs (3) in response to isolation (PND8), and sociability and social preference (4) were measured before (PND28) and after weaning (PND35), respectively. Adult males (PND75–90) were also tested for behavioral emotionality (5), HPA axis functionality as indicated by corticosterone response to stress (6) and hippocampal mRNA levels of the glucocorticoid and mineralcorticoid receptors (7) were also measured. In addition, respiratory responses (8) to a 6% CO2-enriched air mixture were evaluated in young and adult animals.

Maternal Behavior

Maternal behavior was observed daily from PND2 to PND7 by an observer unaware of the litter's manipulation (H, N-H, RCF and N-RCF) in two daily sessions (12.00–12.30 and 16.00–16.30) in the facility room. The first daily session took place at least 1 h after the cross-fostering/maternal separation procedures, in order to facilitate the dams' acclimatization. Maternal behavior encompassing: (a) NURSING, including the arched-back and blanket postures; and (b) GP/L: grooming and licking pups was monitored with an instantaneous sampling method (1 sample every 2 min),

for a total of 16 sampling points/session (Shoji and Kato 2006a).

Ultrasonic Vocalizations (PND8)

Pups' behavior was evaluated at PND8, by measuring USVs emitted during 5 min of isolation (Moles et al. 2004; Cryan & Holmes 2005). Experimental animals were transferred in their home cage to the experimental rooms for USVs assessment, 1 h prior to testing. After this period of acclimatization, the mother was removed and transferred into a clean cage, while pups were left in the home cage standing on a warm plate set at the temperature of 35 °C to prevent cooling. Each pup was individually placed for 5 minutes into a beaker containing clean bedding and the vocalizations were recorded. Four pups of each litter were tested. USVs were recorded using an UltraSoundGate Condenser Microphone (CM16, Avisoft Bioacoustics, Berlin, Germany) lowered 1 cm above the top of the isolation beaker containing the pup. The microphone was sensitive to frequencies of 15–180 kHz with a flat frequency response (± 6 dB) between 25–140 kHz. It was connected via UltraSoundGate USB Audio device to a personal computer, where acoustic data were recorded as wav files at 250,000 Hz in 16 bit format. Sound files were transferred to SasLab Pro (version 4.40; AvisoftBioacoutics) for sonographic analysis and a fast Fourier transformation was conducted (512 FFTlength,

100% frame, Hamming window and 75% time window overlap). Spectrograms were produced at 488 Hz of frequency resolution and 0.512 ms of time resolution. To detect ultrasonic vocalizations, an automatic threshold-based algorithm and a hold time mechanism (hold time: 20 ms) were used. Signals below 30 kHz were truncated to reduce background noise to 0 db. Inaccurate detections were adjusted manually by an experienced user before running the automatic parameter analysis. The total number of vocalizations emitted in 5 minutes was measured.

Sociability and Social Preference

Sociability and social preferences were evaluated in male mice at PND28 (before weaning), and at PND35 (1 week after weaning), respectively, in different animals (Cinque et al. 2012). Measures of interest in an unknown conspecific vs. an unknown object were employed as indicators of sociability. Indices of social preference were acquired to test whether H and RCF affected siblings' recognition. The social preference test was performed 1 week after weaning to reduce the impact of the mother on sibling's olfactory cues. Both tests used a gray Plexiglas rectangular box (60X40X24 cm) consisting of three interconnected chambers. Each of the two lateral compartments contained a circular transparent Plexiglas cylinder (diameter: 8 cm, height: 15 cm) with multiple holes (diameter:1.2cm) yielding olfactory cues. Mouse

behavior was recorded by a video camera and analyzed with the SMART video tracking system. Each subject was placed inside the central compartment and explored the apparatus for a 10 minutes habituation period, with the doors on either side left open. During the 10 min social session of the test, the tested animal was exposed to an unfamiliar animal and a white object of similar size (Sociability test), or was simultaneously exposed to an unfamiliar (same strain, age and treatment) and a familiar male mouse (sibling) (Social preference test). Each partner and object was confined in one of the two Plexiglas cylinders located in the lateral compartments, for 10 min. The position of stimuli (partners and objects) in the apparatus was equally distributed between the left and the right compartment. Collected measures included time spent: (a) in each one of the three compartments; and (b) in the immediate proximity (2 cm: Time Close) of each cylinders.

Emotionality

Male mice were tested in the elevated plus maze at PND75–90 for emotionality. No more than 2 males X litter for group were sampled. The elevated plus maze consisted of 2 open (5 cm wide, 30 cm long) and 2 closed arms (5 cm wide, 30 cm long, enclosed by a wall of 14 cm in height) arranged in a plus configuration, joined by a central square of 5 cm X 5 cm. The apparatus was made of opaque Plexiglas and kept on a base 40 cm above the

floor. All animals were exposed to a test of 5 min duration. At the beginning of the test each mouse was placed individually in the center of the maze, with the head facing an open-arm (the same for all mice). All tests were conducted between 13:00 h and 15:00 h and recorded by a video camera. The animals were initially accustomed to the experimental room for at least 1 hour before the experiment.

HPA Axis Functionality: Corticosterone response to novelty

Corticosterone levels were measured in H, N-H, RCF and N-RCF male mice, at different time intervals from novelty exposure. Apart from the postnatal manipulation, these animals have never been exposed to other experimental procedures. Novelty consisted in exposing the animals to a novel environment: each mouse was removed from its home cage and placed in the center of an open circular arena (60 cm diameter) for 20 minutes. Trunk blood samples were collected at different time intervals after the novelty test. One group of animals for each treatment was not manipulated at all and blood collected represented the group baseline (Time 0'). Immediately at the end of the novelty exposure, 50% of mice were sacrificed to measure the stress response to the open arena (Time 20'), while the other 50% was reintroduced in their home cages and blood was collected after 40 min (Time 60'). After blood centrifugation (20min, 4 °C, 4000

rpm), serum samples were stored at -25 °C until assay were conducted. Corticosterone levels were measured using commercially available EIA kits (Enzo LifeScience, sensitivity 27.0 pg/mL). All corticosterone measures were carried out in duplicate.

Hippocampal mRNA Analyses: **GR and MR expression (Real-time PCR analysis)**

Brains of adult male mice of the Time 0 groups for corticosterone essays were rapidly removed and placed onto an ice-cooled metal plate. Hippocampi were dissected and samples were immediately frozen on dry ice and stored at -80 °C. RNA was extracted from homogenized hippocampi (*N* = 5/7 for each experimental group) using a Total RNA purification kit (Norgen Biotek, Thorold, ON, Canada) following the instructions of manufacturer. RNA quantity was determined by absorbance at 260 nm using a Nano Drop UV-VI Sspectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA was reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Paisley, UK) according to the manufacturer's instructions. Equal amounts of cDNA were then subjected to real-time PCR analysis with an Applied Biosystems 7900 HT thermal cycler, using the SensiMix SYBR Kit (Bioline, London, UK) and specific primers, each at a final concentration of 200nM (Nr3c1: sense: CCTCCCAAACTCTGCCTGG , antisense AGCACAAA

GGTAATTGTGCTGT; Nr3c2: sense CGGCTTCAGCTGACC TTTGA, antisense TGGCTCTTGAGGCCATCTTT; Actb: sense CAATGAGCTGCGTGTGGC, antisense GTACATGGCTGGGGTGTTGA). Each measurement was performed in quadruplicate and each experiment in triplicate. The expression data were normalized using the expression values of Actb gene. Amplification efficiency for each primer pair was determined by amplification of a linear standard curve (from 0.1 ng to 20 ng) of total cDNA as assessed by A260 spectrophotometry. Standard curves displayed good linearity and amplification efficiency for all primer pairs.

Respiratory response to 6% CO_2 enriched air mixture

The assessment of the effect of RCF manipulation on CO_2 sensitivity has been conducted measuring the respiratory responses to 6% air-CO_2 concentration in young (PND 16-20) and adult (PND 75) H, N-H, RCF and N-RCF animals. The changes in tidal volume (i.e., the volume of air displaced between normal inspiration and expiration, TV) during 6% CO_2-enriched air breathing (CO2 challenge) were measured in an unrestrained plethysmograph (PLY4211, Buxco Electronics, Sharon CT) carrying two separate Plexiglas chambers of 450 ml. This allows for the parallel assessment of 2 animals/session. Before any recording, each subject was closed in its chamber for an acclimatization of 40 min. Then, the recording of

respiratory parameters started under air condition (baseline) for 20 min. Next, the challenge began with the administration of 6% CO_2 enriched air, followed by a 20 min recovery period (air). A complete session thus lasted 80 min per animal.

Statistical analysis

Maternal behavior. Data were analyzed by two way ANOVAs, the factors being (1) manipulation (4 levels: H, N-H, RCF and N-RCF); and (2) developmental age (2 levels repeated measure:PND2–4 and PND 5–7). The observation period was split into 2 time-windows: PND2–4 (daily cross—fostering period) and PND5–7 (definitive adoption for the RCF group) to control for the immediate effect of the RCF protocol.

Ultrasonic Vocalizations (PND8). A one-way ANOVA, the factor being manipulation (4 levels: H, N-H, RCF and N-RCF), was used to compare the total number of vocalizations emitted by pups during the 5 min of isolation session. The sex of the pup was not considered as we never observed a male-female difference in 8-day old pups' ultrasonic emission (D'Amato et al. 2011; Cinque et al. 2012)

Sociability and social preference. One-way ANOVAs, the factor being manipulation (4levels: H, N-H, RCF and N-RCF), were conducted on a Sociability and Social Preference index that measured the percentage of time

spent close to unfamiliar partners (Time Close unfamiliar/(Total Time close to both cylinders) X 100).

Emotionality. The time spent in the different arms of the apparatus was evaluated by automatic software analysis (SMART, PanLab) and the percentage of time spent in open arms was used as behavioral index of emotionality (100 X Time Open/(Time Open +Time Closed) in a one-way ANOVA, the factor being the postnatal manipulation (4 levels: H, N-H, RCF and N-RCF).

HPA axis functionality: corticosterone response to novelty. The mean serum corticosterone levels of mice were compared by a two-way ANOVA, the factors being (1) manipulation (4 levels: H, N-H, RCF and N-RCF); and (2) time intervals (3 levels: time 0, 20' and 60').

*Hippocampal mRNA Analyses: GR and MR expression (Real-time PCR analysis).*Expression data were presented, after normalization, as the fold-changes over the expression values of control samples (H vs. N-H and RCF vs. N-RCF). Independent *t*-tests between treated and control delta Cts (H vs. N-H and RCF vs. N-RCF) were used to evaluate significant differences in gene expression.

Respiratory response to 6% CO_2 enriched air mixture. A one way ANOVA, the factor being early manipulation (4 levels: H, N-H, RCF and N-RCF), was used to compare the

mean percentage of increment of tidal volume from baseline (ΔTV %) during 6% CO2 exposure.

RESULTS

This section contains results of the experiment 1 but the figures are contained in the paper already published (Luchetti et al. 2015) and reported in the appendix A.
Here there is a summary table of the all results (table1).

Maternal Behavior

The total amount of nursing and grooming behavior received by pups exposed to different manipulations is shown in Figure 2. The statistical analysis revealed that different manipulations did not affect the total amount of nursing and grooming/licking received by pups during the first week of life (NP: $F(3/48)= 1.00$, ns; GP/L: $F(3/48) = 1.67$,ns) but, while NURSING decreased during the first week of life ($F(1/48) = 14.27$, $p < 0.001$), pups' grooming and licking remained relatively stable ($F.1=48/ = 1.41$, ns) across all 4 experimental groups.

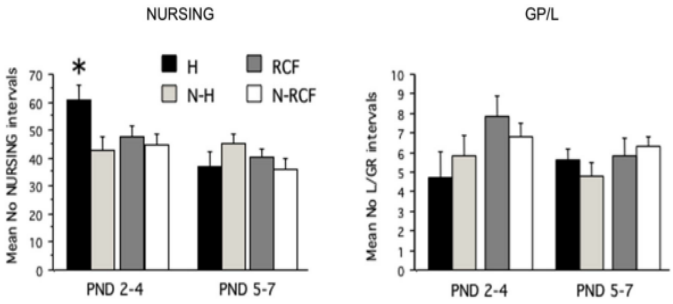


Figure 2. Maternal care received by pups exposed to different post-natal manipulations. Data are presented as mean (+SE) group scores for 3-day intervals (PND2–4 and PND5–7). Experimental groups: H: Handled; N-H: Non-Handled; RCF: Repeated Cross-Fostering; N-RCF: Control. *p < 0.05

The interaction between postnatal manipulation and time reached statistical significance only for NURSING (NP: F (3/48) = 3.80, p < 0.02; GP/L: F(3/48) = 0.98,ns). H pups received more nursing than all others groups, but only during PND2–4. The amount of nurturing received by both control groups (N-H and N-RCF) was very similar.

Ultrasonic Vocalizations

The response to isolation measured in pup on PND8 is shown in Figure 3: the ANOVA indicates a significant difference between groups (F (3/23) = 4.30, p < 0.05). RCF pups emitted the highest number of USVs in comparison with all other groups during the 5 min

session. Again, the 2 control groups (N-H and N-RCF) confirmed similar.

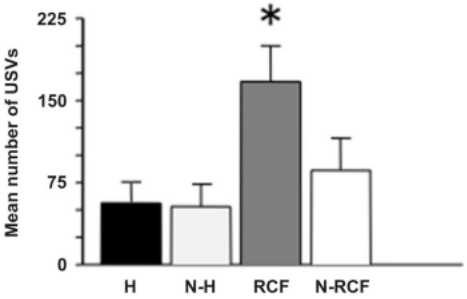


Figure 3. **Mean (+SE) number of ultrasonic calls (USVs) emitted by 8-day old pups of different experimental groups,when isolated in their own home-cage bedding for 5 min.** *$p < 0.05$

Sociability and Social Preference

During the habituation session, when young male mice explored the 3 compartments cage, no difference in the time spent in the different chambers was detected. Neither sociability towards unfamiliar partners ($F(3/42) = 0.77$,ns), nor social preference ($F(3/47) = 1.22$,ns) were affected by early manipulations (results represented respectively in figures 4A and 4B). Considering time spent close to cylinders, more than 50% of this time involved exploration of the unfamiliar mouse and no preference/avoidance of siblings was detected.

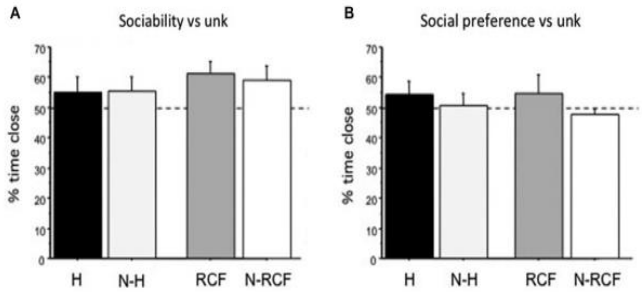


Figure 4. Sociability and Social Preference scores (mean + SE). **(A)** Sociability (preference for conspecific vs. object) and **(B)** Social preference (preference for conspecific vs. littermate) for unfamiliar male mouse (same strain, age and treatment) of juvenile males tested on PND28 and PND35, respectively. Both indices are calculated as the percentage of time spent close to unfamiliar partners (Time Close unfamiliar/(Total Time close to both cylinders) × 100).

Emotionality

Postnatally handled adult males showed, as expected, reduced emotionality in the plus maze test (Figure 5). The one-way ANOVA indicated a significant treatment effect (F (3/33) = 4.43, $p < 0.01$) and *post-hoc* analysis showed that the effect was explained by pups exposed to H manipulation. Indeed they spent more time in open arms than all other groups.

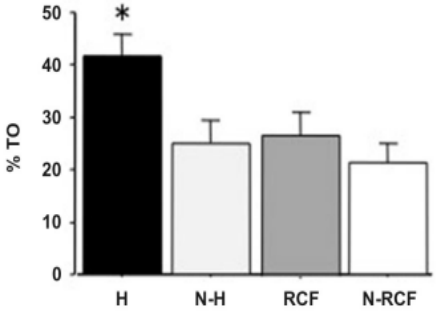
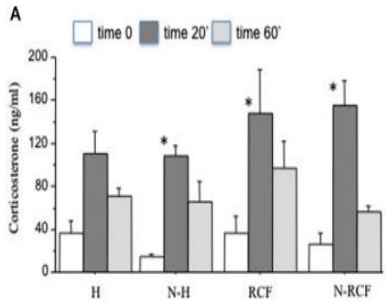


Figure 5. Mean (+SE) percent of time spent in the open arms of an elevated plus maze by adult male mice exposed to different postnatal manipulations. *p < 0.05

HPA Axis Functionality: corticosterone levels after novelty exposure

The corticosterone response to a novel situation in the 4 experimental groups is depicted in Figure 6A. Mice did not differ for the amount of time spent in the central part of the arena ($F(3/45) = 1.72$,ns) during novelty exposure. All groups showed an increase in serum corticosterone at the end of the novelty test (20 min of open field) and a successive reduction of hormone levels during the 40 min of recovery in the home cage. The two-way ANOVA for repeated measures indicated a significant time effect (F (2/63) = 31.59, $p < 0.001$) and no experimental group (F (3/63/ = 1.54,ns),or group X time ($F(6/63) = 0.76$,ns) effects. However, subsequent Tukey *post hoc* analysis revealed that the increase in

corticosterone at the end of the open field exposure (baseline vs. Time 20') was significantly higher in all groups but not in the group exposed to handling during postnatal life.



Fold changes	H vs N-H	RCF vs N-RCF
GR	1.00	0.98
MR	0.98	1.08

Figure 6. (A) Mean (+SE) serum corticosterone levels of male mice from different experimental groups before (Time 0), at the end of novelty (Time 20 0), and 40 min after reintroduction in their home cage (Timer 60 0). **(B)** Fold changes of hippocampal mRNA for Glucocorticoid (GR) and Mineralocorticoid (MR) receptors. * $p < 0.05$

Hippocampal mRNA Analyses: GR and MR Expression

The results of GR and MR gene expression in the hippocampal region, evaluated by real time PCR, indicated no significant differences between groups, either for GR and MR gene expression (Figure 6B). Both GR and MR Delta CTs did not differ either between H and N-H ($t(8) = 0$, ns and $t(8) = 0$, ns, respectively), or

between RCF and N-RCF ($t(12) = 0.28$, ns and $t(12) = -0.79$, ns, respectively).

Respiratory Response to CO_2-Enriched Environment

Adult male mice responses to 6% CO_2-enriched air are shown in Figure 7. The physiological increase in TV was significantly enhanced in RCF subjects (F (3/30) = 3.64, $p < 0.05$) compared to all other groups. Results regarding respiratory response in young animals showed the same effects seen in adults.

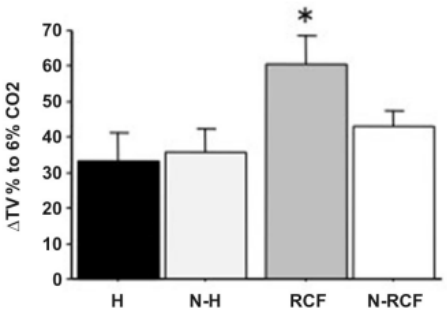


Figure 7. Mean (+SE) percentage of Tidal Volume changes from baseline (Δ TV%) for adult male mice from different experimental groups, in response to 6% CO2 . * p<0.05

BEHAVIOR	H *vs* N-H	RCF *vs* N-RCF
Maternal behavior received	>	ns
USVs response to isolation (m+f)	ns	>
Pups' respiratory Response to CO2 (m+f)	ns	>
Sociability /Social preference (m)	ns/ns	ns/ns
Emotionality in the plus maze (m)	<	ns
Corticosterone response to novelty (m)	<	ns
Hippocampal GR and MR expression (m)	ns	ns
Respiratory Response to CO2 adulthood (m)	ns	>
BODY WEIGHT	ns	ns

Table 1. Summary table of several behaviors evaluated to compare the effects of two different early manipulations: handling and repeated cross-fostering. H: handled; N-H: no-handled; RCF: repeated-cross fostered; N-RCF: no-RCF

EXPERIMENT 1. DISCUSSION AND CONCLUSIONS

Results obtained in this experiment demonstrated that the two different early manipulations used, handling and repeated cross-fostering, have different and specific short- and long- term effects, suggesting that the observed phenotypes depend on characteristics and timings of early adversities that might activate different biological processes.

These results confirm that repeated daily short separation events (Handling) during the first 2 weeks of life promote heightened maternal care and are associated with reduced behavioral and hormonal reactivity to stress (plus maze and restraint stress) in adulthood, according to results from many laboratories, already reported in the literature (Meaney et al. 1996; Schmidt et al. 2003). However differently from previous studies in literature, the increased expression of hippocampal GRs is no detected in adult H mice (Meaney et al. 1985; O'Donnell et al. 1994; Schmidt et al. 2003; George et al. 2013).

On the other hand the RCF procedure, which implies a strong interference with the infant-mother attachment bond, yielded different and significant effects. Indeed, RCF pups did not receive lower amount of maternal care compared to controls, but responded to 5 min of isolation with a higher amount of distress calls showing a separation anxiety response (SAD).

Contrary to handling, RCF protocol did not modify emotionality (plus maze) and hormonal response (corticosterone levels) to stress (Table 1). These results are not surprising considering that differences in emotionality occurring in H adult animals have been explained by the increased levels of maternal care received by these animals. Indeed, the increased level of grooming/licking behavior received by H pups during the first week of life would induce, through epigenetic response, changes in brain and behavior persisting until adulthood (Champagne et al. 2003).

Regarding social behaviors the results suggest that neither H, nor RCF treatment affected social motivation in immature mice. These animals are all interested in conspecifics.

In addition results obtained in this experiment demonstrate that, as already reported in previous study, RCF animals showed higher, stable and specific augmentation of tidal volume in response to 6% CO2-enriched air mixture (D'Amato et al. 2011). This is confirmed here once more, and is specific of RCF subjects as it was not seen among H animals. This hypersensitivity to CO2 can be turned into a remarkable investigational tool and useful endophenotype, allowing modeling PD in the mouse.

Experiment 2a. Molecular investigations of differences in respiratory response to 6% CO_2 between RCF and control mice

INTRODUCTION

The first experiment demonstrated that mice exposed to RCF paradigm of interference with maternal environment and mother-pups bond formation, showed an enhanced separation anxiety and an enhanced hyperventilation in response to 6% CO_2-enriched air mixture. Also in humans, parental instability (early separation or loss) is a risk factor for the development of separation anxiety disorder (SAD) during childhood and panic disorder during adulthood. These two disorders, genetically and developmentally-related anxiety disorders, share the CO_2 hypersensitivity endophenotype (Battaglia et al. 2009) and Battaglia demonstrated that early life adversities interact with genetic factors to enhance human reactivity to hypercapnia condition (Spatola et al. 2011). These evidences suggest that the gene-environment interplay has a role in the development of susceptibility to SAD, PD and CO_2 hypersensitivity as supported by the evidences obtained with the RCF model (D'Amato et al. 2011; Luchetti et al. 2015). The CO_2 hypersensitivity

associated with early-life adversities can be explained by epigenetic mechanisms.

A molecular basis for hypercapnia-associated respiratory diseases has been recently proposed. The amygdala, which is known to play a prominent role in fear circuitry, has been proved to be a chemosensor for the detection of hypercarbia, a function mediated by the acid sensing ion channel-1a subunit (ASIC1a). Although asic1a is expressed throughout the nervous system, particularly high levels are expressed in the amygdala. In rodents, CO_2 inhalation reduces amygdala pH, inducing acidosis and fear behaviors (Ziemann et al. 2009; M. W. Coryell et al. 2007; Wemmie et al. 2003). Conversely, disrupting asic1a in mice decreases acidosis-induced fear behavior, which can be restored through transgenic expression of asic1a in the amygdala (Ziemann et al. 2009). However, CO_2 inhalation was found to induce panic attacks in three individuals with bilateral amygdala damage, suggesting that amygdala chemosensing is not required for the expression of CO_2-triggered panic (Feinstein et al. 2013). Indeed the acid sensing chemoreceptors have been first identified in the brainstem that is an important center of breathing regulation (Nattie 1999) and may play a key role in CO_2 hypersensitivity showed by PD patients and RCF animals.

In addition, several studies exploring the molecular genetic of panic disorder suggest that the Human ortholog of the rodent acid-sensing ion channel gene, ACCN2, is associated with PD and amygdala structure

and function (Gregersen et al. 2012; Smoller et al. 2014) Moreover the most comprehensive neuroanatomical model of PD has suggested an abnormal sensitivity in the brain mechanisms of fear and alarm response involving a network of neuronal pathways and multiple neurotransmitter systems, including serotonin (5-hydroxytryptamine, 5-HT), norepinephrine, gamma-aminobutyric acid (GABA), and others. Accordingly, panic attacks originate from a dysfunction in the brain fear network that integrates various structures such as the brainstem, the amygdala, the hypothalamus, and the cortical regions (E Maron, Hettema, and Shlik 2010).

Taken together these considerations and evidences, we have conducted a genome-wide investigation of altered histone marks (epigenetic investigation) in the brainstems (medulla oblongata) of RCF mice and their controls. Data from this study (submitted) indicate an association between RCF procedure and histone marks in the brainstem and in particular we found modifications correlate with Asic1 gene expression.

Starting from these considerations, to investigate biological bases of enhanced response to hypercapnia in RCF mice, by RT-PCR I evaluated mRNA expression of some candidate genes in animals' brainstems.

MATERIALS AND METHODS

Animals and experimental groups

NMRI outbred mice (Harlan, Italy) were used in all experiments (2a, 2b, 3 and 4). Animals' housing and mating protocol have already been described in Experiment 1.

Experimental groups

As described in experiment 1, animals were manipulated according to the RCF protocol.

A total of two experimental groups resulted from the early manipulation: RCF and their controls (RCF and CT). Animals were weaned when 28 days old, and then separated by sex and left in cage with littermates. A total number of 10 RCF and 10 CT litters were used for all experiments presented here.

Animals were used for:

- ✓ replication and confirmation of the data showed in experiment 1 concerning the respiratory response to 6% CO_2 enriched air mixture (data confirmed but not show here)
- ✓ molecular investigations (experiment 2a);
- ✓ evaluation of new pharmacological treatments for the CO_2 hyperventilation (experiment 2b);
- ✓ assessment of cognitive capabilities of RCF animals (experiment 3);

- ✓ evaluation of heritability of the respiratory endophenotype showed by RCF animals (experiment 4).

Brainstem mRNA Analyses (Real-time PCR analysis)

Adult male mice (90 days old, never tested) were sacrificed and brains were rapidly removed and placed onto an ice-cooled metal plate. Brainstems were dissected and samples were immediately frozen on dry ice and stored at −80◦C. RNA was extracted from homogenized brainstems (N = 4/5 for each experimental group) using a Total RNA purification kit (Norgen Biotek, Thorold, ON, Canada) following the instructions of manufacturer. RNA quantity was determined by absorbance at 260 nm using a NanoDrop UV-VIS spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA was reverse-transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Paisley, UK) according to the manufacturer's instructions. Equal amounts of cDNA were then subjected to real-time PCR analysis with an Applied Biosystems7900HT thermal cycler, using the SensiMixSYBR Kit (Bioline, London, UK) and specific primers, listed in table 2, each at a final concentration of 200 nM. Each measurement was performed in quadruplicate and each experiment in triplicate. The expression data were normalized using the expression values of Actb gene. Amplification efficiency for each primer pair was determined by amplification of a linear

standard curve (from 0.1 ng to 20 ng) of total cDNA as assessed by A260 spectrophotometry. Standard curves displayed good linearity and amplification efficiency for all primer pairs. Genes selected by our preliminary epigenic data and by reports from the literature (E Maron, Hettema, and Shlik 2010; Gregersen et al. 2012) are involved in gabaergic transmission (dbi and Gabrd); in glutamatergic transmission (grik5); or codify for acid-sensing ionic channels (asic1), solute carriers (slc17a7, slc6a13, slc6a4), glycine receptor (gla3), pleiotrophin (ptn), phospholipase C (plcg2), and prostaglandin synthase (ptgds).

PRIMER	SENSE	ANTISENSE
DBI	GGACTCGTGGAACAAGCTGA	TCCACATAGGTCTTCATGGCAC
ASIC1	TTTGTGTCTTGCCAGGAGCAG	TGGTAACAGCATTGCAGGTG
GABRD	ACGGAAAGCCAAGGTCAAGG	GACGATGGCGTTCCTCACAT
GLA3	TGGGCATCACCACTGTACTT	CACAAAAAGGAGGCACACCG
GRIK5	GGCGGTCATGGAGTTCATCTG	TCTCCTGGCACACCGACAC
PLCG2	AGTGAAGACATCGAGCTGGC	CAGTTGGCGACAGGAGGAAT
PTGDS	CCACCTTTAGCAAGGCCCAG	CTGACTTCTCTCACCTGCGT
PTN	AAAACTGTCACCATCTCCAAGC	TCTCCTGTTTCTTGCCTTCCTTT
SLC17A7	CCATCATCGTGGGTGCAATG	TAGTGCACCAGGGAGGCTAT
SLC6A13	TGTTGGCTCTTTTTCACGCC	GTGGCGTGTATTTGATCAGGG
SLC6A4	CTGATCAGCACTCCAGGGAC	GGATGTCCCCACACGGAAT

Table 2. List of genes and related primers used for the analysis of mRNA expression in animals' brainstems.

Statistical analysis

Expression data are shown, after normalization, as fold-changes over the expression values of control samples. Non-parametric tests (Mann-Whitney) between treated and control delta Cts (RCF vs. CT) were used to evaluate significant differences in gene expression between groups.

RESULTS

Brainstem mRNA Analyses (Real-time PCR analysis)

The non-parametric statistics used to compare small samples (Mann-Whitney U test) indicates a significant difference in mRNA between RCF (n = 5) and CONT (n = 4) for the expression of Asic1 (p= 0.05), Dbi (p=0.02), Gla3 (p= 0.05), Ptn (p= 0.02), Grik5 (p= 0.05), Plcg2 (p= 0.05), Gabrd (p= 0.05), Ptn (p= 0.05) and Slc17a7 (p= 0.05) genes (Figure 8A and 8B).

Figure 8A shows the comparison between RCF and CONT ΔCt defined by RT-PCR. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. lower Ct level means a greater amount of target nucleic acid in the sample). Figure 8B shows the fold-changes of RCF over the expression values of control samples.

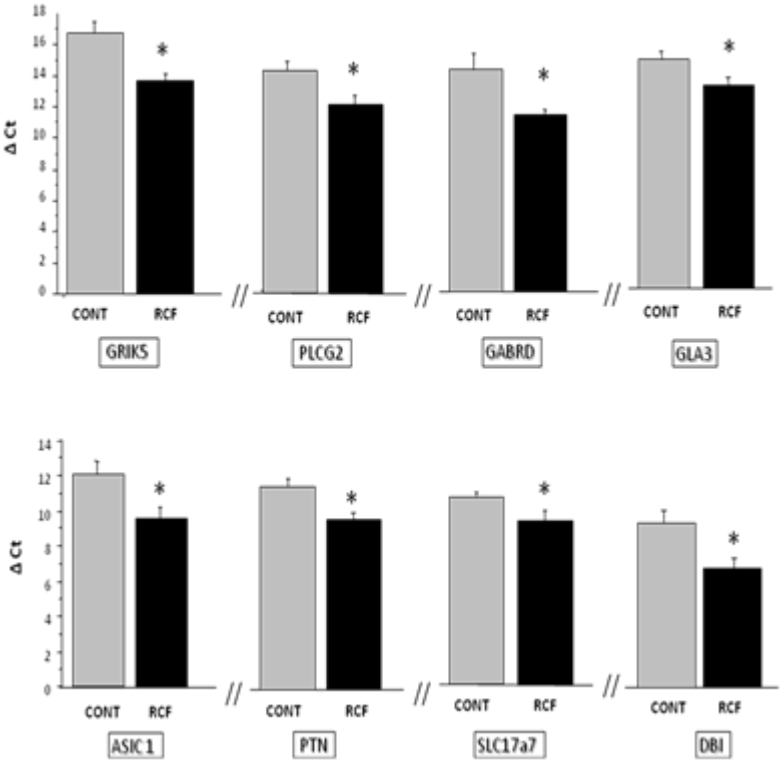


Figure 8A. Gene expression in animals' brainstems presented, after normalization, as ΔCt comparing RCF and CT animals.

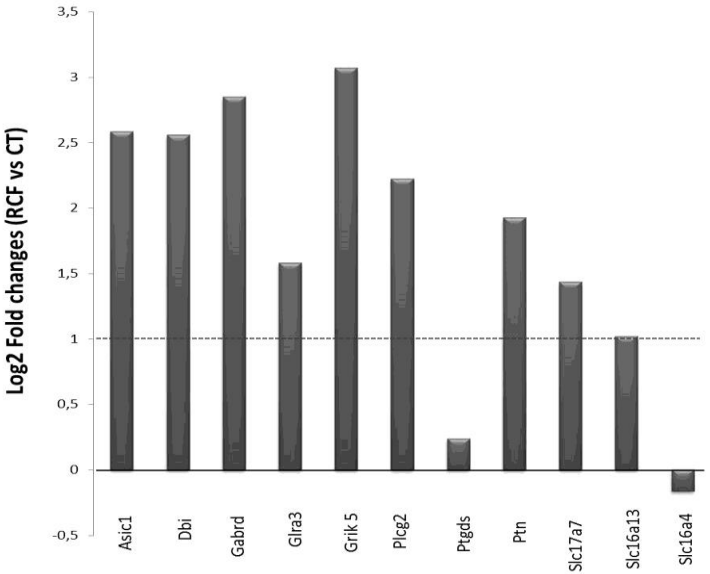


Figure 8B. Gene expression in animals' brainstems presented, after normalization, as fold-changes of RCF over the expression values of control samples.

EXPERIMENT 2a. DISCUSSION AND CONCLUSIONS

Results obtained in the experiment 2a demonstrate that RCF procedure is able to induce significant differences in the mRNA expression of genes suggested to be involved in panic disorder, as previously reported in literature (Maron et al. 2010). In particular RCF animals showed, in brainstem, an increment in the mRNA expression of Asic1, Dbi, Gla3 and Ptn genes. Among the others, the

results concerning Asic1 and Dbi genes are the most interesting.

Asic1, is a gene which codifies for acid-sensing ion channels, has already been related to CO_2 hypersensitivity and fear responses showed by PD patients (Coryell et al. 2007; Ziemann et al. 2009; Wemmie et al. 2003). The reported increment in Asic1 gene mRNA in RCF animals supports the hypothesis of a central role of acid-base balancing mechanisms in the development in panic disorder and also supports the validity of the RCF protocol to model PD in animals.

An increase in Dbi expression, which codifies for diazepam binding inhibitor, suggested an alteration in GABAergic transmission in RCF animals corroborating the hypothesis of an important role of GABAergic neurotransmission in the origin of PD as described in the literature (Maron et al. 2010).

These data obtained by RT PCR well correlate with results obtained analyzing epigenetic marks in the brainstems of these animals. Indeed these latter data (Cittaro et al., submitted) demonstrated epigenetic alterations, related to gene activation, on the same genes analyzed in RT PCR and in particular the alterations were the acetylation of Histone 3 (H3Ac) and the tri-methylation of lysine 4 of Histone 3 (H3K4me3). Taken together these data suggest that RCF is able to induce epigenetic modifications in several genes.

Experiment 2b. New pharmacological rescue treatments for respiratory hypersensitivity to CO_2 in a mouse model of PD

INTRODUCTION

Compounds with reported effectiveness in the treatment of PD include tricyclic antidepressants, benzodiazepines, serotonin selective reuptake inhibitors (SSRIs), serotonin and noradrenaline reuptake inhibitors (SNRIs) and others (Freire et al. 2011). Several studies have demonstrated that symptoms associated with PAs in PD, and laboratory-induced PAs can be rapidly treated with benzodiazepines (Tesar and Rosenbaum 1986; Ballenger et al. 1988) that enhance inhibitory GABAergic tone and represent a fast-acting panicolytic treatment (Baldwin 2005; Borwin Bandelow et al. 2008; Cloos and Ferreira 2009). Indeed most evidence suggests that there is a reduced inhibitory GABAergic tone in patients with PD as indicated by the reduced $GABA_A$ R binding in prefrontal cortex (Nikolaus et al 2010) or deficits in central GABA concentration (Goddard, Mason, et al. 2001). For these reasons benzodiazepines are effective but their use presents some side effects: for instance routine usage makes the drug less effective due to desensitization, and there are many side effects such as sedation and addiction (Johnson, Federici, and Shekhar 2014). On the other hand, some evidences suggest that panic attacks

associated with PD can also be treated with slower-acting pharmacological therapies that enhance monoaminergic (e.g., serotonin, norepinephrine, epinephrine, dopamine, and histamine) activity globally [using tricyclic antidepressants (Ballenger et al. 1988; Bakker, van Balkom, and Spinhoven 2002; Giampaolo Perna, Guerriero, and Caldirola 2011) or monoamine oxidase inhibitors (MAOI) (Kelly, Mitchell-Heggs, and Sherman 1971)], or by specifically targeting serotonergic or noradrenergic systems with reuptake inhibitors (Cloos and Ferreira 2009). Also these treatments exhibit some side effects. The use of MAOIs requires a tyramine-restricted diet and can produce hypertensive crisis. In some cases, TCAs and also SSRIs and NRIs increase anxiety initially, and begin to show anxiolytic and panicolytic properties after 2–3 weeks of daily treatment. Thus, the mechanisms by which these compounds are panicolytic are through compensatory changes that occur with repeated use, and a therapeutic option is to initially co-administer a low dose of a benzodiazepine with SSRIs to PD patients, which has been shown to result in a 41% response rate, compared to 4% response rate for placebo + SSRI group in the first week of treatment (Goddard, Brouette, et al. 2001). Resuming effective anti-panic medications exist but a substantial proportion of patients do not fully respond, the available drugs have several side effects and most medications have a delayed onset of their therapeutic effect. Thus, further advances are needed.

To date, the pharmacological research on PD appears to be relatively limited and many reasons may explain these difficulties, including the heterogeneity of the disorder, the incomplete understanding of its underlying pathophysiological mechanisms and difficulties in the selection of appropriate animal models in preclinical studies. Defining biomarkers and endophenotypes in PD may offer advantages in both understanding the pathophysiology of the disorder and selecting appropriate targets and outcomes for planning future pharmacological research (Perna, Guerriero, and Caldirola 2011).

The experiment 1 demonstrated that RCF manipulation lead to develop CO_2 hypersensitivity typical of PD patients and this abnormal physiological response is a useful endophenotype studying PD and possible new rescue treatments. In addition the experiment 2a showed a possible biological basis responsible for this endophenotype: the acid-sensing ion channels (ASIC) which could be a target for new pharmacological treatments.

For these reasons in the following experiment three different treatments will be evaluated.

The first group of animals will be treated with a benzodiazepine, chlordiazepoxide (CDP), to evaluate whether this treatment, commonly used in anxiety and panic disorders, is effective in reducing the respiratory endophenotype of the RCF mice and thus confirming the

validity of this animal model for panic disorder. In addition, CDP could restore the normal level of transmission, also thanks to decrease levels of DBI (inhibitor of GABAergic transmission) which, as reported in the previous experiment, is enhanced in RCF mice.

The second drug used is the chlorogenic acid (CGA), a polyphenol contained in green coffee and in some vegetables, which has also anxiolytic and antioxidant effects (Bouayed et al. 2007; Hassan et al. 2014). CGA is able to inhibit the functional activity of ASICs decreasing the peak amplitude of proton-gated currents and acidosis-evoked membrane excitability (Qu et al. 2014; Baron and Lingueglia 2015). In addition CGA, like some cathecol-containing dietary polyphenols, is able to inhibit DNA methylation through a non-competitive mechanism (W. J. Lee and Zhu 2006). CGA could be a reliable new pharmacological approach for panic disorder.

The third treatment will be based on amiloride, a previously widely used K^+-sparing diuretic agent that is a nonselective blocker of ENaC. As the member of ENaC superfamily, all ASICs are inhibited by amiloride (Chu et al. 2011; Diochot et al. 2007; Lin, Sun, and Chen 2015). In general, micromolar concentrations of amiloride inhibit ASIC currents in a concentration-dependent manner. Data from the literature demonstrated that amiloride decreased ASIC-mediated increases in intracellular $Ca2^+$, and attenuate acid-induced membrane depolarization (Xiong et al. 2004; Yermolaieva et al. 2004; Wu et al. 2004; Vukicevic and Kellenberger 2004). For its capability

to inhibit ASICs also the amiloride could be a promising new pharmacological treatment for PD.

MATERIALS AND METHODS

Animal experimental groups

Adult males and females NMRI outbred mice were used for this experiment. Animals were subjected to early repeated cross fostering or control manipulations at birth, as described in previous experiments.

From weaning to post-natal day 60-75 (the day of tests) RCF and CT animals were housed in group of four same sex/litter in transparent high temperature polysufone cages (26.7 × 20.7 × 14.0 cm) with water and food available ad libitum, in the animal facility. Room temperature (21 ± 1 ◦C) and a 12:12 h light dark cycle (lights on at 07.00 p.m.) were kept constant.

In this experiment we evaluated the acute effects of three pharmacological treatments, described in the introduction, on the respiratory endophenotype during exposure to 6% CO_2.

RCF and CT adult animals were divided in four groups, according to the acute treatment: animals treated with chlordiazepoxide (CDP), chlorogenic acid (CGA), amiloride (AMI) and saline (SAL). CGA and CDP was administrated by intraperitoneal injection whereas AMI via intranasal administration because it poorly pass the blood brain barrier (Miller et al. 2015; Baron and Lingueglia 2015). SAL was administrated both

intraperitoneally and by intranasal way depending on whether it was the control for CGA/CDP or AMI.
Amiloride treatment was a preliminary experiment so until now is made only in female mice.

Effect of different pharmacological treatments on the respiratory response to 6% CO_2 enriched air mixture

To evaluate the effects of different drugs (chlordiazepoxide, chlorogenic acid and amiloride) on the respiratory response to 6% CO2 enriched air mixture RCF and CT adult animals were tested in the plethysmograph apparatus as described above. Unlike the procedure already described at the end of the baseline period the animals were treated with chlordiazepoxide (5 mg/kg) or chlorogenic acid (20 mg/Kg) or amiloride (10 mg/kg) or saline depending on the experimental group. The treatment was administrated by intra-peritoneal injection for CDP and CGA and by intranasal way for AMI. After the drug administration the animal returned in the plethysmograph chamber and the challenge period (6% CO_2 enriched air mixture) of twenty minutes started. At the end of the challenge period there was a 20 minutes of recovery period (normal air).
Experimental groups for male mice were: RCF SAL (n=7); RCF CGA (n=7); RCF CDP (n=6); CT SAL (n=6); CT CGA (n=5); CT CDP (n=6).

On the other hand experimental groups for female mice were RCF SAL (n=6); RCF CGA (n=8); RCF CDP (n=8); CT SAL (n=5); CT CGA (n=7) and CT CDP (n=9). In addition for females there were the experimental groups for amiloride treatment: RCF SAL (n=4), RCF AMI (n=5), CT SAL (n=5) and CT AMI (n=4). These were very small groups because of the preliminary nature of this experimental treatment.

Statistical analysis

A two way ANOVA, the factors being early manipulation (2 levels: RCF and CT) and pharmacological treatments (2 levels: CDP and SAL or CGA and SAL or AMI and SAL), was used to compare the mean percentage of increment of tidal volume from baseline (ΔTV %) during 6% CO_2 exposure after pharmacological treatment. Males and females were considered in different statistical analysis

RESULTS

Effect of different pharmacological treatments on the respiratory response to 6% CO_2 enriched air mixture in adult male mice

Figure 9 (A-B) shows the effect of pharmacological treatments on the response to hypercapnia in adult (PND 75-90) male mice. Regarding the treatment with CGA (figure 3A) the ANOVA revealed significant interaction between early manipulation and pharmacological treatment ($F(1/21) = 5.44$, $p = 0.29$). Tukey post-hoc analysis revealed significant difference between RCF SAL and CT SAL mice ($p=0.04$) and between RCF SAL and RCF CGA animals ($p=0.05$) suggesting an effect of CGA in restoring the normal respiratory response in RCF animals.

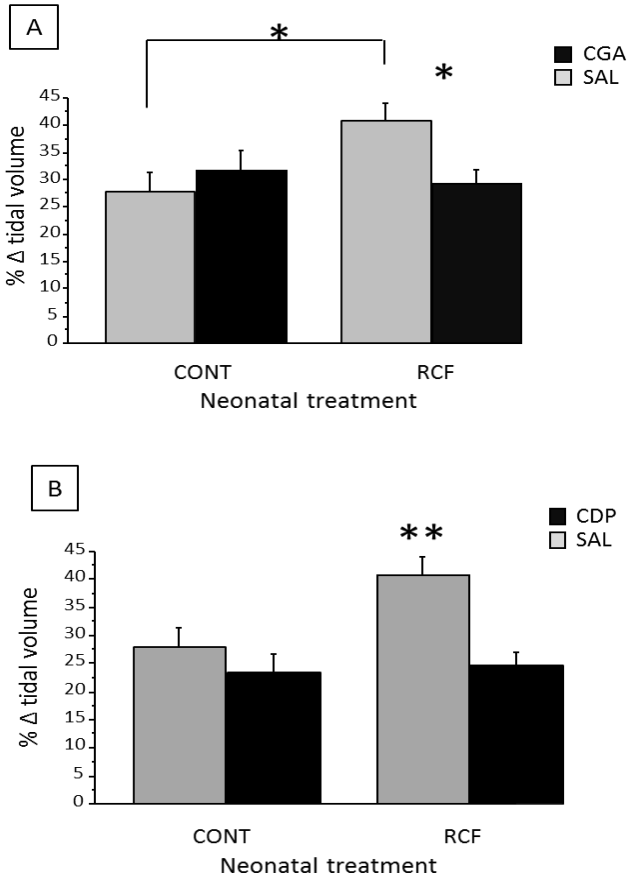


Figure 9. Effect of pharmacological treatment with CGA (A) and CDP (B) on the mean of increment of tidal volume, in response to hypercapnic condition (6% CO_2), in male adult mice. SAL is the control treatment. *p<0.05; **p<0.01

For the treatment with CDP (figure 3B) ANOVA revealed a significant effect of the early manipulation (F (1/21) =

5.16, p = 0.03) and a significant effect of pharmacological treatment (F (1/21) = 11.29, p= 0.003). RCF SAL animals showed a significant enhanced respiratory response in comparison to all other groups. RCF treated with CPD recovered the normal respiratory response as CT animals.

Effect of different pharmacological treatments on the respiratory response to 6% CO_2 enriched air mixture in adult female mice

The Figure 10 (A-B) shows the effect of pharmacological treatments on the response to hypercapnia in adult (PND 75-90) female mice. The ANOVA regarding the treatment with chlorogenic acid (CGA, panel A) revealed a significant effect of the interaction between neonatal manipulation X pharmacological treatments (F (1/22) = 5.1, p= 0.03). The Tukey post-hoc test revealed statistical difference between: RCF SAL animals vs CT SAL animals (p= 0.04) and RCF SAL vs RCF CGA (p= 0.02), indeed RCF SAL animals showed an enhanced response to hypercapnia in comparison with these experimental groups. The analysis regarding the treatment with the benzodiazepine chlordiazepoxide revealed only the main effect of the neonatal treatment (F (1/24) = 6.11, p=0.02) but no significance effect of the pharmacological treatment. In figure 11 are shown the results regarding the effect of the intranasal administration of amiloride on the response to hypercapnia in adult (PND 75-90) female mice. The ANOVA revealed a significant effect of

the neonatal manipulation ($F(1/14)= 5.15$, $p= 0.03$), of the pharmacological treatment ($F(1/114)=14.32$, $p=0,002$) and a significant effect of the interaction between neonatal treatment X pharmacological treatment ($F(1/14)= 8.18$, $p= 0.012$).The Tukey post-hoc test revealed statistical difference between: RCF SAL animals vs CT SAL animals ($p= 0.01$) , RCF SAL vs RCF AMI ($p= 0.001$) and RCF SAL vs CONT AMI ($p= 0.005$).

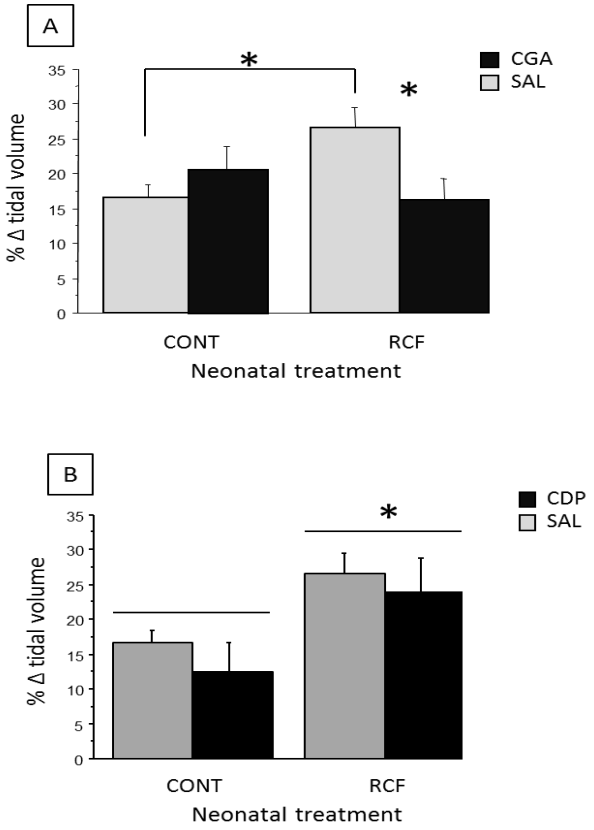


Figure 10. Effect of pharmacological treatment with CGA (A) and CDP (B) on the mean of increment of tidal volume, in response to hypercapnic condition (6% CO_2), in female adult mice. SAL is the control treatment.*p<0.05

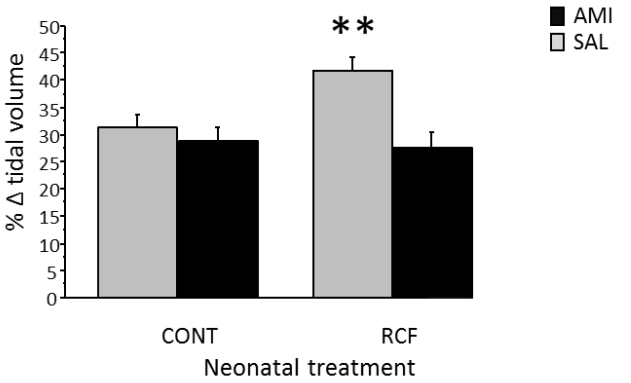


Figure 11. Effect of pharmacological treatment with intranasal amiloride (10mg/kg), on the mean of increment of tidal volume in response to hypercapnia condition (6% CO2), in female adult mice. SAL is the control treatment. *p<0.05, **p<0.01

EXPERIMENT 2b. DISCUSSION AND CONCLUSIONS

In the experiment 2b the effects of different pharmacological treatments on the respiratory response to 6% CO_2 in RCF mice was evaluated. On the basis of gene expression results in brainstem previously obtained, three compounds have been tested: chlordiazepoxide, chlorogenic acid and amiloride.

Specifically, whereas saline treated RCF male mice confirmed the enhanced respiratory response to 6% CO2 enriched air mixture in comparison with controls animals (SAL CT), the three drug treatments were able to reduce CO_2 hypersensitivity in RCF animals. Results

demonstrated that both chlordiazepoxide and chlorogenic acid are able, in RCF male mice, to restore the respiratory response observed in controls.

Both in female and male mice results confirmed once more that the manipulation affects the respiratory response to hypercapnic condition, indeed RCF animals showed a hyper-ventilatory response to 6% CO2. However, unlike males, chlordiazepoxide did not rescue RCF female mice respiratory response. Instead RCF females treated with chlorogenic acid and amiloride recovered the normal respiratory response, suggesting that these treatments are more effective than the previous one.

Taken together these results suggest that common drugs used currently to treat panic disorder (benzodiazepine) could be not always effective, as demonstrated by the different responses to chlordiazepoxide shown by male and female mice.

The very interesting theme of these results is that the proposed pharmacological treatments acting specifically on ASIC channels (chlorogenic acid and amiloride) might be effective treatments for panic disorder, considering the CO2 hypersensitivity as a useful marker to study this disease. Thus these data add further evidence of the possible role of ASIC channels in this disorder. In addition data concerning the effectiveness of chlorogenic acid are very interesting for its role in the inhibition of DNA

methylation. Indeed the dosage of this cathecolic polyphenol is able to modulate the cellular DNA methylation process (W. J. Lee and Zhu 2006). Data obtained in experiment 2a demonstrated an enhanced expression of ASIC gene related to epigenetic hyper-methylation of this gene. The therapeutic effect of chlorogenic acid reported here could be due to the modulation of the methylation process, as well as to its role on ASIC current modulation.

Experiment 3. Assessment of cognitive capability in RCF animals

INTRODUCTION

Historically, philosophers have subdivided the study of the human mind and behavior into two broad categories: the cognitive (how we know the world) and the affective (how we feel about it). This division is, however, arbitrary as cognition — a highly complex construct— and emotion interact; cognitive status can color the processing of emotions, and changes in mood affect cognitive function (Pessoa 2008). It is therefore surprising that changes in emotion are universally recognized as being inherent to psychiatric disorders and their classification, whereas cognitive impairment — which has an equally disabling effect on patients — has been comparatively neglected. Despite this close interrelationship between cognition and mood, the cognitive deficits of psychiatric disorders are not just a secondary consequence of perturbed affect, and their underlying neurobiological substrates differ (Millan et al. 2012).

Among distinct psychiatric disorders there are contrasting patterns of cognitive deficits. Cognitive dysfunction does not just signify poor memory — the range of cognitive impairment is broader and more

complex. In the case of panic disorder, few studies have been published about cognitive dysfunctions; therefore, there is still uncertainty as to which cognitive functions could be affected by the disorder. The cognitive functions expected to be most affected are those related to regions involved in the fear network, i.e., the frontal cortex and limbic regions in particular. This would predominantly involve executive functions and emotional processing (Alves et al. 2013). Some studies reported that cognitive dysfunction in panic disorders is mainly confined to excessive attention and hyper-reactivity to threatening, but not emotionally neutral stimuli (Castaneda et al. 2008; Gordeev 2008). However, often in PD patients an emotionally neutral stimulus, if is present during an aversive panic attack, became a threatening stimulus able, in turn, to trigger anticipatory anxiety for, or an actual occurrence of, panic attacks through classical conditioning(Bouton, Mineka, and Barlow 2001). Conditioned stimuli contributing to the onset and maintenance of panic disorder are thought to extend to exteroceptive and interoceptive stimulus events resembling those co-occurring with panic (Bouton, Mineka, and Barlow 2001; Susan Mineka and Zinbarg 2006) via stimulus generalization—a learning mechanism whereby fear responses extend to a range of stimuli resembling the original conditioned stimuli (Pavlov 1927). For example, conditioned fear to the environment/situation where a panic attack occurs might transfer, or generalize, to similar environments and

situations. Similarly, fear associated with the autonomic constituents of panic may generalize to everyday activities that elicit similar changes in physiology (e.g., exercise or climbing stairs). This conditioned fear overgeneralization could allow an initial panic attack to evolve into panic disorder through the proliferation of cues that trigger anticipatory anxiety and could be a pathogenic marker for panic disorder itself (Lissek et al. 2010).

Starting from data available from the literature in this experiment I investigated the cognitive capability of RCF animals. We investigated both the "classical" memory in the novel object recognition test and the associative learning and retention for conditioning events in two different conditions: 1) presentation of general aversive stimulus (a foot-shock) in the active avoidance test and the 2) presentation of a possible aversive and emotionally relevant stimulus (CO_2) in a tone fear conditioning test.

MATERIALS AND METHODS

Animal experimental groups

Adult males NMRI outbred mice were used for this experiment. Animals were subjected to early repeated cross-fostering or control manipulations at birth as described in the first experiment.

Since weaning until post-natal day 90 (the day of tests) RCF and CT mice were housed in group of 4 animals of the same sex and litter in transparent high temperature polysufone cages (26.7 × 20.7 × 14.0 cm) with water and food available ad libitum. Room temperature (21 ± 1 ◦C) and a 12:12 h light dark cycle (lights on at 07.00 p.m.) were kept constant.

Males mice were used for the assessment of cognitive capability in three different cognitive tests: a) active avoidance test; b) object recognition test and c) classical conditioning test (tone+CO_2).

Active avoidance test

The active avoidance test evaluated associative learning and retention for conditioning events (Bovet et al., 1969). Briefly, mice learn to avoid a noxious stimulus by a specific locomotor response driven by a conditioning stimulus which is presented few seconds before the noxious stimulus. The apparatus was computer-controlled and consisted of two sets of eight shuttle boxes (acrylic boxes; 40×10 cm) divided into two 20×10 cm compartments connected by a 3×3 cm opening. A light (10 W) was alternately switched on in the two compartments and used as conditioned stimulus (CS). The CS precedes the onset of the unconditioned stimulus (US) by 5 sec, and overlaps it for 25 sec. Using this procedure the light is present in the compartment for 30 sec (5 sec alone and 25 sec together with the US). After

30 sec both CS and US are terminated and the cycle immediately begin in the other compartment. The US is an electric shock (0.2 mA) continuously applied to the grid floor (stainless steel rods spaced 0.4 cm apart). Over extensive training, mice learn to associate CS and US, and to avoid US by running into the dark compartment. An avoidance response is recorded when mice avoid US by running into the dark compartment within 5 sec of the onset of CS. If mice fail to avoid the US they could however escape it. In such case, mice responses are recorded as simple escape responses. Mice were subjected to five daily, 100-trial avoidance sessions. Failure of escape response seldom occurred.

Novel object recognition test

The object recognition task uses the mice's natural tendency to explore novel objects and assesses recognition memory by measuring its preference for a novel object (Ennaceur and Delacour 1988). When the mouse shows a preference for the new object (i.e., spends more time exploring it) in the presence of a familiar object, it can be inferred that the mouse has a memory for the familiar object.

The test took place in an open-field box (58×58×46 cm) of Plexiglas with dark floor. The objects used in the task varied in shape and color and were made of water-repellant materials such as plastic.

The procedure took place in three consecutive days. The first day the animals underwent a 5-min habituation

period in which time they were free to explore the empty arena. On the second day two identical objects were placed in arena and each animal was placed at the center of the arena and left free for 5 minutes to explore the objects. We considered this session as training session.
After 24 hours from the training session, in the test session, two different objects were placed in the box. One was a copy of the objects used during the training period; the other object was a novel one. A copy of the familiar object was used to ensure that the object had not been scent-marked during the training period.
The location of the novel object was counterbalanced, so that the novel object was located in the left site of the arena for half of the mice and in the right site for the other half.
The box and the objects were cleaned with 10 % ethanol solution between trials.

CO_2 Fear conditioning paradigm

Fear conditioning (FC) is the most common model of aversive memory in rodents. Main characteristics include development of classical conditioning associations with emergence of non-associative hyperarousal reactions (Sauerhöfer et al. 2012) and generalization of fear to situations sharing less common features with the original one (Balogh et al.,2002; Winocur et al.,2007). The FC paradigm consists in the association of a conditioned stimulus (CS: 9,5 kHz tone) with an aversive unconditioned stimulus (US).

Unlike the classical fear conditioning where the US is a foot shock, in this protocol we used as US the exposure to CO_2 air mixture. We used 6 % CO_2 because we questioned whether RCF, in comparison with CT animals, could be more responsive to the aversive valence of this US, and thus more easily conditioned to an associated tone. The conditioned behavior evaluated was the respiratory profile.

The procedure consisted in:

a) animals' exposure to the context for familiarization with the plethysmograph apparatus (D1);

b) pairing of CS with US during the training session (D2) ;

c) animals' exposure to the CS only during the test session (D3) to assess the conditioned behavior.

During the habituation (D1) the animals were placed in plethysmograph chamber two times (11 a.m. and 15 p.m.) for 10 minutes to familiarize with the context.

The day after (D2: training session) the animals were placed in plethysmograph apparatus. After 10 minutes of habituation (baseline measurement of respiratory response) a 20 sec tone (9,5 kHz) paired with 3 minutes of 6% CO2 enriched air mixture exposure was delivered two times with 2 minutes of recovery interval (normal air). The test session (D3) consisted of 10 minute of baseline condition followed by 5 minute of 9,5 kHz tone presentation.

During both training and test session respiratory parameters as tidal volume and breathing frequency have been recorded.

Statistical analysis

Active avoidance test

A repeated measures ANOVA, the factor being early manipulation (2 levels: RCF and CT) and daily session as repeated measure (from D1 to D5), was used to compare the mean percentage of conditioned responses displayed by animals.

Novel object recognition test

A repeated measures ANOVA, the factor being early manipulation (2 levels: RCF and CT) and the repeated variable being zones of arena (center or periphery), was used to compare the mean of time (sec) spent by RCF (n=8) and CT (n=8) animals in each zone of the arena during the first day of habituation.

Regarding the day of training (Day 2) a repeated measures ANOVA, the factor being early manipulation (2 levels: RCF and CT) and the repeated variable being zones of arena (object on the left, object on the right), was used to compare the mean of time (sec) spent by RCF and CT animals in each zone of the arena exploring the objects.

Regarding the day of the test (day 3) repeated measures ANOVA, the factor being early manipulation (2 levels: RCF and CT) and the repeated variable being zones of arena (familiar object, new object), was used to compare the mean of time (sec) spent by animals in each zone of the arena exploring the new and familiar objects.

CO_2 fear condition paradigm

In the training session the ventilatory response (TV) to administration of 6% CO2 air mixture combined to 9,5 kHz tone was analyzed by repeated measures ANOVA using as independent factor early manipulation (2 levels: RCF and CT) and as repeated variable the five sessions of plethysmograph test. A one-way ANOVA was used to analyze the presence of conditioned hyperventilation (increment of tidal volume) in response to tone only during the test using as independent factor early manipulation (2 levels: RCF and CT).

RESULTS

Active avoidance test

In Figure 12 is shown the % of corrected responses shown by RCF (n=7) and CT (n=7) animals to avoid the shock during the five days of active avoidance test. The ANOVA revealed no difference in the mean of percentage of conditioned responses in the two experimental groups ($F(1/48) = 3.61$, $p = 0.08$).

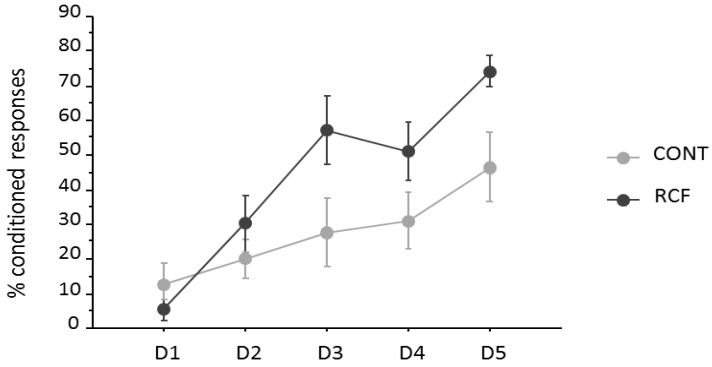


Figure 12. Percentage of conditioned responses shown by RCF and CT adult animals during active avoidance test.

Novel object recognition test

In the statistical analysis for the first day of habituation to a new environment (Figure 13), there is no effect of the neonatal manipulation on the permanence time in the different zones of the apparatus. Indeed RCF (n=8) and CT (n=8) animals spent the same time in the periphery and in center of the arena ($F(1/14) = 1.0$, $p=0.33$). All the animals had a preference for the periphery in comparison to the center.

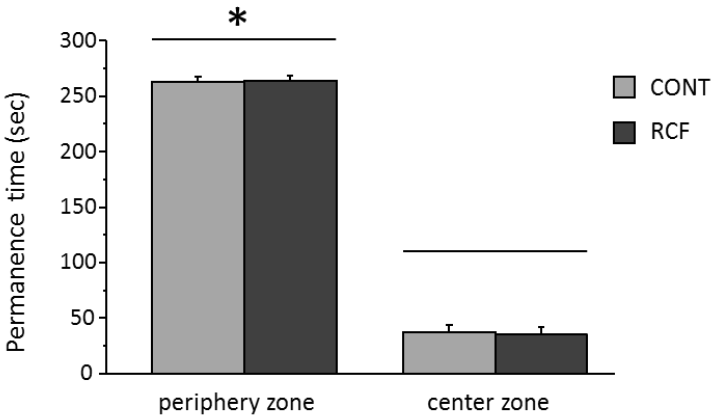


Figure 13. Permanence time in each zone of the apparatus during 5 minutes of habituation in the novel object recognition test.

Data obtained during the training session of the novel object recognition test are shown in Figure 14. ANOVA revealed no significant effect of the neonatal manipulation on the mean percentage of time spent in exploring the two object inside the arena ($F(1/14) = 0.63$, $p = 0.43$).

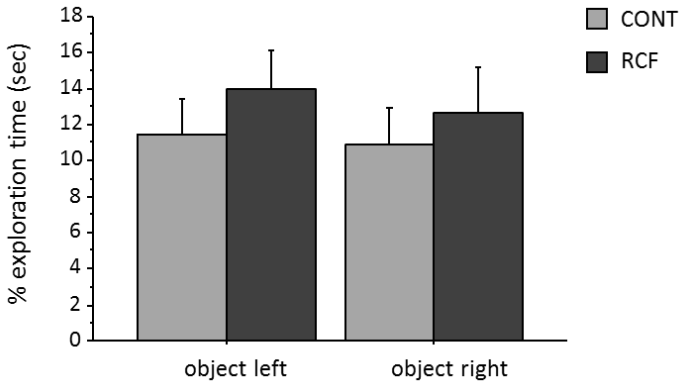


Figure 14. Mean percentage of time spent in exploring the two objects inside the arena during the 5 minutes of training session of novel object recognition test.

In Figure 15 are shown the results of the test session of the novel object recognition test. All animals recognized the new object: both RCF and CT mice spent more time close to the new object in comparison to the familiar one ($F(1/14)= 23.9$, $p= 0.0002$). There is no difference between the RCF and CT animals in exploration time of the two objects (new and familiar) inside the arena ($F(1/14)=0.24$, $p=0.6$).

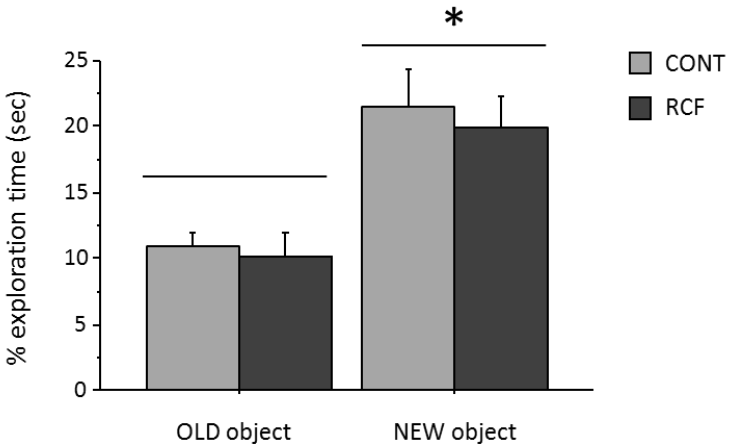


Figure 15. Mean percentage of time spent in exploring the two objects inside the arena during the 5 minutes of test session of novel object recognition test.

CO_2 Fear conditioning paradigm

Results of the training session are shown in Figure 16.

Data revealed that both CT and RCF animals responded to CO_2 plus tone during the two challenges presented in this session. Indeed tidal volume during the challenges was higher than during baseline or recovery period ($F(4/40) = 13.8$, $p < 0.0001$).

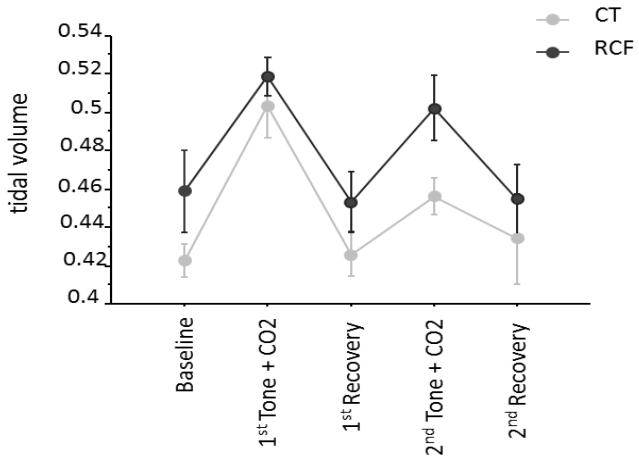


Figure 16. Tidal volume of RCF and CT animals during training session of CO_2 fear conditioning paradigm.

The Figure 17, in the right section, shows conditioned hyperventilation response to the tone, represented by the percentage of increment of tidal volume during 5 minutes of tone presentation over baseline tidal volume. There is an effect of the neonatal manipulation ($F(1/13) = 6.7$, $p = 0.02$); indeed RCF (n=7) animals showed a stronger conditioned hyperventilation in comparison to CT (n=8) animals. In the left section of the same figure is represented the timeline of respiratory response during baseline and tone exposure in test session.

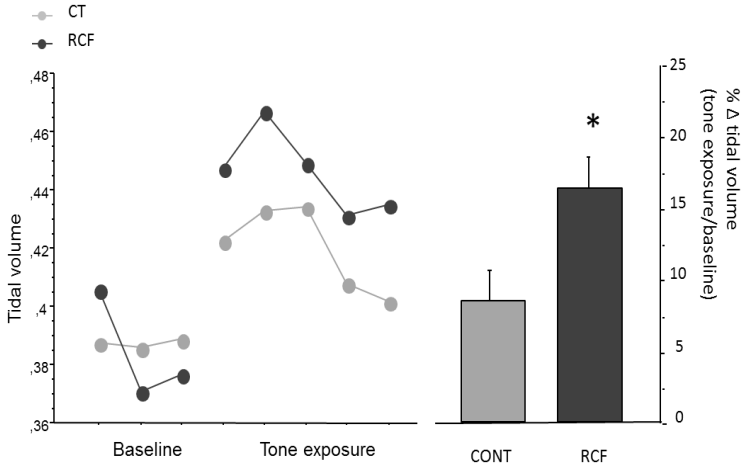


Figure 17. On the left is represented the timeline of respiratory response during baseline and tone exposure in test session. On the left is displayed the mean percentage of Tidal Volume changes from baseline (ΔTV %) from different experimental groups, in response to 5 min tone presentation in CO_2 fear conditioning paradigm. Experimental groups: RCF: Repeated Cross-Fostered Dams; CT: Control Dams. *p < 0.05

EXPERIMENT 3. DISCUSSION AND CONCLUSIONS

In this experiment I tested the cognitive capability of RCF animals in comparison with controls. Indeed, often patients with anxiety disorders (including panic) also show cognitive deficits (Alves et al. 2013; Castaneda et al. 2008). In particular it has been suggested that fear overgeneralization, caused by deficit in conditioning

processes, could allow an initial panic attack to evolve into panic disorder (Lissek et al. 2010).

Both novel object recognition test and active avoidance test suggested that RCF animals are able 1) to recognize the novelty as controls, 2) learn to avoid the aversive stimulus as controls animals, excluding the possibility that RCF mice suffered from major cognitive defects.

In the CO2 fear conditioning test the stimulus is the CO2 which is an aversive stimulus emotionally relevant for RCF animals. During the training session both RCF and control mice showed hyper-ventilatory response to CO2 plus tone exposure. Instead only RCF animals showed a CO2 conditioned respiratory response during the test session, when animals were exposed to the tone alone. This suggests that in RCF animals, but not in controls, CO2 is able to condition the respiratory endophenotype as panic attack does in humans, even in absence of real dangers.

Experiment 4. Trans-generational transmission of respiratory endophenotype typical of Panic Disorder

INTRODUCTION

According to twin studies in humans, shared genetic determinants appear to be the major underlying cause of the developmental continuity of childhood SAD into adult PD, and of the association of both disorders with altered sensitivity to CO_2 (Battaglia et al. 2009; Battaglia et al. 2008). In addition to genetic determinants, environmental risk factors affect the liability to these traits, indeed several life events that influence the susceptibility to PD also predict heightened CO_2 reactivity (Ogliari et al. 2010). Thus there is now the evidence that genetic and environmental determinants may not simply add, but also interact, to influence human responses to CO_2 (Battaglia and Ogliari 2005).

Several studies in humans have documented inheritance of the effects of early experiences. Indeed stressful events can strongly impact an individual's development, physiology and behavior, and are major risk factors for mental health disorders later in life and across generations (Heim et al. 2008; Perepletchikova and Kaufman 2010). For example children of women with post-traumatic stress disorder (PTSD), are more often

affected by PTSD and have increased susceptibility to a lower level of plasma cortisone like their parents (Yehuda et al. 2007). As well as some studies support the idea that first-degree relatives of PD patients are more responsive to the CO_2 challenge than control subjects and thus to the panic disorder itself (van Beek and Griez 2000; Giampaolo Perna et al. 1995; Giampaolo Perna et al. 1999; W. Coryell 1997).

Several studies in mice demonstrated that both negative and positive early experiences of one generation are transmissible to the subsequent generations by epigenetic mechanisms (Franklin et al. 2010; Weiss et al. 2011; Arai et al. 2009).

In this experiment I evaluated the transmission of the respiratory endophenotype, resulting by the early repeated cross fostering manipulation in parental generation, to the first no-manipulated generation. Preliminary studies conducted in my laboratory (not yet published) have shown maternal transmission only, of the respiratory endophenotype. For this reason in this experiment I replicated previous data, mating RCF and CT females only, with control males.

MATERIALS AND METHODS

Animals

Animals used in this experiment were the first generation (F1) derived from F0 generation. F0 generation had been subjected to postnatal repeated cross fostering manipulation or control manipulation, as described for the first experiment. F0 RCF and CT females were mated when they were 12 weeks old. Mating protocol consisted in housing 2 females with 1 male in transparent high temperature polysufone cages (26.7 × 20.7 × 14.0 cm) with water and food available ad libitum. Room temperature (21 ± 1 ◦C) and a 12:12 h light dark cycle (lights on at 07.00 p.m.) were kept constant. After 15 days, males were removed and pregnant females were isolated, left in clean cages, and inspected twice a day for live pups. F1 litters were not manipulated at all, with the exception of litters' culling to 8 pups (4males and 4 females) on PND1.

Maternal behavior (F0 dams)

Maternal behavior was observed daily from PND1 to PND7 in two daily sessions (12.00–12.30 and 16.00–16.30) in the facility room. Maternal behavior encompassing: (a) NURSING, including the arched-back and blanket postures; and (b) GP/L: grooming and licking pups was monitored with an instantaneous sampling method (1 sample every 2 min), for a total of 16 sampling

points/session (Shoji and Kato 2006b). The analyses of maternal behaviors were based on the observation of NURSING and GP/L on 15 litters of RCF, 16 litters of CT.

F1 Respiratory response to 6% CO_2 enriched air mixture

The ventilatory response to 6% air-CO2 concentration in twenty days F1 animals was evaluated. The changes in tidal volume (i.e., the volume of air displaced between normal inspiration and expiration, TV) during 6% CO_2-enriched air breathing (CO_2 challenge) were measured in unrestrained plethysmograph (PLY4211, Buxco Electronics, Sharon CT) as already described in previous experiments. Each subject was closed in its chamber for an acclimatization of 40 min. Then, the recording of respiratory parameters started under air condition (baseline) for 20 min. Next, the challenge began with the administration of 6% CO_2 enriched air, followed by a 20 min recovery period (air). A complete session thus lasted 80 min per animal.

Statistical analysis

Maternal behavior

Data were analyzed by repeated measures ANOVA, the independent factor being mothers' early manipulation (2 levels: RCF and CT); and the repeated variable being maternal behaviors during early postnatal days (PND2–7).

Respiratory response to 6% CO_2 enriched air mixture

A one-way ANOVA, the factor being mothers' manipulation (2 levels: RCF and CT), was used to compare the mean percentage of increment of tidal volume from baseline (ΔTV %) during 6% CO_2 exposure.

RESULTS

Maternal behavior (F0 dams)

The analysis of maternal behaviors did not show significant differences in maternal care toward the offspring between F0-RCF dams and F0-CT dams (Figure 18).

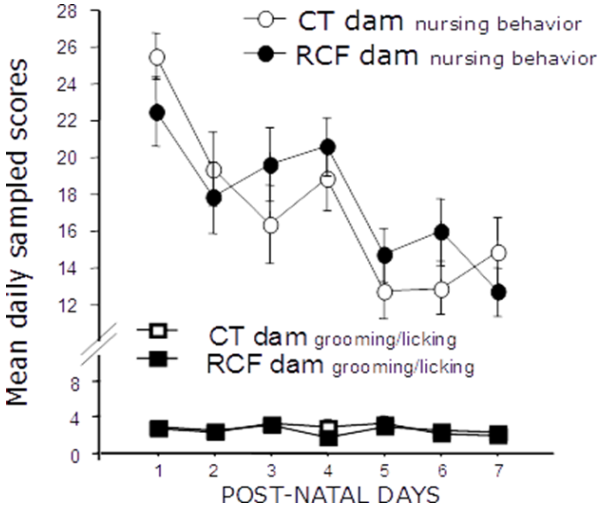


Figure 18. Amount of maternal care (nursing and grooming) displayed by F0 RCF and CT dams toward their offspring during the first week of pups' life.

F1 Respiratory response to 6% CO2 enriched air mixture

The analysis of respiratory response (tidal volume) to 6% CO_2 enriched air mixture in F1 generation is shown in Figure 19. The physiological increase in TV was significantly enhanced among pups (n=11) of RCF dams ($F(1/17) = 6.51$, $p= 0.02$) compared to pups (n=8) of CT dams.

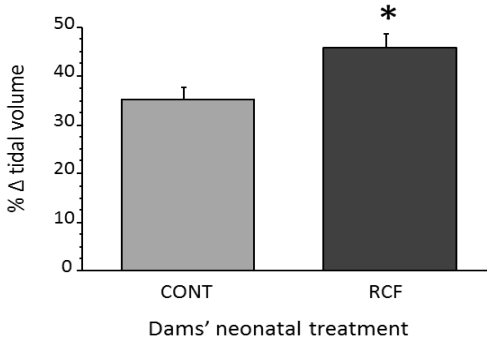


Figure 19. Mean percentage of Tidal Volume changes from baseline (ΔTV %) for young male and female F1 mice from different experimental groups, in response to 6% CO2. Experimental groups: RCF: Repeated Cross-Fostered Dams; CT: Control Dams. *$p < 0.05$

EXPERIMENT 4. DISCUSSION AND CONCLUSIONS

In this experiment I evaluated the transgenerational transmission of the respiratory endophenotype. Pups of first generation/not manipulated, born from RCF and control dams have been exposed to 6% CO2 and their respiratory profile was evaluated. Pups from RCF mothers showed the hyperventilatory response similar to their manipulated mother in comparison with pups born from control mothers. Observing the maternal behavior we were not able to find any differences in maternal cares displayed by RCF and CT mothers towards their offspring. Taken together these results suggest the heritability of the respiratory endophenotype (van Beek & Griez 2000) maybe trough an epigenetic mechanism (Franklin & Mansuy 2010; Weiss et al. 2011).

GENERAL DISCUSSIONS AND CONCLUSIONS

First of all, results reported in this study suggest that the behavioral and physiological phenotypes observed during development and adulthood depend on characteristics and timings of early adversities capable of activate different biological processes. Reasonably, the response of the animal to the early manipulations is different and aimed at maximizing individual fitness: the early environment could exert its programming role during this developmental plastic period, through specific epigenetic modifications. Short, even if repeated, separations from the mother (Handling protocol) induce habituation to a relatively low stressing environment, enhancing the capability of the subject to face new stressful situations. By contrast, the disruption of the infant attachment bond (RCF protocol) is associated to a modification in the respiratory response to high CO_2 in breathing air, an endophenotype these animals share with PD patients.

The disruption of infant-mother bond in RCF animals suggested by the enhanced separation anxiety at 8 days age supports the relation between SAD and PD already reported in literature (Battaglia et al., 2009). In addition the CO_2 reactivity showed by these animals represents a useful tool to study PD in pre-clinical research. Models of PD used in pre-clinical research measure the defensive

behaviors showed by the animals in response to a real aversive stimulus (as described in chapter III) and not spontaneous fear response in the absence of real dangerous situation, as in PD. Not being able to interview the animal, about its symptoms, such as fear of dying or going crazy as in human PD patients, CO_2-hypersensitivity, observed in patients with panic and their unaffected relatives, represents a valid endophenotype to model this disorder in animals. Thus RCF protocol acquires relevance in the field of animal model of panic disorder for its capability to induce this CO_2 hypersensitivity and to measure fear response in absence of real dangerous situation differently from others animal model of panic disorder.

Using the RCF protocol in this study I analyzed possible molecular mechanisms underlying the CO_2 susceptibility (exp 2a), evaluated new pharmacological treatments to cure the hyperventilation (exp 2b), explored the cognitive capabilities of these animals (exp 3) and evaluated the transmission of the respiratory endophenotype to the subsequent generation (exp 4).

Molecular alterations found in RCF animals (experiment 2a) supported the involvement of acid-base balance dysregulation in development of CO_2 hypersensitivity. Indeed RCF animals showed a higher expression in ASIC1 gene that codifies for acid sensing ion channels. These channels are sensitive to lower levels of pH being able to

detect changes in CO_2 concentration in the body and adjust the respiratory function to receive enough O_2 not to compromise biological processes. Molecular investigations in addition revealed alterations in GABAergic transmission in RCF animals supporting the idea of an involvement of this neurotransmitter in the development of PD. RCF animals showed an increased expression of Dbi, an inhibitor of GABAergic transmission. In addition data not presented in this thesis (Cittaro et al., submitted) revealed epigenetic regulation of these genes expression (Asic1, Dbi and others), involved in the respiratory endophenotype showed by RCF animals.

These molecular findings suggest that a possible rescue treatment for PD patients should consist in reducing CO_2 hypersensitivity. Lowering of this increased respiratory response to modest increase in CO_2 could reduce the negative feeling associated to condition, reducing the conditioning potentiality that favor the development of panic disorder, after repeated panic attacks. It is well known, that panic attacks are able to condition behaviors of PD patients. They indeed tend to avoid situations and places similar to those where a panic attack previously occurred. Similarly RCF animals showed, in experiment 3, behavioral conditioning to the situation previously paired with CO_2 (tone exposure). It should be now explored whether RCF animals generalize the conditioned fear,

suggesting how an initial panic attack can evolve into panic disorder in humans (Lissek et al. 2010).

The use of benzodiazepine such as chlordiazepoxide was able to restore the normal respiratory response to CO_2 as well, giving pharmacological validation to RCF model. However, benzodiazepines have several contraindications, especially for chronic treatments and their sedative effect should also be taken into consideration. Even if I only present few data on the effects of chlorogenic acid and amiloride on RCF animals, I think these results are very interesting and need further and deeper evaluation. Both these compounds interacted with the pH sensitive channels (Asics) and their administration was able to restore the respiratory response observed in control animals. In addition chlorogenic acid might acts at epigenetic level being able to modulate DNA methylation. It is possible that CGA administration leads to a decrease in DNA methylation in those genes hyper-methylated in RCF animals (Asic, Dbi and so on). The two different levels of action of CGA make it a fascinating rescue treatment to be better investigated. In addition the use of the polyphenol chlorogenic acid is very interesting because it could be assumed stably in the diet, avoiding unspecific side effects of common pharmacological treatment for PD. Future investigations are aimed at investigating the effect of a diet enriched in chlorogenic acid on the development of CO_2 hypersensitivity in vulnerable

individuals (RCF animals and PD patients). Not only the diet could prevent the development of the hyperventilation, it is also possible that the diet could reduce the endophenotype itself, at adulthood, without severe side effects. The use of the amiloride is very interesting for other reasons. Amiloride was given intranasally and seems to have, due to the route of administration, very immediate effects. This drug could represent a "first-aid self-administrable" treatment for PD patients perceiving in advance the negative sensations of a panic attack. It could be a strategy to help these individuals to face unpleasant situations possibly eliciting a PA, helping them to improve the quality of their life.

Finally RCF model demonstrated a transgenerational transmission of the respiratory endophenotype (experiment 4) supporting the hypothesis of gene-enviroment interplay role to predisposition to panic disorder (Spatola et al., 2011). The epigenetic mechanisms responsible for this trans-generational transmission are under investigation as well as possible strategies to prevent this phenomenon.

In conclusion, the Repeated Cross-Fostering protocol seems a valid mouse model of Panic Disorder in humans: RCF mice show typical features of this disorder such as separation anxiety during childhood, CO_2 hypersensitivity and CO_2 conditioned and avoidance

behaviors. Acid sensing ion channels are interesting molecular markers which can be used as new targets for pharmacological treatments and can help to explain hyper-responsiveness to CO_2 in PD patients as well.

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Appendix A

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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Early handling and repeated cross-fostering have opposite effect on mouse emotionality

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Early life events have a crucial role in programming the individual phenotype and exposure to traumatic experiences during infancy can increase later risk for a variety of neuropsychiatric conditions, including mood and anxiety disorders. Animal models of postnatal stress have been developed in rodents to explore molecular mechanisms responsible for the observed short and long lasting neurobiological effects of such manipulations. The main aim of this study was to compare the behavioral and hormonal phenotype of young and adult animals exposed to different postnatal treatments. Outbred mice were exposed to (i) the classical Handling protocol (H: 15 min-day of separation from the mother from day 1 to 14 of life) or to (ii) a Repeated Cross-Fostering protocol (RCF: adoption of litters from day 1 to 4 of life by different dams). Handled mice received more maternal care in infancy and showed the already described reduced emotionality at adulthood. Repeated cross fostered animals did not differ for maternal care received, but showed enhanced sensitivity to separation from the mother in infancy and altered respiratory response to 6% CO_2 in breathing air in comparison with controls. Abnormal respiratory responses to hypercapnia are commonly found among humans with panic disorders (PD), and point to RCF-induced instability of the early environment as a valid developmental model for PD. The comparisons between short- and long-term effects of postnatal handling vs. RCF indicate that different types of early adversities are associated with different behavioral profiles, and evoke psychopathologies that can be distinguished according to the neurobiological systems disrupted by early-life manipulations.

Keywords: early adversities, mice, maternal behavior, HPA axis, respiratory response to hypercapnia, panic disorder, attachment behavior

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Introduction

The developmental programming hypothesis suggests that the early environment, whether by nutritional, hormonal or behavioral processes, can give rise to persistent modifications of the adult phenotype. In particular, when facing a challenging environment, epigenetic modifications may occur that modify the behavioral, physiological, hormonal and neurobiological profile of the developing individual, to optimize its future coping strategies (Bock et al., 2014). Several studies

in rodents have investigated the effects of a challenging environment, experimentally altering the external or internal pup's milieu, and various postnatal manipulations, differing for severity, time and duration schedules have been applied to developing animals. In the majority of studies (but see also Moles et al., 2004b, 2008), pups were directly stressed exposing them to low temperature, poor mothering, saline injection, unfamiliar odors and others (Oddi et al., 2015). The most common manipulation applied to developing rodents consisted in exposing young animals to daily sessions of separation from the mother during the first 1–2 weeks of life (Pryce and Feldon, 2003). Maternal separation is adversative and pups search for the mother by emitting calls and by seeking olfactory and thermal cues of her presence. This indicates the establishment of an attachment bond between the infant and the mother in the first 2 weeks of life, with signs of distress (e.g., ultrasonic vocalizations (USVs)) following maternal separation that are already detectable in the first few postnatal days (PND0). According to the duration of the stress sessions and the age of pups, different aversive experiences were recruited: pups experienced body temperature loss, starvation, absence of familiar and presence of new odors, absence or excess of tactile stimulation and so on. Moreover, according to the duration of separation sessions, different effects have been observed in the mother and, during development and at adulthood, in the offspring. The hypothalamus-pituitary-adrenal (HPA) axis functioning is greatly affected by these early separation sessions, with opposite behavioral and hormonal responses to stress observed in adult age, according to the duration of the separation events (Anisman et al., 1998; Nishi et al., 2013; but see also Faturi et al., 2010). In many studies pups, repeatedly subjected to long separations from the mother, showed depression- and anxiety-like behaviors in adulthood (Newport et al., 2002; Daniels et al., 2004; Lee et al., 2007; Ryu et al., 2009).

Rather than repeated separations, unpredictability of the early environment may represent a stressful condition for pups. Repeated cross-fostering (RCF) has been used in mice as a postnatal manipulation to model human early environmental instability, a risk factor for internalizing disorders (including separation anxiety disorder—SAD-, panic disorder—PD- and CO_2 hypersensitivity, Kendler et al., 1992; Forman and Davies, 2003; Battaglia et al., 2009). Even though animal models are not expected to reproduce clinical disorders exactly, a translational model of PD should allow to differentiate panic attack (PA) from fear, on the basis of respiratory symptoms (over-reaction to hypercapnia) and lack of increments in stress hormones (Schenberg et al., 2014). Cross-fostering is a routine procedure used in many laboratories that consists in giving pups to a lactating female different from the biological mother, usually within 24–48 h from birth (Oddi et al., 2015). RCF consists in repeating the same procedure every day for the first 4 days of life. Changes in maternal (olfactory, gustatory, tactile, thermal, etc.) cues connected with the RCF procedure may disrupt the associative learning process that is necessary for establishment of the attachment bond in the developing infant (Landers and Sullivan, 2012).

The temporary separation from the mother, or the absence/malfunctioning of the attachment bond (RCF protocol) may act on different molecular system and differently affect the development of emotionality and vulnerability to specific psychopathologies. For example, it is known that separation protocols in rodents strongly affect the HPA axis, whereas corticosterone baseline levels are not affected by the RCF protocol, at least in young animals (D'Amato et al., 2011). Consistent with these data, depression and anxiety disorders, as modeled by separation protocols, are usually associated with alterations in HPA axis functioning, whereas PD, modeled by RCF, is not, at least during the first phases of illness (Klein, 1993).

Here, we evaluated the short- and long-term behavioral effects of two different manipulations of the early environment. In one case pups experienced short separations (Handling) from the mother, which interferes with continuity of the bond; in the other case, pups experienced the Repeated Cross-Fostering procedure, which is aimed at interfering with bond formation. Some data suggest that handled pups should show reduced behavioral and hormonal response to stress at adulthood (Meaney et al., 1996), whereas RCF mice should develop deficit in the attachment and reward system, together with hyper-responsiveness to CO_2 in inhaled air (Oddi et al., 2015). However, the effects of maternal separation in rodents-mice especially-yield little agreement among laboratories and strains (see for example Millstein and Holmes, 2007).

To help resolving these issues, we analyzed the specificities of the RCF vs. Handling protocols effects on behavioral readouts and on the panic-related respiratory responses to CO_2 among outbred strains in the same laboratory. Different response to these manipulations would support the relative selectivity of behavioral and molecular mechanisms involved in response to different types of adversities.

Methods

Animals

NMRI outbred mice (Harlan, Italy) were used in all experiments. Mice were mated when they were 12 weeks old. Mating protocol consisted in housing 2 females with 1 male in transparent high temperature polysufone cages (26.7 × 20.7 × 14.0 cm) with water and food available *ad libitum*. Room temperature (21 ± 1°C) and a 12:12 h light dark cycle (lights on at 07.00 p.m.) were kept constant. After 15 days, males were removed and pregnant females were isolated, left in clean cages, and inspected twice a day for live pups.

All animal used procedures were in strict accordance with standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609/EEC) and the Italian legislation on animal experimentation (Decreto L.vo 116/92).

Experimental Manipulations

On PND1 litters were culled to 8 pups (4 males and 4 females) and assigned to handling (H) or repeated cross-fostering (RCF) procedure.

Handling

According to the well-validated paradigm called "handling" (e.g., Pryce et al., 2005), pups were briefly handled and separated from the dam for 15 min daily. This procedure took place from PND1 to PND14 between 9:30 and 11:00 am. Control litters (N-H), once completed the culling procedure, were left undisturbed for the first 2 weeks of life.

Repeated Cross-Fostering

After having spent the first postnatal day (PND0) with the biological mother, on PND1 culled litters were assigned to experimental Repeated Cross Fostering or control (N-RCF) treatment. Differently from the "classical" cross-fostering procedures (Bartolomucci et al., 2004), RCF pups changed caregiver every 24 h: 4 times in the PND1-PND4 time interval by following a rotation scheme, each dam shifted to 4 different litters and each litter was shifted to 4 different dams (see also **Figure 1**). The daily procedure consisted of first removing the mother from the cage, then removing its entire litter, and immediately introducing this litter into the home-cage of a different dam whose pups had just been removed. The RCF pups were then semi-covered with the home-cage bedding of the adoptive mother, which was then reintroduced in the cage and left with this litter for the next 24 h. The entire procedure lasted about 30 s and took place every day between 10.30 and 11.00 a.m. This was repeated daily, four times until reaching the fourth adoptive mother, with which pups were left until weaning (PND0: biological mother, PND1-PND4: adoptive mother 1–4, PND4-PND28: fourth adoptive mother- **Figure 1**). Adoptive dams were lactating females with pups of the same age as fostered litters. Control litters (N-RCF) were picked up daily and reintroduced in their home-cage, covered with home-cage bedding and had their biological mothers returned within 30 s; this procedure took place from PND1 to PND4 in order to control the possible effect of manipulation necessarily required by RCF procedure.

A total of four experimental groups resulted from the early manipulations: handled and their controls (H and N-H), RCF and their controls (RCF and N-RCF). Animals were weaned when 28 days old, and then separated by sex and left in cage with littermates. Only male mice were tested from weaning onwards.

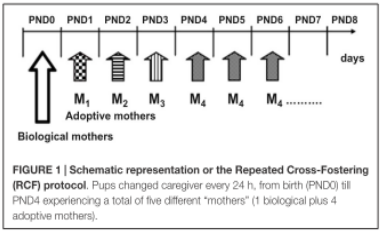


FIGURE 1 | Schematic representation or the Repeated Cross-Fostering (RCF) protocol. Pups changed caregiver every 24 h, from birth (PND0) till PND4 experiencing a total of five different "mothers" (1 biological plus 4 adoptive mothers).

Short and Long-Term Effects of Postnatal Manipulations

The effects of H and RCF on offspring were compared according to eight different physiological, molecular, and behavioral parameters collected during development and adulthood. Body weight (1) was measured in infancy (PND8) and adulthood (PND90). Maternal behavior (2) was observed during the first week of life to exclude the action of poor nurturing on offspring's responses. USVs (3) in response to isolation (PND8), and sociability and social preference (4) were measured before (PND28) and after weaning (PND35), respectively. Adult males (PND75–90) were also tested for behavioral emotionality (5), HPA functionality as indicated by corticosterone response to stress (6) and hippocampal mRNA levels of the glucocorticoid and mineralocorticoid receptors (7). In addition, respiratory responses (8) to a 6% CO_2-enriched air mixture were evaluated in adult animals. No more than 2 animals per litter were tested on the same task.

Behavioral Measures on Infant-Mother Bond

Maternal Behavior

Maternal behavior was observed daily from PND2 to PND7 by an observer unaware of the litter's manipulation (H, N-H, RCF and N-RCF) in two daily sessions (12.00–12.30 and 16.00–16.30) in the facility room. The first daily session took place at least 1 h after the cross fostering/maternal separation procedures, in order to facilitate the dams' acclimatization. Maternal behavior encompassing: (a) NURSING, including the arched-back and blanket postures; and (b) GP/L: grooming and licking pups was monitored with an instantaneous sampling method (1 sample every 2 min), for a total of 16 sampling points/session (Shoji and Kato, 2006). The analyzes of maternal behaviors were based on the observation of NURSING and GP/L on 15 litters of RCF, 16 litters of N-RCF, 10 litters of H and 11 litters of N-H pups.

Data were analyzed by two way ANOVAs, the factors being (1) manipulation (4 levels: H, N-H, RCF and N-RCF); and (2) developmental age (2 levels repeated measure: PND2–4 and PND 5–7). The observation period was split into 2 time-windows: PND2–4 (daily cross—fostering period) and PND5–7 (definitive adoption for the RCF group) to control for the immediate effect of the RCF protocol.

Ultrasonic Vocalizations

Pups' behavior was evaluated at PND8, by measuring USVs emitted during 5 min of isolation (Moles et al., 2004a; Cryan and Holmes, 2005). Experimental animals were transferred in their home cage to the experimental rooms for USVs assessment, 1 h prior to testing. After this period of acclimatization, the mother was removed and transferred into a clean cage, while pups were left in the home cage standing on a warm plate set at the temperature of 35°C to prevent cooling. One randomly chosen pup was placed into a beaker, containing own-cage bedding and the vocalizations were recorded. No more than 1 pup/litter was employed. USVs were recorded using an UltraSoundGate Condenser Microphone (CM16, Avisoft

Bioacoustics, Berlin, Germany) lowered 1 cm above the top of the isolation beaker containing the pup. The microphone was sensitive to frequencies of 15–180 kHz with a flat frequency response (± 6 dB) between 25–140 kHz. It was connected via a UltraSoundGate USB Audio device to a personal computer, where acoustic data were recorded as wav files at 250,000 Hz in 16 bit format. Sound files were transferred to SasLab Pro (version 4.40; Avisoft Bioacoutics) for sonographic analysis and a fast Fourier transformation was conducted (512 FFT-length, 100% frame, Hamming window and 75% time window overlap). Further details on this procedure, the device used and the analysis of data can be found in D'Amato et al. (2011).

A one-way ANOVA, the factor being manipulation (4 levels: H, N-H, RCF and N-RCF), was used to compare the total number of vocalizations emitted by pups during the 5 min of isolation session. The sex of the pup was not considered as we never observed a male-female difference in 8-day old pups' ultrasonic emission (D'Amato et al., 2011; Cinque et al., 2012).

Sociability and Social Preference

Sociability and social preferences were evaluated in male mice at PND28 (before weaning), and at PND35 (1 week after weaning), respectively, in different animals (Cinque et al., 2012). Measures of interest in an unknown conspecific vs. an unknown object were employed as indicators of sociability. Indices of social preference were acquired to test whether H and RCF affected siblings' recognition. The social preference test was performed 1 week after weaning to reduce the impact of the mother on sibling's olfactory cues. Both tests used a gray Plexiglas rectangular box (60 × 40 × 24 cm) consisting of three interconnected chambers. Each of the two lateral compartments contained a circular Plexiglas cylinder (diameter: 8 cm, height: 15 cm) with multiple holes (diameter: 1.2 cm) yielding olfactory cues. Mouse behavior was recorded by a video-camera and analyzed with the SMART video-tracking system. Each subject mouse was placed inside the central compartment and explored the apparatus for a 10-min habituation period, with the doors on either side left open. During the 10 min social session of the test, the tested animal was exposed to an unfamiliar animal and a white object of similar size (Sociability test), or was simultaneously exposed to an unfamiliar (same strain, age and treatment) and a familiar male mouse (sibling) (Social preference test). Each partner and object was confined in one of the two Plexiglas cylinder located in the lateral compartments, for 10 min. The position of stimuli (partners and objects) in the apparatus was equally distributed between the left and the right compartment. Collected measures included time spent: (a) in each one of the three compartment; and (b) in the immediate proximity (2 cm: Time Close) of each cylinders.

One-way ANOVAs, the factor being manipulation (4 levels: H, N-H, RCF and N-RCF), were conducted on a Sociability and Social Preference index that measured the percentage of time spent close to unfamiliar partners (Time Close unfamiliar/(Total Time close to both cylinders) × 100).

Emotionality

Male mice were tested in the elevated plus maze at PND75–90 for emotionality. No more than 2 males × litter for group were sampled. The elevated plus maze consisted of 2 open (5 cm wide, 30 cm long) and 2 closed arms (5 cm wide, 30 cm long, enclosed by a wall of 14 cm in height) arranged in a plus configuration, joined by a central square of 5 cm × 5 cm. The apparatus was made of opaque Plexiglas and kept on a base 40 cm above the floor. All animals were exposed to a test of a standard 5-min duration. At the beginning of the test each mouse was placed individually in the center of the maze, with the head facing an open-arm (the same for all mice). All tests were conducted between 13:00 h and 15:00 h and recorded by a video camera. The animals were initially accustomed to the experimental room for at least 1 h before the experiment.

The time spent in the different arms of the apparatus was evaluated by automatic software analysis (SMART, PanLab) and the percentage of time spent in open arms was used as behavioral index of low emotionality (100 × Time Open/(Time Open + Time Closed) in a one-way ANOVA, the factor being the postnatal manipulation (4 levels: H, N-H, RCF and N-RCF).

HPA Axis Functionality

Corticosterone response to novelty

Corticosterone levels were measured in H, N-H, RCF and N-RCF male mice, at different time intervals from novelty exposure. Apart from the postnatal manipulation, these animals have never been exposed to other experimental procedures. Novelty consisted in exposing the animals to a novel environment: each mouse was removed from its home cage and placed in the center of an open circular arena (60 cm diameter) for 20 min. Trunk blood samples were collected at different time intervals after the novelty test. One group of animals for each treatment was not manipulated at all and blood collected represented the group baseline (Time 0′). Immediately at the end of the novelty exposure, 50% of mice were sacrificed to measure the stress response to the open arena (Time 20′), while the other 50% was reintroduced in their home cages and blood was collected after 40 min (Time 60′). After blood centrifugation (20 min, 4°C, 4000 rpm), serum samples were stored at −25°C until assay were conducted. Corticosterone levels were measured using commercially available EIA kits (Enzo Life Science, sensitivity 27.0 pg/mL). All corticosterone measures were carried out in duplicate. The mean serum corticosterone levels of mice were compared by a two-way ANOVA, the factors being (1) manipulation (4 levels: H, N-H, RCF and N-RCF); and (2) time intervals (3 levels: time 0, 20′ and 60′).

Hippocampal mRNA Analyses

GR and MR expression (Real-time PCR analysis)

Brains of adult male mice of the Time 0 groups for corticosterone essays were rapidly removed and placed onto an ice-cooled metal plate. Hippocampi were dissected and samples were immediately frozen on dry ice and stored at −80°C. RNA was extracted from homogenized hippocampi (N = 5/7 for each experimental group) using a Total RNA purification kit (Norgen Biotek, Thorold, ON, Canada) following the instructions of

manufacturer. RNA quantity was determined by absorbance at 260 nm using a NanoDrop UV-VIS spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

RNA was reverse-transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Paisley, UK) according to the manufacturer's instructions. Equal amounts of cDNA were then subjected to real-time PCR analysis with an Applied Biosystems 7900HT thermal cycler, using the SensiMix SYBR Kit (Bioline, London, UK) and specific primers, each at a final concentration of 200 nM (Nr3c1: sense: CCTCCCAAACTCTGCCTGG, antisense AGCACAAA GGTAATTGTGCTGT; Nr3c2: sense CGGCTTCAGCTGACC TTTGA, antisense TGGCTCTTGAGGCCATCTTT; Actb: sense CAATGAGCTGCGTGTGGC, antisense GTACATGGCTGGG GTGTTGA). Each measurement was performed in quadruplicate and each experiment in triplicate. The expression data were normalized using the expression values of Actb gene. Amplification efficiency for each primer pair was determined by amplification of a linear standard curve (from 0.1 ng to 20 ng) of total cDNA as assessed by A260 spectrophotometry. Standard curves displayed good linearity and amplification efficiency for all primer pairs.

Expression data were presented, after normalization, as the fold-changes over the expression values of control samples (H vs. N-H and RCF vs. N-RCF). Independent *t*-tests between treated and control delta Cts (H vs. N-H and RCF vs. N-RCF) were used to evaluate significant differences in gene expression.

Respiratory Response to CO_2 Enriched Environment

We evaluated the ventilatory responses to 6% air-CO_2 concentration in adult H, N-H, RCF and N-RCF animals. Since the RCF procedure evokes enhanced respiratory responsiveness to hypercapnia, an endophenotype of human PD (D'Amato et al., 2011; Battaglia et al., 2014), we were interested in assessing to what extent H may alter CO_2 sensitivity. We measured the changes in tidal volume (i.e., the volume of air displaced between normal inspiration and expiration, TV) during 6% CO_2-enriched air breathing (CO_2 challenge) in an unrestrained plethysmograph (PLY4211, Buxco Electronics, Sharon CT) carrying two separate Plexiglass chambers of 450 ml. This allows for the parallel assessment of 2 animals/session. Before any recording, each subject was closed in its chamber for an acclimatization of 40 min. Then, the recording of respiratory parameters started under air condition (baseline) for 20 min. Next, the challenge began with the administration of 6% CO_2 enriched air, followed by a 20 min recovery period (air). A complete session thus lasted 80 min per animal. In a previous study (D'Amato et al., 2011) the complete procedure consisted in two subsequent challenges per animal with 6% CO_2 enriched air, but since the correlation of the respiratory responses between the two challenges was found very high (>0.80), in the present study we relied on 1 challenge only.

A one way ANOVA, the factor being manipulation (4 levels: H, N-H, RCF and N-RCF), was used to compare the mean percentage of increment of tidal volume from baseline (ΔTV%) during 6% CO_2 exposure (D'Amato et al., 2011).

TABLE 1 | Mean body weight (gr.) of animals exposed to different postnatal manipulations, after USVs (PND8) and at adulthood, after respiratory parameters evaluation (PND90).

	PND8	PND90
H	6.52 + 0.08	45.70 + 1.52
N-H	6.67 + 0.29	49.98 + 2.09
RCF	6.57 + 0.10	50.20 + 1.13
N-RCF	6.23 + 0.15	46.24 + 2.21
One-way ANOVA	$F_{(3/23)} = 1.27$	$F_{(3/30)} = 1.79$
N	6–8	6–11

Results

Body Weight

No effect of postnatal manipulation on body weight at PND8 and PND90 emerged (**Table 1**).

Behavioral Measures on Infant-Mother Interactions

Maternal Behavior

The total amount of nursing and grooming behavior received by pups exposed to different manipulations is shown in **Figure 2**. The statistical analysis revealed that different manipulations did not affect the total amount of nursing and grooming/licking received by pups during the first week of life (NP: $F_{(3/48)} = 1.00$, ns; GP/L: $F_{(3/48)} = 1.67$, ns) but, while NURSING decreased during the first week of life ($F_{(1/48)} = 14.27$, $p < 0.001$), pups' grooming and licking remained relatively stable ($F_{(1/48)} = 1.41$, ns) across all 4 experimental groups. The interaction between postnatal manipulation and time reached statistical significance only for NURSING (NP: $F_{(3/48)} = 3.80$, $p < 0.02$; GP/L: $F_{(3/48)} = 0.98$, ns). As is clearly depicted in **Figure 2**, H pups received more nursing than all other groups, but only during PND2–4. The amount of nurturing received by both control groups (N-H and N-RCF) was very similar.

Ultrasonic Vocalizations

The response to isolation measured in pup on PND8 is shown in **Figure 3**: the ANOVA indicated a significant difference between groups ($F_{(3/23)} = 4.30$, $p < 0.05$). RCF pups emitted the highest number of USVs in comparison with all other groups during the 5 min session. Again, the 2 control groups (N-H and N-RCF) confirmed similar.

Sociability and Social Preference

Young male mice explored the 3-compartment cage during the habituation sessions and no difference in the time spent in the different chambers was detected (data not shown, available from authors on request). Neither sociability towards unfamiliar partners (**Figure 4A**: $F_{(3/42)} = 0.77$, ns), nor social preference (**Figure 4B**: $F_{(3/47)} = 1.22$, ns) were affected by early manipulations. Considering time spent close to cylinders, more than 50% of this time involved exploration of the unfamiliar mouse and no preference/avoidance of siblings was detected.

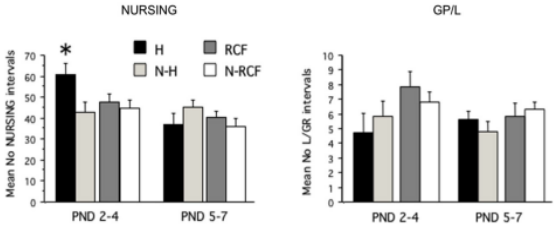


FIGURE 2 | Maternal care received by pups exposed to different postnatal manipulations. Data are presented as mean (+SE) group scores for 3-day intervals (PND2–4 and PND5–7). Experimental groups: H: Handled ($n = 10$); N-H: Non-Handled ($n = 11$); RCF: Repeated Cross-Fostering ($n = 15$); N-RCF: Control ($n = 16$). *$p < 0.05$ in comparison with other groups of the same time interval (Fisher test).

Emotionality

Postnatally-handled adult males showed, as expected, reduced emotionality in the plus maze test (**Figure 5**). The one-way ANOVA indicated a significant treatment effect ($F_{(3/33)} = 4.43$, $p < 0.01$) and *post-hoc* analyzes showed that the effect was explained by pups exposed to H manipulation.

HPA Axis Functionality

Corticosterone Levels After Novelty Exposure

The corticosterone response to a novel situation in the 4 experimental groups is depicted in **Figure 6A**. Mice did not differ for the amount of time spent in the central part of the arena ($F_{(3/45)} = 1.72$, ns) during novelty exposure. All groups showed an increase in serum corticosterone at the end of the novelty test (20 min of open field) and a successive reduction of hormone levels during the 40 min of recovery in the home cage. The two-way ANOVA for repeated measures indicated a significant time effect ($F_{(2/63)} = 31.59$, $p < 0.001$) and no experimental group ($F_{(3/63)} = 1.54$, ns), or group × time ($F_{(6/63)} = 0.76$, ns) effect. However, subsequent Tukey *post hoc* analysis revealed that the increase in corticosterone at the end of the open field exposure (baseline vs. Time 20′) was significantly higher in all groups but not in the group exposed to handling during postnatal life.

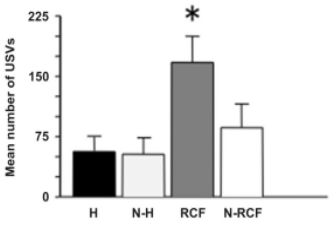


FIGURE 3 | Mean (+SE) number of ultrasonic calls (USVs) emitted by 8-day old pups of different experimental groups, when isolated in their own home-cage bedding for 5 min. Sample sizes: 6–8 per group. Experimental groups: H: Handled; N-H: Non-Handled; RCF: Repeated Cross-Fostering; N-RCF: Control. *$p < 0.05$ in comparison with all other groups (Fisher test).

Hippocampal mRNA Analyses

GR and MR Expression in the Hippocampus

The results of GR and MR gene expression in the hippocampal region, evaluated by real-time PCR, indicated no significant differences between groups, either for GR and MR gene expression (**Figure 6B**). Both GR and MR Delta CTs did not differ either between N and N-H ($t_{(8)} = 0$, and $t_{(8)} = 0$, ns, respectively), or between RCF and N-RCF ($t_{(12)} = 0.28$ and $t_{(12)} = -0.79$, ns, respectively).

Respiratory Response to CO_2 Enriched Environment

Adult male mice responses to 6% CO_2-enriched air are shown in **Figure 7**. The physiological increase in TV was significantly enhanced among RCF subjects ($F_{(3/30)} = 3.64$, $p < 0.05$) compared to all other groups.

Discussion

The aim of this study was to compare the short (i.e., maternal behavior and pup's body weight and USVs) and long lasting (i.e., sociability at weaning and adults' emotionality, glucocorticoids mRNA and respiratory response to 6% CO_2) neurobiological outcomes of two different postnatal manipulations, namely the H (whereby maternal separation is short and repeated) and the RCF (where maternal separation consists of repeated cross-fostering to lactating females other than the biological mother) protocols in NMRI outbred mice. Rodent studies of maternal separation and/or pup isolation in the first weeks of life were typically designed to approximate early-life adversities, to assess their psychobiological impact, and build proxies for human developmental psychopathology. The instability of the early environment induced by the RCF manipulation, represents an early aversive condition that has long-term effects, but does

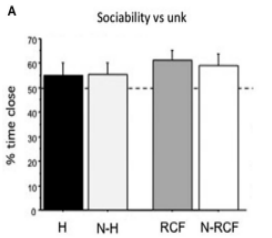


FIGURE 4 | Sociability and Social Preference scores (mean + SE). **(A)** Sociability (preference for conspecific vs. object) and **(B)** Social preference (preference for conspecific vs. littermate) for unfamiliar male mouse (same strain, age and treatment) of juvenile males tested on PND28 and PND35, respectively. Both indices are calculated as the percentage of time spent close to unfamiliar partners (Time Close unfamiliar/(Total Time close to both cylinders) × 100). Sample sizes: 9–14 per group. Experimental groups: H: Handled; N-H: Non-Handled; RCF: Repeated Cross-Fostering; N-RCF: Control.

not to interfere with the HPA axis functioning. RCF pups were exposed to maternal cues from four different dams, a condition that might have prevented or disrupted infant-mother attachment bond. By studying rats, R. Sullivan found that the somatosensory, olfactory and gustatory stimulation associated with suckling, pups learned to recognize their mother (e.g., Sullivan et al., 2011). It is not clear whether the pups are able to recognize their mother or rather "a mother" as the presence of maternal signature odors (pheromone) has been reported for the rabbit and hypothesized in the mouse (Logan et al., 2012). We suggest that 8–10 day-old pups are able to recognize their own mother/nest environment, but the RCF protocol disrupts this preference. This deficit/malfunctioning of the attachment process, may simulate human separation events, that are risk factors for PD.

Results reported here are summarized in **Table 2**. Two points should be stressed before discussing results: (a) these studies were conducted on outbred mice a condition that can more closely be associated to the human situation; and (b) to facilitate comparisons with the previous, sometimes inconsistent, mouse studies of H (see for example Millstein and Holmes, 2007), and further extend knowledge on the recently-introduced RCF procedure (D'Amato et al., 2011; Ventura et al., 2013; Battaglia et al., 2014), in the table each manipulated group (H and RCF) was compared to the corresponding own control group (N-H and N-RCF animals).

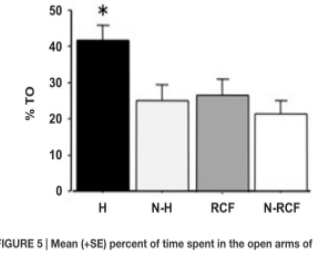


FIGURE 5 | Mean (+SE) percent of time spent in the open arms of an elevated plus maze by adult male mice exposed to different postnatal manipulations. Sample sizes: 9–10 per group. Experimental groups: H: Handled; N-H: Non-Handled; RCF: Repeated Cross-Fostering; N-RCF: Control. *$p < 0.05$ in comparison with other experimental groups (Fisher test).

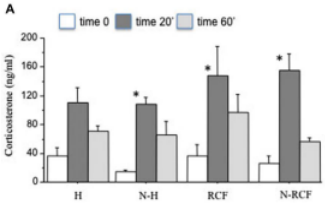
Body Weight

First of all, data show that neither H nor RCF affected body weight during development or in adulthood. This suggests that neither manipulation induced generalized developmental perturbations.

Maternal Cares, Emotionality and Glucocorticoids

Our results confirm that repeated short separation events (Handling) during the first 2 weeks of life promote heightened maternal care and are associated with reduced behavioral and hormonal reactivity to stress (plus maze and restraint stress) in adulthood, confirming data from many laboratories (e.g., Meaney et al., 1996; Schmidt et al., 2003). We were however not able to find, in our adult H mice, the increased expression of hippocampal GRs reported in the literature (Meaney et al., 1985; O'Donnell et al., 1994; Schmidt et al., 2003; George et al., 2013).

The RCF procedure, which implies a strong interference with the infant-mother attachment bond, yielded different short and long-term effects. **Table 2** shows that RCF pups did not receive lower amount of maternal care compared to N-RCF, but responded to 5 min of isolation with a higher amount of distress calls. The fact that the RCF experimental procedure is based on the introduction of to-be-adopted pups into the adoptive dam's home-cage covered with the adoptive mother's bedding, may indeed explain the consistence of maternal cares across biological and adoptive mothers. No effect of RCF treatment on basal corticosterone levels in PND27 offspring and their



B

Fold changes	H vs N-H	RCF vs N-RCF
GR	1.00	0.98
MR	0.98	1.08

FIGURE 6 | HPA axis functionality. (A) Mean (+SE) serum corticosterone levels of male mice from different experimental groups before (Time 0), at the end of novelty (Time 20'), and 40 min after reintroduction in their home cage (Timer 60'). Sample sizes: 6–7 per group. *$p < 0.05$ in comparison with same experimental group, different time interval. **(B)** Fold changes of hippocampal mRNA for Glucocorticoid (GR) and Mineralocorticoid (MR) receptors. Sample sizes: 5–7 per group. Experimental groups: H: Handled; N-H: Non-Handled; RCF: Repeated Cross-Fostering; N-RCF: Control.

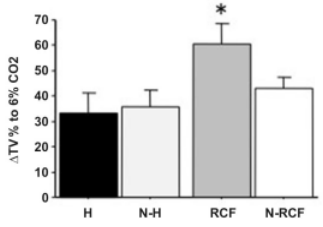


FIGURE 7 | Mean (+SE) percentage of Tidal Volume changes from baseline (ΔTV%) for adult male mice from different experimental groups, in response to 6% CO_2. Sample sizes: 6–11 per group. Experimental groups: H: Handled; N-H: Non-Handled; RCF: Repeated Cross-Fostering; N-RCF: Control. *$p < 0.05$ in comparison with other experimental groups (Fisher test).

mothers was found (D'Amato et al., 2011), in spite of the higher USV response to separation of RCF pups. This suggests that the RCF manipulation may hamper the pups' ability to recognize the scent of their home cage and/or be reassured by being in an olfactory known-environment (D'Amato et al., 2011).

Contrary to handling, RCF protocol did not modify emotionality (plus maze) and hormonal response (corticosterone levels) to stress (**Table 1**). These results are not surprizing considering that differences in emotionality occurring in H adult animals have been explained by the different levels of maternal care received by these animals that would induce, through epigenetic response, changes in brain and behavior that persist until adulthood (Champagne et al., 2003). RCF pups received similar amount of care than controls and were never separated or isolated from a lactating female as H pups did, supporting absence of differences in emotionality between these two groups of animals. In addition H pups were exposed to fluctuation in corticosterone levels in the absence of the mother during the handling procedure, as well as to mother's corticosterone levels changes that concomitantly occurred (D'Amato et al., 1992; Moles et al., 2008). By contrast, RCF pups and mothers were never separated and probably not exposed to such hormonal variations in early life.

Social Preference

To reduce the impact of sexual hormones on sociability and social preferences parameters, we tested mice at weaning. Since both H and RCF may interfere with attachment, they may impact on social behavior (social preference/recognition of siblings) later in development. This was not the case, and neither H, nor RCF treatment affected social behaviors in young male mice. These animals are all interested in conspecifics and the postnatal treatment seems not to affect social motivation in immature mice.

Sensitivity to Carbon Dioxide

As already reported in our previous study, RCF animals showed higher, stable and specific augmentation of tidal volume in response to 6% CO_2-enriched air (D'Amato et al., 2011). This was confirmed here, as it was not seen among H animals, and unrelated to body weight between experimental groups.

TABLE 2 | Summary table reporting results of comparisons between each manipulated and its control group.

Neurobiological/Behavioral indices	H vs. N-H	RCF vs. N-RCF
Maternal behavior received	>	ns
USVs response to isolation (m + f)	ns	>
Sociability (m)	ns	ns
Social preference (m)	ns	ns
Emotionality in the plus maze (m)	<	ns
Corticosterone response to novelty (m)	<	ns
Hippocampal GR and MR expression (m)	ns	ns
Respiratory response to CO_2 adulthood (m)	ns	>
Body weight (m)	ns	ns

H: Handled; N-H: Non Handled; RCF: Repeated Cross-Fostering; N-RCF: Controls for RCF; m: males; f: females.

This hypersensitivity to CO_2 can be turned into a remarkable investigational tool and useful endophenotype, allowing to model PD in the mouse. Animal models of PA in rats and mice are usually based on behavioral observations in classical anxiety tests, using pharmacological treatment (lactate) or electrical stimulation of the dorsal periaqueductal gray (dPAG) to exaggerate the response (e.g., Johnson and Shekhar, 2012; Andrews et al., 2014; Canteras and Graeff, 2014). Panic and anxiety disorders are usually discernible in humans on the basis of psychological and physical feelings, respiratory responses, and panic in the absence of real dangers that are largely independent of HPA axis activation (Abelson et al., 2007). The altered respiratory response to CO_2 that characterized PD patients, as well as unaffected relatives, might represent a shared endophenotype that allow to investigate, in the mouse, the gene × environment interplay, the molecular mechanisms and functional alterations that characterize the psychopathology. The experience of early adversities that in humans influence—in addition to genetic factors-the risk for PD in adulthood, seem to be convincingly modeled by the RCF protocol in the mouse.

A methodological point is worth mentioning concerning the use of repeated cross-fostering as an early aversive event. Cross-fostering is a routine procedure in many laboratories and consists in giving pups to a lactating female different from the biological mother, usually within 24–48 h from birth (Oddi et al., 2015). This experimental protocol is used in several studies to improve maternal cares, to separate the effects of prenatal from postnatal treatments, to evaluate the effects of different amount of maternal care on offspring behavioral and physiological development, and to ameliorate sanitary condition in animal house by removing colony infections (see for example Barros et al., 2006; Buxbaum et al., 2011; Schmauss et al., 2014; Wattez et al., 2014). Few studies have investigated the effects of this procedure *per se* on later development. However, earlier adoptions exert smaller impacts than later ones, stressing that changes in mother's cues are perceived by neonates and older may probably better detect differences than jounger pups, (Barbazanges et al., 1996; Hickman and Swan, 2011). These studies indicate that even if essential cares are provided by adoptive mothers, by substituting the biological mother in rodents one may affect body weight, emotional behavior and nociception in the offspring, and induce consistent metabolic and cardiovascular changes (Bartolomucci et al., 2004; Malkesman et al., 2008; Dickinson et al., 2009; Leussis and Heinrichs, 2009; Lu et al., 2009; Matthews et al., 2011).

Conclusions

Generally speaking, results reported in this study, comparing in the same laboratory and in the same outbred mouse strain the short and long term effects of two different early treatments, suggest that the observed phenotypes depend on characteristics and timings of early adversities that might activate different biological processes. Reasonably, the response of the animal to the early manipulations is different and aimed at maximizing individual fitness: the early environment could exert its programming role during this developmental plastic period through specific epigenetic modifications. Short, even if repeated, separations from the mother (Handling protocol) induce habituation to a relatively low stressing environment, enhancing the capability of the subject to face new stressful situations. By contrast, the disruption of the infant attachment bond (RCF protocol) induces a modification in the respiratory response to high CO_2 in breathing air, an endophenotype these animals shared with PD patients. The molecular mechanisms responsible for this increased response to acidosis in RCF mice are under investigation, as well as the impact of this early adversity on different genetic background, using genetic inbred lines of mice. This information will provide additional validity to this animal model of panic that shares etiology and respiratory endophenotype with the human PD.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.