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**Pre-clinical modelling of psychopathological processes
in Borderline Personality Disorder:
environmental and genetic models in the mouse**

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Introduction

Borderline Personality Disorder (BPD) is a severe and complex behavioral pathology which etiology is thought to result from developmental and/or acquired brain dysfunctions associated with early trauma such as abuse or neglect (Gunderson and Sabo, 1993; van Reekum, 1993; Zanarini, 1997; Zerkowicz et al., 2001). Recent studies have described physiological alterations of brain anatomy, particularly at the level of the prefrontal cortex (van Reekum et al., 1993; van Elst et al., 2001), and abnormalities of the hypothalamo-pituitary-adrenals (HPA) axis, a system responsible for the hormonal response to stress (Grossman et al., 1997; Gunnar and Donzella, 2002; Heim and Nemeroff, 1999). Such alterations may result from a genetic vulnerability predisposing individuals to react more strongly to stressful experiences and increase the impact on brain development. Thus, the interaction between gene and environment appears as a strong factor in the occurrence of BPD symptoms (Horsfall, 1999; Paris, 1998).

Goals

Our research goal is to better understand the influence of environmental and genetic factors in the etiology of BPD using animal models. The aim of our approach is to establish models for early stress in the mouse to mimic as closely as possible some BPD symptoms, with a focus on anxiety and stress-induced analgesia, two prominent features of BPD (Gunderson and Singer, 1975; McCown et al., 1993; Bohus et al., 2000).

The first step of this project is to develop a protocol of maternal separation (MS) in the mouse and assess the impact of such manipulation on adult behavior. This work is performed in close collaboration with the laboratory of Bruce McEwen at Rockefeller University and our common objective is to establish a robust mouse model in both laboratories. The availability of such a model will allow us to perform further behavioral, genetic, molecular and endocrinological studies that will be complementary across labs.

The second step of our project consists in generating transgenic animals overexpressing the stress hormone corticotropin-releasing factor (CRF) in forebrain in an inducible and reversible manner. We will assess the impact of perturbed hormonal homeostasis on behavior at various developmental stages. Then we will combine the CRF genetic mutation with environmental stress by subjecting the CRF-overexpressing transgenic mice to MS. This approach will provide us with a unique means to examine the consequence of the interaction of these factors on adult behavior.

Achievements

The first year of research in the lab (February 2001-February 2002) was dedicated to the establishment of a model of MS in the mouse. In order to obtain a robust model, several MS treatments combined or not with additional stress to increase the overall severity of the manipulation were tested in close collaboration with the lab of Dr McEwen. The effects of the various MS paradigms on adult behavior were evaluated using several behavioral tests that were newly established in our laboratory for that purpose. These tasks included the open field test, the elevated plus maze, the hot-plate and tail flick tests. For each procedure, a particular effort was made to use paradigms and experimental conditions as similar as possible to those used in the lab of Dr McEwen to insure reproducibility across sites. Several adjustments on the protocols and on the design of behavioral tasks were done along these experiments (not described in detail in this report).

It is important to note that for these experiments, due to legal restrictions imposed by the Swiss Authorities for Animal Welfare at the beginning of this project, our initial study was restricted essentially to the Balb/c strain and to a low number of animals. A small group of C57Bl/6J animals

was tested but will not be reported here because too small. Recent approval of a full animal experimentation license has now allowed us to initiate a larger experiment with C57Bl/6J animals.

In parallel to the establishment of the MS model, we worked over the past year on the generation of CRF-overexpressing transgenic mice. We initiated the cloning of a DNA construct carrying the doxycycline-responsive tetO promoter linked to the rat CRF gene. This DNA construct is currently being completed and will soon be microinjected into fertilized eggs.

A) An environmental model for early stress in the mouse

1) Establishment of maternal separation in the mouse

A) MS

A first experiment (April-July 2001) was conducted using a protocol consisting in separating pups from their mother for 3 h per day at the same time daily (predictable **MS**) from post-natal day (PND) 1 to 14. During separation, the pups were kept either at room temperature (21-22°C; MSrt) or at nest temperature (28-30°C; MSwarm). Controls were non-maternally separated (**NMS**) animals reared in normal-husbandry conditions. This experiment was performed on 32 Balb/c (14 males and 18 females) for MSrt, 32 Balb/c (12 males and 20 females) for MSw, and 27 Balb/c (11 males and 16 females) for NMS. Due to a low rate of birth in C57Bl/6J mice, the experiment was performed only on a total of 18 animals and will therefore not be reported upon here.

B) Variations on the MS protocol

Two additional protocols were assayed (August-December 2001) for which the conditions of MS were rendered more stressful in two ways:

- a) MS was made unpredictable by being conducted at different time of the day at room temperature. This paradigm is named MS unpredictable or **MSU** hereafter. MSU: 7 males and 5 females, NMS: 5 males and 10 females.
- b) MSU was combined with an additional stress applied to the lactating mothers during the period of separation (performed at room temperature). Two stressors were used in random order : restraint stress for 20 min or forced swim in cold water (18°C) for 5 min. This protocol is named MS unpredictable-stress or **MSUS** hereafter. MSUS: 8 males and 9 females, NMS: 5 males and 10 females.

The effects of MSU and MSUS were assessed on adult behavior in 3-4 month old mice.

2) Behavioral evaluation of MS in the open field test

A) The open field environment



The open field test was used to assess overall locomotor and exploratory activity as well as neophobia (Denenberg, 1969). Animals were placed in the open field (70 x 70 x 30 cm) for 10 min. Distance travelled (cm), entries and time spent in the center of the open field, and rearings were recorded.

B) Activity / neophobia in MS animals

The results of the first experiment showed that in Balb/c mice, MS has only a minor impact on adult behavior whether performed at room or nest temperature. As illustrated in Figure 1, MSrt and MSw slightly increased the time spent in the center of the open field when compared to NMS in both males and females, suggesting a trend for reduced fear/anxiety. This effect may be explained by increased maternal care during and/or after the period of separation and may be specific to the Balb/c line of mice. In future experiments, maternal scoring will be performed to examine this possibility and experiments will be repeated in C57Bl/6J mice.

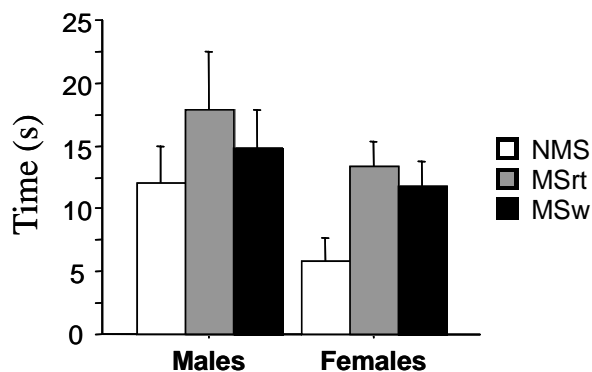


Figure 1: Open field test. Mean \pm SEM time spent in center (sec) for NMS (n = 11 males and 16 females), MSrt (n = 14 males and 18 females) and MSw (n = 12 males and 20 females) mice.

C) Activity / neophobia in MSU and MSUS animals

As illustrated in Figure 2, MSUS females showed a trend for a decrease in the number of entry (Figure 2A) and time spent in the center of the open field (Figure 2B) when compared to NMS females, indicating enhanced fear in this group. By contrast, MSU had no effect with MSU females being indistinguishable from NMS females. In males while MSUS had no effect, MSU induced a trend for increased number of entry (Figure 2A) and time spent in the center (Figure 2B), indicating reduced fear in this group compared to NMS males similarly to that observed with the MS manipulation (Figure 1). Finally, there was no effect of MSUS or MSU on exploratory behaviour (assessed by rearing) in any of the groups (not shown).

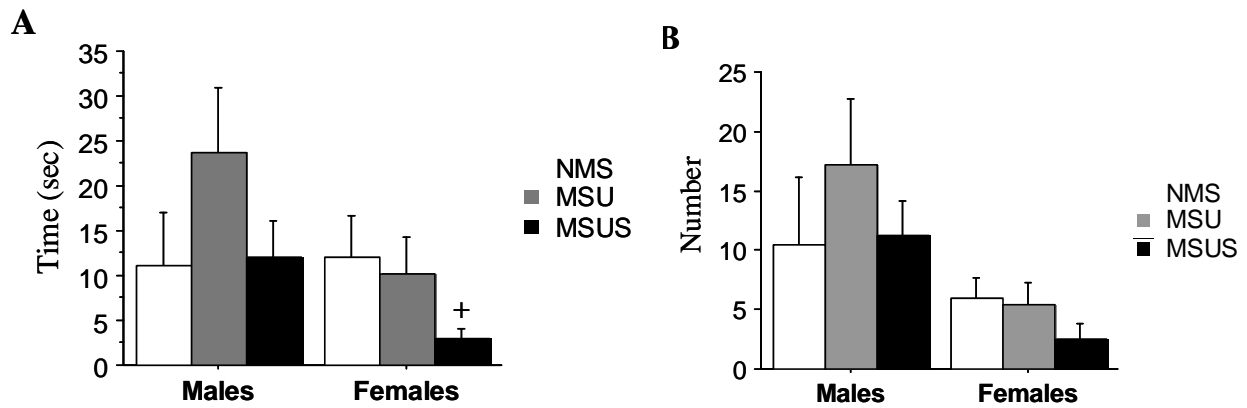


Figure 2: Open field test. **A.** Mean \pm SEM time (sec) spent in the center of the open field for NMS (n = 5 males and 10 females), MSU (n = 7 males and 5 females), and MSUS (n = 8 males and 9 females) mice. **B.** Mean \pm SEM entries in the center of the open field for the same NMS, MSU and MSUS male and female mice. + p=0.08

In summary among the different MS treatments, MSUS appears to be the best to produce increased anxiety/neophobia in an open field and this specifically in females. Again, the requirement for more stressful MS conditions to induce fear in the Balb/c strain may be specific to this strain of in mice from the C57Bl/6J strain, MS alone affects behavior as observed by the lab of Dr McEwen.

3) Behavioral evaluation of MS in the elevated plus maze test

A) *The elevated plus maze*



The elevated plus maze was used to evaluate anxiety (Lister, 1987; Pellow et al., 1985). The maze consists in two open and two closed arms shaped like a plus sign. Animals are placed at the center of the maze facing an open arm and are allowed 5 min of exploration.

B) Anxiety in MS animals

Consistent with that observed in the open field test, MSrt reduced anxiety in males as revealed by a significant increase in entries in the open arms of the elevated plus maze (MSrt versus NMS) (Figure 3), but MSw had no effect. No effect was observed in females.

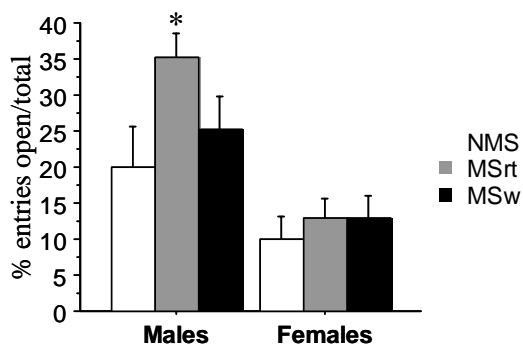


Figure 3: Elevated plus maze test. Anxiety index (% open arm entries / total arm entries) for NMS (n = 11 males and 16 females), MSrt (n = 14 males and 18 females) and MSw (n = 12 males and 20 females) mice.
* p<0.05

C) Anxiety in MSU and MSUS animals

While MSU and MSUS did not affect the level of anxiety in males, MSUS induced an anxiogenic profile in females manifested by a reduced number of entries in the open arms. In contrast, MSU induced an anxiolytic profile as revealed by an increased number of entries in the open arms of the maze compared to NMS females, consistent with the open field results in males (Figure 4). Scoring risk assessment behaviours (Rodgers and Cole, 1993) confirmed this effect and the dissociation between MSUS and MSU on anxiety in females. MSUS females displayed fewer risk assessment behaviors than NMS females, but MSU females showed more of such behaviors than NMS females in unprotected areas of the maze (Figure 5, left panel). No difference was observed in the closed arms (Figure 5, right panel). Here again, the behavior of females subjected to MSU may be explained by increased maternal care during development.

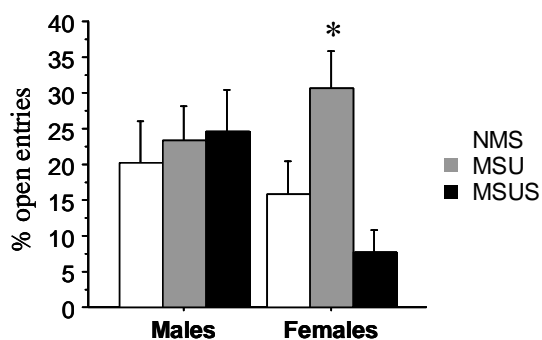


Figure 4: Elevated plus maze test. Anxiety index (% open arm entries / total arm entries) for NMS (n = 5 males and 10 females), MSU (n = 7 males and 5 females), and MSUS (n = 8 males and 9 females) mice.
* p<0.05

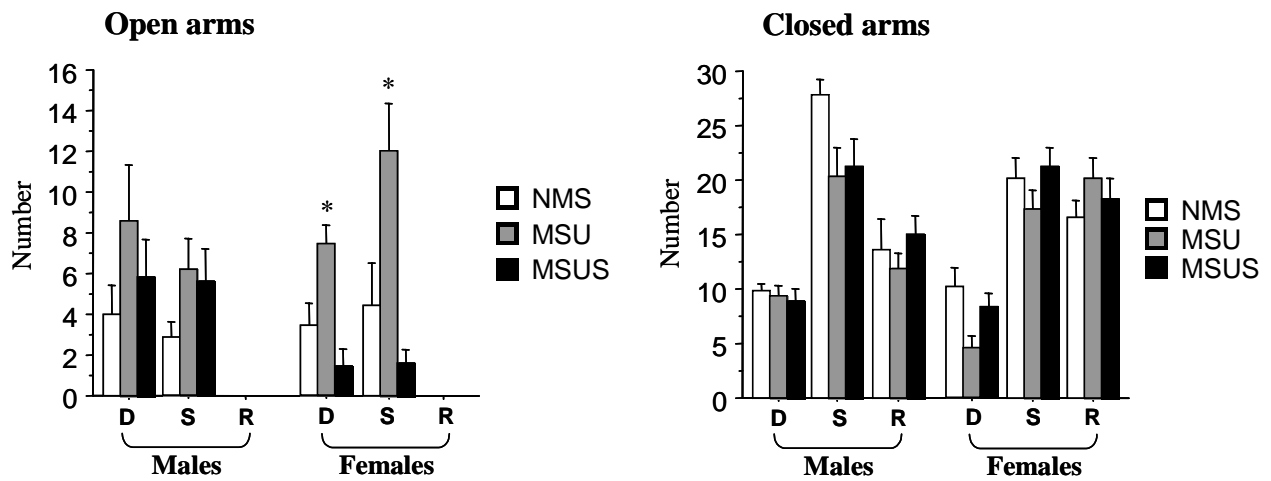


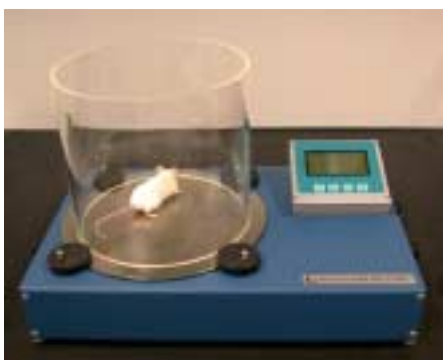
Figure 5: Risk assessment behaviors in the elevated plus maze test. Head dippings (D), stretch attends (S) and rearings (R) monitored when the mice were in the open (left panel) or closed arms (right panel) of the maze. Mean \pm SEM for each behavior for NMS (n = 5 males and 10 females), MSU (n = 7 males and 5 females), and MSUS (n = 8 males and 9 females) mice. *p < 0.05

4) Behavioral evaluation of MS on analgesia tests

A) The hot plate and tail flick tests

The hot plate and tail flick tests were used to assess pain sensitivity and analgesia. In the hot-plate test (Eddy and Leimbach, 1953), a mouse is placed on a heated platform (55°C) and the latency to show either forelimbs licking, hindlimbs licking or jump is scored (see left picture below).

In the tail-flick test (D'Amour and Smith, 1941), a mouse is gently maintained in a towel and its tail is placed above an infra-red beam (see right picture). The latency for tail withdrawal is scored..



Hot-plate test



Tail-flick test

B) Pain sensitivity and analgesia in MSU and MSUS animals

In the hot-plate test, MSUS females showed a significantly lower pain threshold than NMS females (Figure 6A). No change was observed in males. In the tail-flick test however, neither MSU nor MSUS affected basal pain sensitivity (Figure 7). This discrepancy may be explained by the fact that the hot-plate test implicates spinal cord and brain stem reflexes while the tail-flick test involves only reflexes at the spinal level. The sensitivity of these circuits may be different. No measures were performed in MS animals because these tasks were not available at the time the MS experiment was completed.

Stress-induced analgesia was assessed on the hot plate using 5-min forced swim in cold water (18°C) as stress treatment. The hot-plate was chosen for this assay because several protocols are available in the literature (Marek et al., 1992; Panocka et al., 1986). A stress-induced analgesia protocol is currently being established in the lab using the tail flick system and will be used in future experiments.

On the hot-plate test, application of a stress increased pain threshold in MSUS females 10 min after stress, suggesting marked stress-induced analgesia in this group (Figure 6B). A trend towards a similar effect was also observed in males but no significant effect was observed in MSU animals.

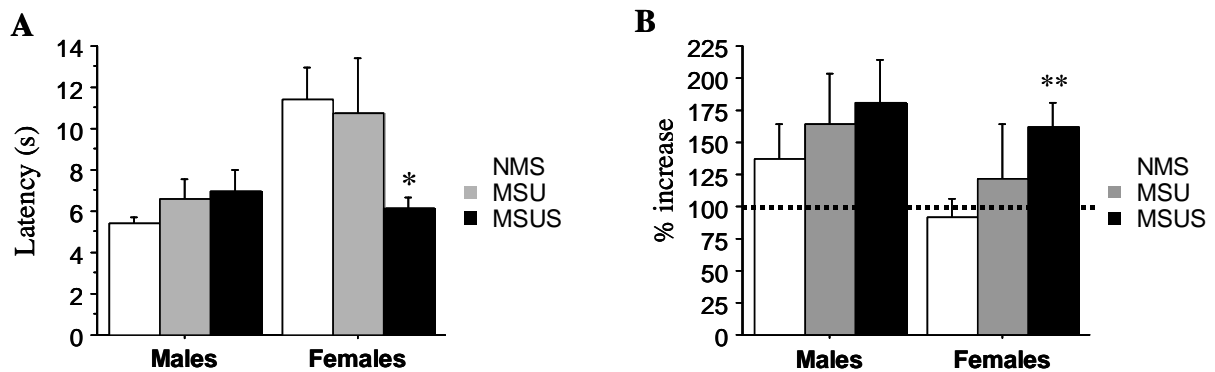


Figure 6: Hot-plate test. **A.** Mean \pm SEM basal pain threshold measured in NMS, MSU and MSUS males and females. **B.** Mean \pm SEM percentage change in pain threshold after stress and relative to basal threshold for NMS (n = 5 males and 10 females), MSU (n = 7 males and 5 females), and MSUS (n = 8 males and 9 females) mice. * p<0.05 ** p<0.01

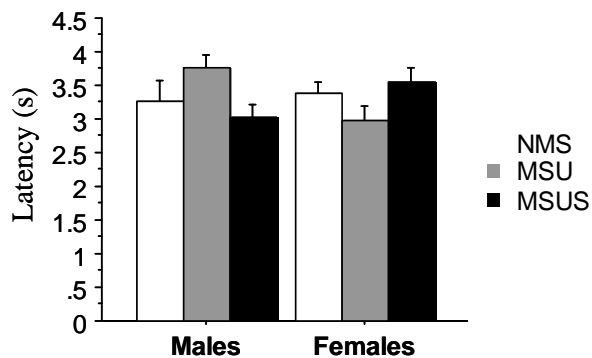


Figure 7: Tail-flick test. Basal pain sensitivity for NMS (n = 5 males and 10 females), MSU (n = 7 males and 5 females), and MSUS (n = 8 males and 9 females) mice.

5) Additional measures

The effect of MSU and MSUS was assessed on growth rate by measuring body weight during development and in adulthood. MSU induced an increase in body weight (10 %, $p < 0.05$; see Figure 8, left panel) at 3 weeks of age. This effect suggests the possibility that mothers may have provided extra maternal care in response to separation. Maternal behavior would need to be scored to verify this hypothesis, such scoring will be performed in the future. In contrast to MSU, MSUS induced a reduction in body weight at weaning (14%, $p < 0.001$; see Figure 8, right panel) but that effect was only transient as adult MSU, MSUS and NMS mice had similar weight.

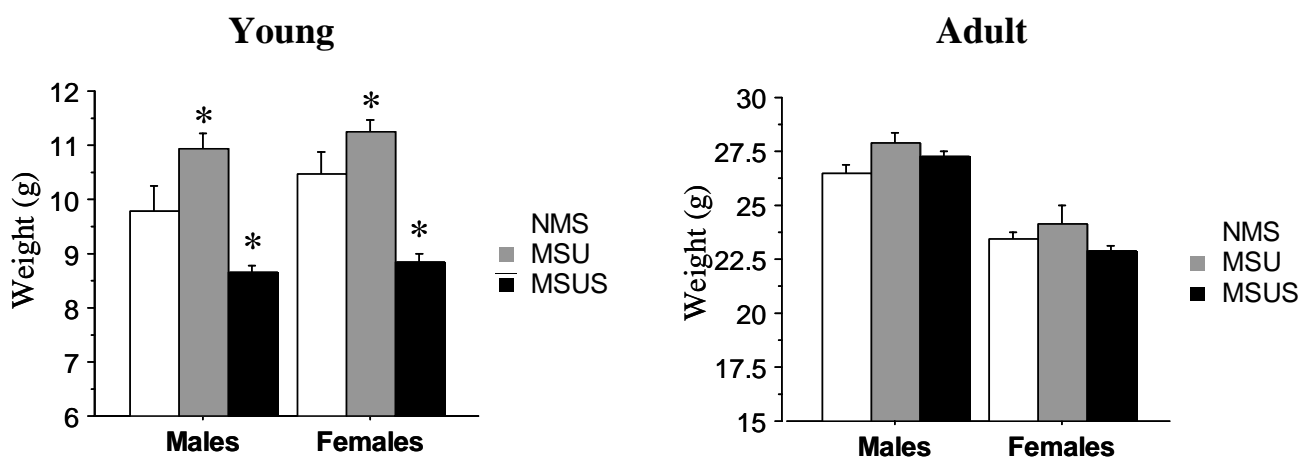


Figure 8: Weight of NMS (n = 5 males and 10 females), MSU (n = 7 males and 5 females), and MSUS (n = 8 males and 9 females) mice at weaning age (3 weeks) and in adulthood (3 months).

* $p < 0.05$

Summary

Although restricted to the Balb/c strain of mice, our initial studies yielded promising results towards the establishment of a mouse model based on MS manipulations common to the lab of Dr McEwen. We observed that unpredictable MS combined with stress to lactating mothers is the most appropriate paradigm to induce fear, anxiety and stress-induced analgesia in female Balb/c mice. Females Balb/c were generally more timid than males Balb/c, an interesting finding that will be yet studied in the C57Bl/6 strain by controlling the estrous cycle of females during behavioral testing.

The establishment of all the settings necessary to perform MS using various experimental paradigms in the mouse and of several behavioral tasks to assess the effect of MS in adult mice will now allow us to pursue our effort to understand the impact of early stress on behavior. We are currently working on a novel MS experiment using C57Bl/6J mice and three treatment groups: NMS (normal-husbandry controls), MS (predictable 3h MS) and MSS (predictable 3h MS + stress of the lactating mothers). To obtain an additional read-out of the effect of MS, specifically on maternal care, maternal scoring is being performed. These measures will allow us to evaluate the effect of the different MS treatments on maternal care and later correlate these data with physiological (weight, hormonal response to stress) and behavioral (anxiety, stress-induced analgesia, learning) parameters.

B) A genetic model for early stress in the mouse

1) Generation of a transgenic mouse model

In parallel to the environmental approach, we are currently working on the generation of a transgenic mouse model overexpressing the stress hormone CRF in forebrain. CRF overexpression will be spatially restricted to forebrain by using the specific CaMKII α promoter and temporally controlled by the tetracycline transactivator (tTA) system. The tTA system will allow us to activate the expression of CRF at specific time during development or adulthood to mimic stressful experience early and/or late in life (Figure 9). In these transgenic mice, the excess of CRF should result in anxiogenic behaviour (Stenzel-Poore et al., 1994; Heinrichs et al., 1997; Holsboer, 1999) but unlike existing transgenic CRF models, its inducibility will provide a unique way to assess the role of CRF at specific times during development.

2) Construct

A DNA construct carrying the rat CRF gene under the control of the tTA-dependent tetO promoter is currently being cloned (Figure 9). This construct will soon be microinjected into fertilized mouse eggs to produce transgenic mice. When available, tetO-CRF transgenic mice will be crossed with transgenic mice expressing tTA under the control of the CaMKII α promoter (Mayford et al., 96) to produce double mutant animals (Figure 9). In these animals, CRF expression will be activated by tTA and suppressed by doxycycline administered in the drinking water in a reversible manner (Figure 9).

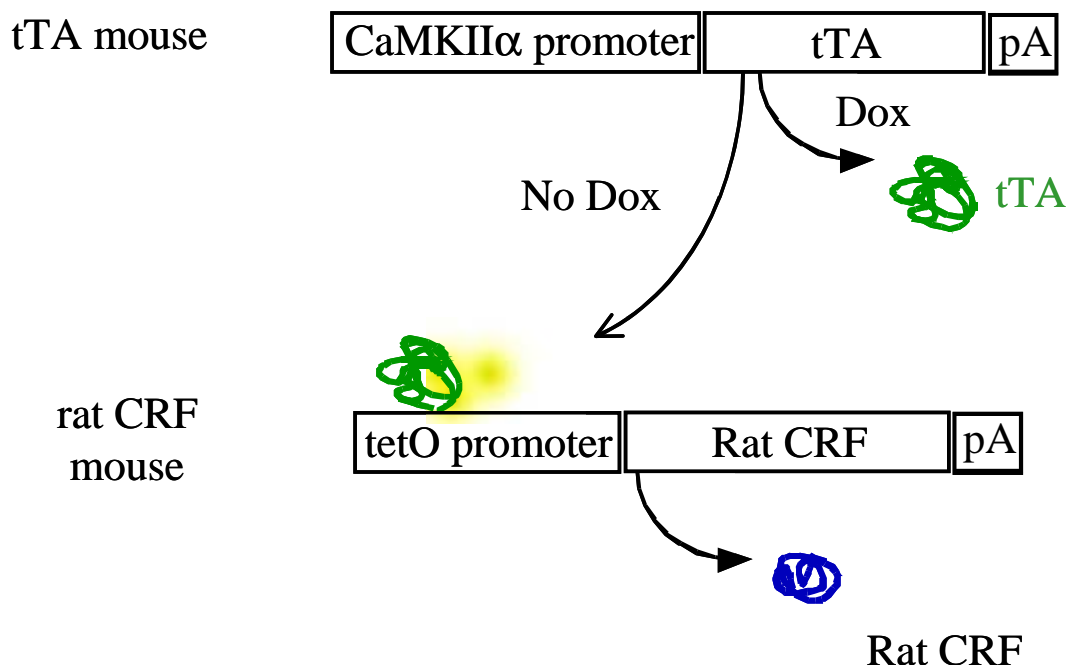


Figure 9: Inducible and reversible CRF-overexpression in the mouse brain. Crossing of tTA expressing transgenic mice with mice harbouring the rat CRF gene under the control of the tetO promoter will yield double mutant mice. In these animals, tTA will activate CRF gene expression. Administration of doxycycline (dox) will suppress this expression. pA = polyadenylation site.

OUTLOOK

Maternal separation

In the coming year, we will firmly establish the model of maternal separation to obtain robust, stable and reproducible behavioral changes in adult animals. Four types of manipulations are and will be assayed in C57Bl/6J mice for that purpose a) MS, b) MSU c) predictable MS combined with additional stress to the lactating mothers (MSS) and d) MSUS. For these experiments, maternal care is being scored before and after separation (according to Bruce McEwen's laboratory protocol) and a full behavioral characterization will later be performed using the open field, elevated plus maze, hot-plate and tail flick tests. Molecular characterization will also be carried out to examine the levels of CRF in the separated animals. For this, *in situ* hybridization will be performed using a rat CRF cDNA probe. Additional probes currently being developed in the lab of Dr McEwen after DNA microarray analyses will be used when available.

Transgenic mice

The transgenic CRF construct will be completed and microinjected into fertilised mouse eggs. The resulting progeny will be screened and founder animals will be backcrossed to C57Bl/6J mice to establish independent lines of transgenic mice. The level and pattern of CRF transgene expression will be examined in each line and the most appropriate line will be used for further experiments.

Development of new tests and assays

Additional behavioural tests including the fear conditioning test, startle habituation and prepulse inhibition, and the resident-intruder task are currently being established in the laboratory. These tests will be used to further evaluate the model in future experiments.

Endocrinological assays will be established to monitor blood levels of ACTH and corticosterone in the mouse. These assays will provide additional biological markers for the model.

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