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General Hypothesis Adolescent stress causes life-long impairment disrupt the cortical-limbic circuit by dampening the glucocorticoid signaling in the infralimbic cortex and affecting the molecular machinery involved in the conditioning and sensitization of the contextual fear response.

Specific Aims: 1- To analyze the effects of chronic variable stress (CVS) during adolescence and adulthood on the expression of the glucocorticoid receptor, and to co-localize the immunoreactivity of GR with the transcription factor ΔfosB as a marker of chronic activation under stress in the infralimbic and prelimbic areas of the prefrontal cortex.
2- To evaluate the changes in the expression of the GR in particular types of neurons in the above mentioned areas (Glutamatergic, GABA-ergic)
3- To study the expression of molecules associated with the process of learning such as Cdk5, p35, PSD-95 and the subunits NR2A and NR2B of the NMDA receptor and to relate them with the activity of the glucocorticoid receptor.

Summary of Results

For this project we modified the original plan following previous results. As we had previously observed, adolescent CVS has short and long-term effects on a contextual fear conditioning paradigm. For that experiment, animals that were submitted to adolescent CVS (2 weeks, starting at postnatal day 40) were first habituated to the conditioning chamber for 3 min. Then 3 shocks were applied 3, 1mA, 1s duration, separated by 1 minute. The time animals spent doing freezing was scored for 3 minutes. Subsequently, the animals were placed in the same chamber for 4 days without being shocked and freezing and was recorded for 5 minutes. Freezing was defined by the total lack of movement except for breathing. In Figure 1 we observed how the animals that were stressed during adolescence both at short and long-term present greater freezing behavior both in response to series of shocks during the conditioning phase as well as the subsequent exposure to the context.

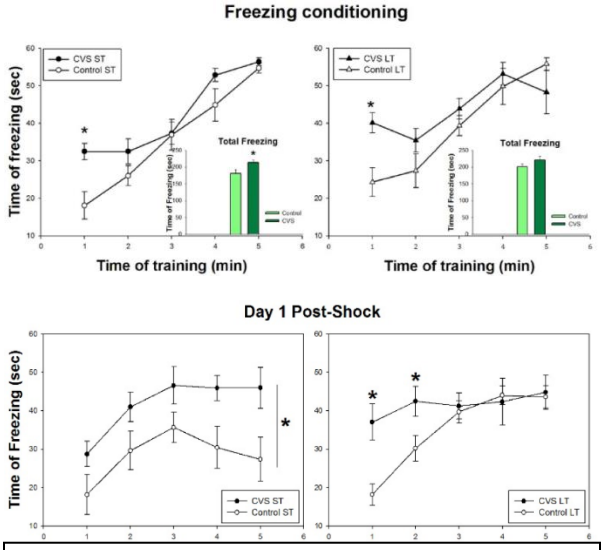

Fig1: Upper panel: Freezing behavior during training (day 1) in animals stressed during adolescence that were evaluated at 60 and 90 days old. Bottom: Freezing behavior on evaluation day (day 2). * Significant difference versus. Corresponding control p< 0.05 fig. 2.
p<0,05

On the contrary, there were no changes in the expression of this behavior during the rest of the duration of the extinction procedure of extinction, neither during the recall session after a reminder shock.

These results indicate that the stress in adolescence is able to modify the adult behavioral phenotype, resulting in sensitivity and increased capacity of generate contextual conditioning to an aversive event. Nevertheless, there were no deficits in the learning process during the extinction procedure. This effect is long-term maintained, as it is observed not only 1 week after stress but also even 5 weeks after having been subjected to the chronic stress protocol.

Based on the results, we decided to determine the expression of proteins associated with the process of formation of memories of association such as Cdk5, p35, PSD-95 and subunit NR2A and B of NMDA receptors by biochemical after the animals were trained in the conditioning chamber. This experiment is an adaptation of what was planned for aim number 3.

For this, we evaluated the effects of the conditioning training on the animals that were submitted to CVS during adolescence and then evaluated at 60 or 90 days of age in the contextual fear conditioning paradigm. To do this, the animals were trained in the conditioning chamber the same way mentioned in the first experiment and then

they were returned to their home cage for 1 hour, after which the animals were decapitated to obtain the fresh brains. We subsequently dissected tissue samples from the hippocampus, a brain structure associated with contextual learning to later obtained protein samples from the total homogenates and membrane, cytoplasmic and nuclear fractions for the determination of the proteins of interest using the Western Blot technique. Partial results are displayed because these samples are still being processed. In Figure 2 we can observe the results for the membrane fraction of the animals stressed during adolescence and trained in a contextual fear conditioning paradigm at 90 days of age. **The results suggest that animals with a previous history of stress during adolescence have higher recruitment of the NMDA glutamate receptor subunit NR2B to the membrane**, which would be associated to the highest levels of freezing behavior observed in experiment 1 after conditioning, participating in the consolidation of the conditioned memory. In this way, we can suggest that one of the mechanism for the long-term effect of stress in adolescence would be promoting the facilitation this type of learning, at the level of the synapses in the hippocampus. This effect is long-term maintained, as it is observed even 5 weeks after having been subjected to the chronic stress protocol.

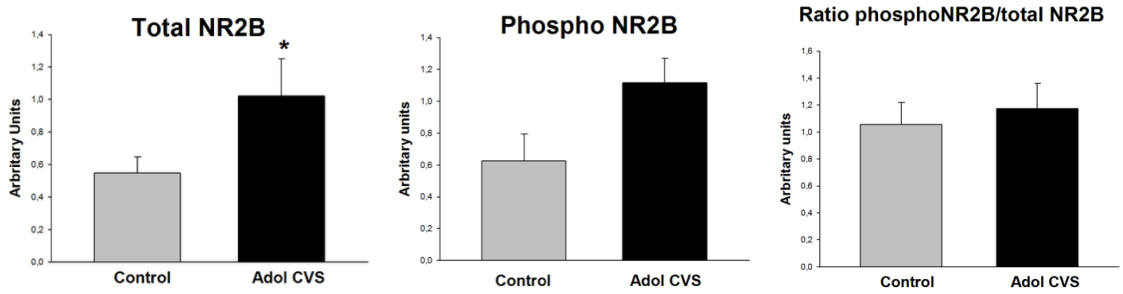

Fig2: Expression levels of the NR2B subunit of the glutamate NMDA type receptor, its phosphorylated form and ratio between both in hippocampus of the animals submitted to variable chronic stress during the adolescence and evaluated at 90 days of age. The animals were trained in a contextual fear conditioning paradigm and decapitated an hour after to dissect the brains. *p<0.05

Based on these results, we decided to focus the study of the effects at this time and to evaluate other aspects related to the response to stress in the adult. This constituted a new aim that was added to those already raised in the original project.

Focusing on the long-term effects observed after 5 weeks of being submitted to stress during adolescence, the goal was to determine the HPA axis response of the axis to an acute stressor. In addition, behaviors associated with stress such as anxiety and depressive type behaviors were assessed. Following being stressed for 2 weeks during late adolescence, at PND 90, the animals were exposed 5 minutes to an elevated plus maze to determine the anxiety-like behavior. 5 days later, the animals were restrained in plastic tubes to determine the HPA stress response. For this, a series of blood samples were taken by tail clip. The first sample was drawn, as soon as the animal was placed in the restrainer without taking more than 3 minutes. This sample was considered the baseline secretion point. Another sample was taken 30 min after right before releasing the animals to their home cages. After this another two sample without restraining the animals were taken at 60 and 120 minutes. ACTH and corticosterone were determined before, during and after being exposed to an acute stressor. Lastly, 5 days after, animals were evaluated in the forced swim test during 10 minutes. In this case it was observed that **chronic stress in the adolescence did not have effect on the stress hormones response to an acute stimulus, while there was a behavioral effect as these animals displayed more immobility in the forced swim test indicating a phenotype of passive behavioral adaptation when facing a stressful situation** (figure 3)

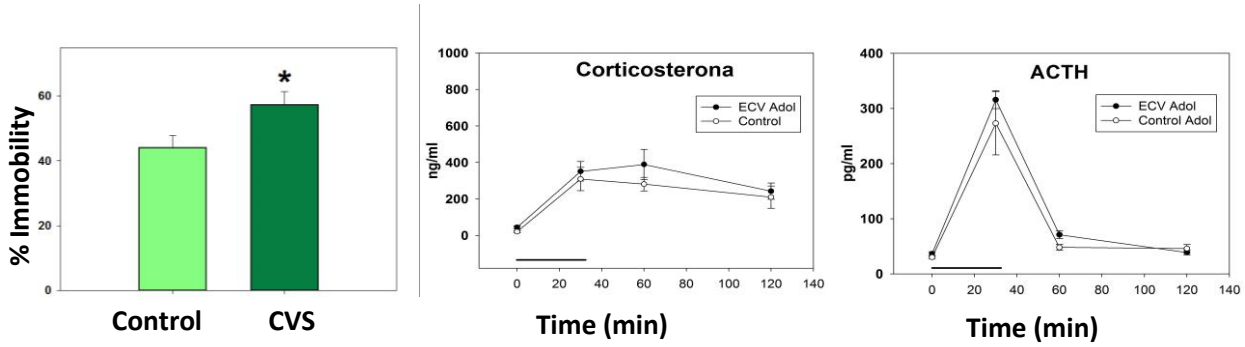

Fig 3: Immobility in the forced swim test and corticosterone and ACTH after 30 minute restraint (bar). * p<0.05

In order to determine if this long term effect specifically were caused by stress in the adolescence, the same design was repeated but this time applying the protocol of chronic stress once the animals had reached adulthood (PND60) and they were also evaluated 5 weeks after finished the CVS protocol (110 days).

In figure 4 these results are displayed. Interestingly, **the animals that were stressed in adulthood showed long-term effects on the HPA axis response to an acute stressor, whereas the behavior during the forced swim test was not modified.** This experiment not only shows that the effects observed previously on coping behavior during the forced swim test in the animals stressed during adolescence is specific of the age of application of stress, but that also it suggests that during adolescence the animals would adapting to chronic stress (either by resistance or resilience) in a way that would prevent the dysregulation of the activity of the axis HPA that was observed in the adult stressed animals and that have been reported previously by other groups using this and other models of chronic stress in the adult. It remains to elucidate the mechanisms involved in the prevention of such effect in the long term.

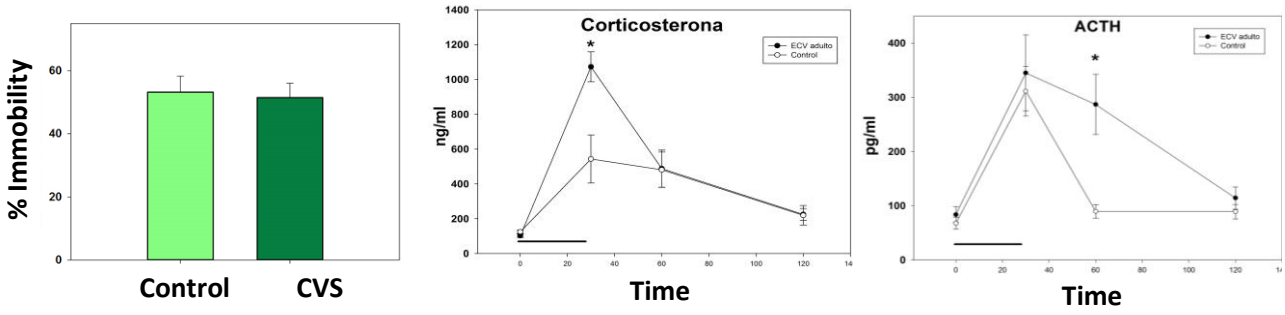

Fig 4: Immobility in the forced swim test and corticosterone and ACTH after 30 minute restraint (bar). * p<0.05

The day after the forced swim test, animals were perfused and the brains were collected to analyze the effects of the CVS in adolescence and adulthood, on the expression the ΔFosB transcription factor and other fos related genes (FRA). These early genes products are considered markers of neuronal recruitment in the response to stress, since the expression starts after the neuron was activated by a stressful event and the proteins accumulate for hours and even days in the cell, allowing us to determine

the brain regions that were activated during the last stressor that remain participating in the response after the stressor has ceased. These results are currently being processed. Some pictures of the areas to analyze are shown if Fig. 5

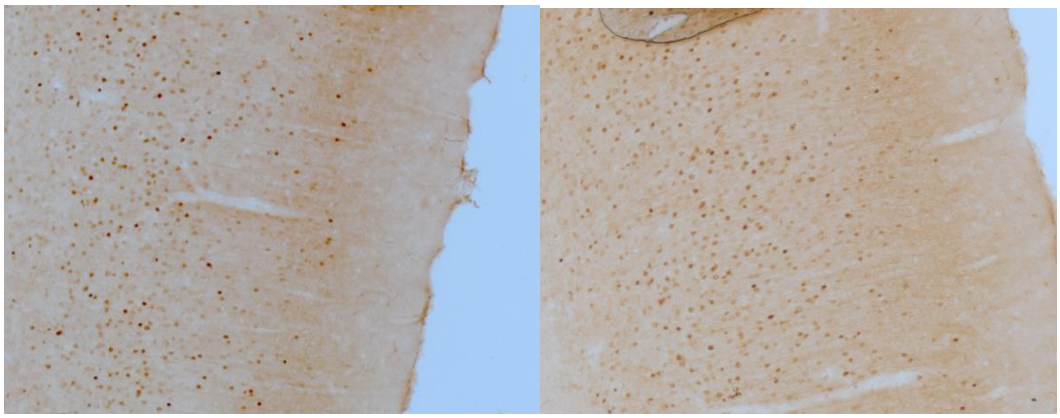

Fig 5. Representative micrograph of fos related antigens (FRA) in prefrontal cortex. Antibody c-fos sc-253 (Santa Cruz) 1:3000

Some of the results here presented are still being processed and altogether they will be soon prepared for publication and we want to acknowledge the International Society for Neurochemistry for funding this project.

Expenses on the grant:
Antibodies for western blot and immunohistocehmistry: GR, FosB, c-fos, PSD95, NR2B, p-NR2B, biotinylated secondary antibodies mouse and rabbit.
Western blot supplies: Kit for protein quantification
Hormones determination: RIA kit for corticosterone