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TITLE: “Early Life Stress and Sleep Restriction as Risk Factors in PTSD: An Integrative Pre-Clinical Approach”

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The project proposes a novel integrative preclinical approach to study risk factors for and neurobiology of post-traumatic stress and depression. Why risk factors? Most models of PTSD concentrate on what would constitute a trauma in the studied animals. However, as most people exposed to traumatic experiences do not develop PTSD, the exposure to the trauma is not a sufficient condition to induce PTSD. We focus on both distal (Childhood adversities) and proximal (Sleep restriction) potential risk factors, with high relevance to soldiers. The primary aims of the project are thus.

1) To establish an effective animal model of PTSD that would take into consideration risk factors to the induction of trauma
2) To examine the role of sleep restriction as an immediate risk factor in PTSD
3) To establish the role of childhood adversity as a long-term risk factor in PTSD, particularly in association with sleep restriction
4) To develop the model as a platform for pharmacological testing of novel targets for drug development
5) As an additional aim – once an effective animal model is established - to use in order to identify novel targets for drug development
Title:  **Early life stress and sleep restriction as risk factors in PTSD – An integrative preclinical approach**

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Introduction

The project proposes a novel integrative preclinical approach to study risk factors for and neurobiology of post-traumatic stress and depression.

Why risk factors? - Because PTSD is the only psychiatric disorder to which there is seemingly a clear etiological agent – a traumatic event that triggers it, most models of PTSD concentrate on what would constitute a trauma in the studied animals. However, because the majority of people exposed to traumatic experiences actually do not develop PTSD the exposure to the traumatic experience is necessary, but not a sufficient condition to induce the disorder. We focus on both distal (Childhood adversities) and proximal (Sleep restriction) potential risk factors, with high relevance to soldiers. The primary aims of the project are thus:

1) To establish an effective animal model of PTSD that would take into consideration the contribution of risk factors to the induction of the trauma.
2) To examine the role of sleep restriction as an immediate risk factor in PTSD.
3) To establish the role of childhood adversity as a long-term risk factor in PTSD, particularly in association with sleep restriction.
4) To develop the model as a platform for pharmacological testing of novel targets for drug development.
5) As an additional aim – once an effective animal model is established – to use it in order to identify novel targets for drug development.

Body

Task 4: The impact of sleep restriction on the outcome of exposure to UWT -

The effect of scattered sleep restriction on the long term behavioral response to underwater trauma

Our results so far indicated that sleep restriction (SR) protocol had immediate physiological effects. Some long term behavioral effects on the reaction to Underwater trauma (UWT) were found only when we used a severe protocol (only 2 hrs rest instead of 4 hrs) – fewer entries to the center of the open field test, but this was accompanied with a surprisingly stronger habituation in the acoustic startle response test.

Following a discussion with colleagues at the Walter Reed Army Institute of Research (a discussion led by Dr. Thomas Balkin), we set out to examine an additional variation of the model; in order to make our SR protocol less predictable, and hence
possibly more effective, we decided to scatter the hours of sleep restriction as well as the resting hours across the day. In this experiment there was no longer one resting phase, but 3 separated, each of 1 hr. Our aim was to examine whether scattered SR will exacerbate the long term behavioral reaction to UWT. In addition, and in order to potentially increase the sensitivity of the behavioral testing battery, an additional behavioral test was added – the performance in a contextual fear conditioning task.

**Aim:**

To examine whether scattered SR exacerbates the long term reaction to UWT, when it is applied prior to the UWT.

**Methods**

**Animals**

Male Sprague Dawley rats (~8 weeks old, 250-275g) were used for the experiment. Animals were group housed at 22 ± 2°C under 12-h light/dark cycles. Water and food were available ad libitum. The experiment was approved by the University of Haifa Ethics and Animal Care Committees.

**Experimental groups**

All rats were randomly assigned to one of the following experimental conditions:

1. **SR prior to UWT (SRU)** – Rats exposed to SR, followed by UWT.
2. **SR Control prior to UWT (CU)** – Rats exposed to the control procedure of SR, followed by UWT.
3. **SR Control with no UWT (C)** – Rats exposed only to the control procedure of SR but this was not followed by UWT.
Experimental design

Following delivery and acclimation to the vivarium, all rats were habituated to the SR apparatus by placing them on the wheels for 1 h on 3 consecutive days (slowly or voluntary rotating wheels, according to the experimental group). Following wheels habituation, group SRU was exposed to SR for 8 days and the other groups were exposed to the control procedure of SR. After 8 days of SR, rats from groups SRU and CU were exposed to UWT. Approximately 4 weeks following UWT, rats were assessed for anxiety level using open field (OF), elevated plus maze (EPM) and contextual fear conditioning test (FC). Timeline of procedures is presented in figure 1.

![Figure 1: Timeline of procedures.](image)

Procedures and assessments

Sleep restriction

In this protocol (adapted from Meerlo et al., 2002) SR was performed by confining the rats in slowly rotating wheels (diameter 35.5 cm, approximately 1.5 meter per minute, model 80860A; Lafayette Instruments Company, Lafayette, IN, USA). Control rats were placed in voluntary rotating wheels (model 80860W). The rats had continuous access to food and water at the side of the wheel. 21 hrs of the day, the sleep restricted rats were placed in the motorized rotating wheels and control rats were placed in the voluntary rotating wheels. 3 hours, scattered randomly across the light phase, rats were placed in home cage.

Underwater Trauma

In this protocol (adapted from Richter-Levin, 1998) rats were placed in a plastic tank (diameter 40 cm, height 45 cm) containing 30 cm of water at 22±2°C, and held under
water for 45 sec, using a special metal net (20X20X15cm). This session was taken place immediately following the last day of SR.

The open field test

The open field consists of a wooden box 90cm × 90cm × 38cm, positioned in a dimly-lit room. The walls and floor are painted black. Following 2 min habituation period for the testing room, rats were placed at a corner of the open field for 5 min of free exploration. Distance moved in the central and peripheral areas of the box, as well as number of entries to the center and duration of stay, were recorded using Ethovision XT 8.

The elevated plus maze test

This maze consists of a plus-shaped platform with two open arms and two closed arms surrounded by 38-cm high opaque walls on three sides, with arms of the same type located opposite each other (File, 1993). Following 2 min habituation to the testing room, each rat was placed on the central-platform facing an open arm and was allowed to explore the maze for 5 min. Distance moved, duration of stay and number of entries to open and closed arms were recorded using Ethovision XT 8.

Contextual fear conditioning

Fear conditioning was measured using a standard chamber (Panlab SLU, Spain). Rats were trained to associate a foot shock (UCS) to a chamber’s context (CS) and on the subsequent days the retrieval and extinction was tested. On the conditioning day the rats were placed in the chamber and after 2min of exploration, they were given three foot shocks (0.5mA for 0.5s) with 2min intervals between them. Rats that had over 50% freezing during the first exploration (before the shocks) were excluded. Conditioning was measured according to percent of freezing during the 2 min exploration after the last foot shock.
The next 3 days rats were placed in the camber for 10min. to assess also extinction of the fear response. Percent of freezing was measured on each day.

**Statistical Analysis**

Differences were determined using one way analysis of variance (ANOVA) or repeated measures ANOVA.

**Results**

No significant differences were found between groups, in none of the long term behavioral measures. As shown in figure 2, 3 & 4, in the majority of the measures, rats that were exposed to SR prior to UWT showed the same trend as rats that were exposed to UWT alone. Apparently SR did not exacerbate the long term reaction to UWT.

**Figure 2:** Open field test results. No significant differences between groups. [N: SRU – 8, CU – 7, C - 7]
Figure 3: Elevated plus maze test results in open arms. No significant differences between groups.
[N: SRU – 8, CU – 7, C - 7]

Figure 4: Contextual fear conditioning test results. No significant differences between groups.
[N: SRU – 8, CU – 7, C - 7]
The effect of sleep restriction on the short term behavioral response to a mild stressor – preliminary results

Another issue that was raised during the discussion with colleagues at the Walter Reed Army Institute of Research (a discussion led by Dr. Thomas Balkin), was the possibility that our results so far, indicating no substantial effects of SR on the long term reaction to UWT, could result from the UWT being such a severe stressor, that SR cannot further exacerbate the effects. Hence we decided to test the effect of SR on the short term reaction to a milder stressor – forced swim (FS).

**Aim**

To examine whether SR will exacerbate the short term reaction to UWT.

**Methods**

**Animals**

Male Sprague Dawley rats (~8 weeks old, 250-275g) were used for the experiment. Animals were group housed at 22 ± 2°C under 12-h light/dark cycles. Water and food were available ad libitum. The experiment was approved by the University of Haifa Ethics and Animal Care Committees.

**Experimental groups**

All rats were randomly assigned to one of the following experimental conditions:

1. **SR prior to FS** (SRS) – Rats exposed to SR, followed by FS.
2. **SR Control prior to FS** (CS) – Rats exposed to the control procedure of SR, followed by FS.
3. **SR Control with no stressor** (control) – Rats exposed only to the control procedure of SR.
Experimental design

Following delivery and acclimation to the vivarium, all rats were habituated to the SR apparatus by placing them on the wheels for 1 h on 3 consecutive days (slowly or voluntary rotating wheels, according to the experimental group). Following wheels habituation, group SRS was exposed to SR for 8 days and the other groups were exposed to the control procedure of SR. After 8 days of SR, rats from groups SRS and CS were exposed to FS. 3 days later, rats were assessed for anxiety level using open field, elevated plus maze and acoustic startle testing. Timeline of procedures is presented in figure 5.

Procedures and assessments

Sleep restriction

The same procedure as described in the first experiment, only SR was for 20 hours, and resting in home cage was not scattered. Rats are allowed to sleep in their home cages between 10:00-14:00, the first 4 hrs of the light phase. The remaining 20 hrs of the day, they were on the wheels.

Forced swim stressor

15 min forced swim in a plastic tank (diameter 40 cm, height 45 cm) containing 40 cm of water at 22±2°C.

Acoustic startle testing

Unconditioned startle response to an acoustic stimulus was measured using a standard startle chamber (Panlab SLU, Spain). Rats were held using a plastic cylinder in the startle apparatus. The apparatus is equipped with a speaker for producing sound bursts and with a high sensitivity weight transducer system that allows recording and
analysis of the signal generated by the rat movement during each sound burst. Output
from the transducer is led to a computer for sampling.

The protocol was adapted from Adamec et al. (2012). Prior to startle testing, animals
were acclimatized to the darkened apparatus for 5 min with a background white noise
level of 60 dB. Following acclimatization, rats received a 50 ms burst of 120 dB
rising out of the 60 dB background noise once every 30 s for 15 min. The 30 trials
were conducted in the dark.

**Statistical Analysis**

Differences were determined using one way analysis of variance (ANOVA) or
repeated measures ANOVA.

**Preliminary Results**

Data presented here is preliminary with only 4 animals in each group. At the moment
the results indicate no significant differences between the groups. It seems that all rats
that were stressed with FS show the same behavioral trend, whether they were sleep
restricted prior to FS or not. Preliminary results are shown in Figures 6, 7 & 8.

In the acoustic startle test, as shown in figure 8, neither the control group, nor any
other group showed habituation to the sound bursts, as was found in previous
experiments. In these experiments the acoustic startle test was conducted on a
separate day. It is possible that here the animals did not habituate because this was
the third test on the same day. We planned all behavioral tests on the same day,
because we wanted to examine the effect of SR on the short term reaction to FS.
Figure 6: Open field test results. No significant differences between groups. [N: control – 4, CS – 4, SRS - 4]

Figure 7: Elevated plus maze test results in open arms. No significant differences between groups. [N: control – 4, CS – 4, SRS - 4]

Figure 8: Average of maximal amplitude of startle response and maximal amplitude along trials. No significant differences between groups. [N: control – 4, CS – 4, SRS - 4]
Summary

Taken together, the current results demonstrate no clear effect of relatively severe sleep restriction for 8 days. These results join previous results we have obtained that have similar outcome. This lack of effect of SR as a potential proximal risk factor is in sharp contrast to our results with the juvenile stress as a distal risk factor (previous reports, this report below and the resulting publications). With juvenile stress as a distal risk factor we clearly find that such pre-exposure, one month prior to the adulthood trauma severely exacerbated the outcome of exposure to UWT, even when tested four weeks following the exposure to the adulthood trauma. These results come to show that if the SR protocol could have a similar effect on the response to the UWT – this could be detected.

Based on Meerlo et al (2008) paper one could expect significant short and long-term effects of severe sleep restriction. Our cumulative data does not support this expectation. We have made various efforts to verify whether the lack of effects was due to some procedural effects but despite variations in the protocols the results remained negative. We believe that even if the exact details of our protocols were not optimal for an as yet unforeseen reason, at least a trend should have been noted if SR in this format is indeed a risk factor for exacerbating the impact of an exposure to a traumatic event. While we will finalize the currently running experiment we have decided to end this part of the project and to focus on those aspects that show very consistent and clear results –

A) The model of Childhood exposure to stress as a risk factor

B) The PTSD model as was developed here – to be used both as a drag testing platform and as a model to identify novel targets for drug development in PTSD.
Task 3: **Behavioral and neurobiological investigation of the impact of the underwater trauma (UWT) with or without a reminder cue –**

**The short and long term effects of exposure to reminders of underwater trauma:**

Recollection of emotional memories is attributed in part to the activation of the amygdala and the hippocampus. Recent hypothesis suggests a pivotal role for the ventral hippocampus (VH) in traumatic stress processing and emotional memory retrieval. Persistent re-experiencing and intrusive recollections are core symptoms in acute and posttraumatic stress disorders (ASD; PTSD). Such intrusive recollections are often triggered by reminders associated with the trauma. Study 1 assessed the effects of trauma reminder cues on the activation of the amygdala, dorsal and ventral hippocampus 24h after the trauma.

Often, freezing and startle behaviors in the context of a previously experienced stress are taken as an indication of post-traumatic stress disorder (PTSD)-like symptoms in rats. However, PTSD is characterized by large individual variations of symptoms. In order to take into consideration the complex and long term distinctive variations in effects of trauma exposure study 2 designed a novel test for measuring long term effects of trauma exposure in rats.

In study 3 we have implemented a behavioral profiling approach that is based on two novel behavioral tests, the Water Associated Zero Maze (WAZM) that was developed in study 2, and the continuous saccharine preference test.

1. **Differential activation of amygdala, dorsal and ventral hippocampus following an exposure to a reminder of underwater trauma**

This project examined the impact of exposure to a trauma reminder (under water trauma (UWT)) on the activation of the basolateral amygdala (BLA), dorsal and
ventral hippocampus (DH and VH respectively). Rats were exposed to UWT and 24 h later were re-exposed to the context of the trauma. Phosphorylation of the extracellular signal-regulated kinase (ERK) was used as a marker for level of activation of these regions. Significant increase in ERK activation was found in the VH and BLA following exposure to the context of the UWT 24h following the trauma itself. Such pattern of activation was not found in animals exposed only to the trauma or in animals exposed only to the trauma reminder (Figure 1). Additionally, the dissociative pattern of activation of the VH sub-regions positively correlated with the activation of the BLA (Figure 2). Our findings suggest a specific pattern of neural activation during recollection of a trauma reminder, with a unique contribution of the VH. Measured 24 h after the exposure to the traumatic experience, the current findings relate to relatively early stages of traumatic memory consolidation. Understanding the neural mechanisms underlying these initial stages may contribute to developing intervention strategies that could reduce the risk of eventually developing PTSD.
FIGURE 1 | Mean ± standard error of the mean (SEM) ERK2 activation 30 min after the exposure to reminder of a traumatic experience (n = 12 in each group). (A) ERK2 activation in the dorsal CA1 did not differ significantly between the groups. (B) ERK2 activation in the dorsal DG did not differ significantly between the groups. (C) ERK2 activation in the ventral CA1 of the UWT + R group was significantly higher than the rest of the groups. (D) ERK2 activation in the ventral DG of the UWT + R group was significantly higher than the rest of the groups. (E) ERK2 activation in the BLA of the UWT + R group was significantly higher than the rest of the groups. (F) Diagram of analyzed regions. DH = dorsal hippocampus; VH = ventral hippocampus. ** p < 0.01.
**FIGURE 2** | Behavior and Pearson correlations of ERK2 activation of rats exposed to the reminder. (A) Mean ± SEM of time spent wall climbing during the reminder (n = 9 in each group). (B) Correlation between the time spent wall climbing during the reminder and ERK2 activation in the BLA (n = 9 in each group). (C) Correlation between ERK2 activation in the BLA and ERK2 activation in the dorsal CA1 (n = 12 in each group). (D) Correlation between ERK2 activation in the BLA and ERK2 activation in the dorsal DG (n = 12 in each group). (E) Correlation between ERK2 activation in the BLA and ERK2 activation in the ventral CA1 (n = 12 in each group). (F) Correlation between ERK2 activation in the BLA and ERK2 activation in the ventral DG (n = 12 in each group). *p < 0.05; **p < 0.01.
2. Water associated zero maze: a novel rat test for long term traumatic re-experiencing

The current study used a novel behavioral test, the water associated zero maze (WAZM; Figure 1). This test was planned to enable a formation of an association between the context of the maze and an underwater trauma (UWT) or swim stress in order to examine the impact of exposure to the context which immediately precedes a stressful or a traumatic experience on rat’s complex behavior. Rats were exposed to the WAZM and immediately after to an UWT or short swim. One month later rats were re-exposed to the context of the WAZM while their behavior was video recorded. Furthermore, c-Fos expression in the amygdala was measured 90 min after this exposure. The results of the current study indicate that the WAZM can be used to discern behavioral changes measured a long time after the actual traumatic or stressful events (Figure 3). Furthermore, the behavioral changes detected were accompanied by changes of c-Fos expression in the amygdala of exposed rats (Figure 4). We suggest that the WAZM can be used to model the long-term traumatic memories re-experiencing in rodent models of human stress-related pathologies such as PTSD.
FIGURE 3: Rate behavior during the contextual exposure to the WAZM on the 30th day. Rats that were exposed to the underwater stress spent significantly less time (A) and traveled shorter distance (B) in the open quadrants of the WAZM. No significant difference was found in the distance traveled in the closed quadrants of the maze between the groups (C). Significant difference was found in total time of freezing in the maze (D). *p < 0.05; **p < 0.01.

FIGURE 4: c-Fos expression 90 min after the WAZM test on the 30th day. (A) Representative images of c-Fos-IR (red) and DAPI (blue) in the central amygdala (CeA) and basolateral amygdala (BLA). All groups’ c-Fos expression differed significantly in the CeA (B) and BLA (C). All bars represent the mean ± s.e.m. **p < 0.01.
3. Modeling of complex PTSD-like behaviors in rats

In this study we have implemented a behavioral profiling approach that is based on two novel behavioral tests, the Water Associated Zero Maze (WAZM) and the continuous saccharine preference test. The WAZM enables the formation of an association of the maze with an underwater trauma, and by that, the assessment of complex behaviors following an exposure to the context that immediately precedes a traumatic experience. The continuous saccharine preference test enables a fine measurement of consumption (hedonic) behavior over a long period of time without stressing the rats by the need for water deprivation, single housing or interruption of transfer to a test cage.

Study design (Figure 1) included exposure to the WAZM for 4 days. Immediately after the 4th exposure, UWT rats were held under water for 45 sec. 27 days later rats were re-tested in the WAZM. During these 27 days rats were placed in a home cage suited for continuous saccharine preference test. In this cage animals are held 2 in a cage, with a perforated partition separating between them. The perforated separation enables a level of social interaction but separates the use of two sets of two burettes (one filled with water and the other with 0.03 mg/ml Saccharine). This setting makes it possible to continuously follow individually saccharine preference for each animal.

After establishing that UWT and Control conditions have a differential effect over rats behavior (Figure 2), behavioral profiling was used in order to establish distinct profiles of altered anxiety-like behaviors. In this analysis the classification is based on the upper or lower 20th percentiles of the Control group behavior. Four fear-derived behaviors (time spent in the open parts, distance traveled in the open and closed parts and total freezing) were used in order to set criteria of anxious-like Affected diagnosis for the rats (i.e- falling in the edge of the distribution in 3 out of 4 measurements).
This classification showed that rats that went through the UWT a month before the test were at significantly higher risk of falling into the “Affected” category (Figure 3). When this classification analysis was implemented for the cumulative data obtained through the continuous saccharine preference test, Affected animals category was found to dissociate into 2 different sub populations (Figure 4). These findings indicate that even after one month, re-exposure to a contextual reminder cue, which immediately precedes the traumatic experience, does initiate PTSD-like symptoms in the WAZM. Combining these altered behaviors with the continuance saccharin preference assessment yielded significant different sub-categories of affected profiles. The distribution of these affected profiles resembles the human psychiatric literature. We now analyze the functions of the regions associated with these behaviors, such as the amygdala, ventral and dorsal hippocampus.

![Experimental design](image)

**Figure 1: Study design**
Figure 2: Rats behavior during the exposure to the contextual reminder on day 31. Rats that went through the UWT spent significantly less time and traveled less distance in the open parts of the WAZM (A; C). No significant difference was observed in distance traveled in the closed parts (B). Significant difference was found for total freezing behavior (D).

Figure 3: Individual classification for rats one month after the exposure to the UWT. Rats that were exposed to the UWT were at significantly higher risk of falling into the “Affected” category.
Figure 3: Individual classification by the continuous saccharine preference test (A; B). Rats that were exposed to the UWT were at significantly higher risk of falling into the “Hedonic” and “Anhedonic” categories (C). Affected animals category of UWT exposed rats was further found to dissociate into 2 different sub populations (D). These sub populations seem to develop in accordance to time passing since the trauma exposure (E).

The long term biochemical effects of exposure to underwater trauma in the ventral and dorsal hippocampus:

Chronic infusion of mice with a $\beta$2 adrenergic receptor ($\beta$2AR) analog was shown to cause long-term DNA damage in a pathway which involves $\beta$ Arresin-1-mediated activation of Mdm2 and subsequent degradation of the tumor suppressor protein p53. In study 4 we assessed the interaction of Mdm2 with p53, $\beta$2AR, and $\beta$ Arrestin-1 in the dorsal and ventral hippocampal CA1.

The pro-inflammatory enzyme cyclooxygenase-2 (COX-2) is regularly expressed in the hippocampal neurons, but its role in emotional trauma is not known. In study 5 we tested whether heightened anxiety-like behavior one month after the UWT will be accompany with changes in the expression of cyclooxygenase-2 (COX-2) in the hippocampus.
4. **Selective increase in the association of the β2 adrenergic receptor, β Arrestin-1 and p53 with Mdm2 in the ventral hippocampus one month after underwater trauma**

The objective of this study was to test whether a single acute stress, which manifests long lasting changes in behavior, affects the interaction of Mdm2 with p53, β 2AR, and β Arrestin-1 in the dorsal and ventral hippocampal CA1. Adult rats were subject to underwater trauma (UWT) and tested a month later for behavioral and biochemical changes. Elevated plus maze test confirmed that animals that experienced the threat of drowning present heightened levels of anxiety one month after trauma (Table 1). An examination of the CA1 hippocampal areas of the same rats showed that underwater trauma caused a significant increase in the association of Mdm2 with β 2AR, β Arrestin-1, and p53 in the ventral but not dorsal CA1 (Figure 1). Our results provide support for the idea that stress-related events may result in biochemical changes restricted to the ventral ‘emotion-related’ parts of the hippocampus.

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<td><strong>Time (s)</strong></td>
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<tr>
<td>Center</td>
<td>54.66 ± 7.03</td>
<td>27.16 ± 5.05</td>
<td>p &lt; 0.01**</td>
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<tr>
<td>Open arms</td>
<td>36.75 ± 7.32</td>
<td>17.72 ± 5.06</td>
<td>p &lt; 0.05*</td>
</tr>
<tr>
<td>Closed arms</td>
<td>205.14 ± 13.76</td>
<td>253.29 ± 9.97</td>
<td>p &lt; 0.01**</td>
</tr>
<tr>
<td><strong>Distance (cm)</strong></td>
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<tr>
<td>Center</td>
<td>359.56 ± 40.84</td>
<td>196.15 ± 37.38</td>
<td>p &lt; 0.01**</td>
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<tr>
<td>Open arms</td>
<td>262.40 ± 54.46</td>
<td>140.98 ± 39.72</td>
<td>n.s.</td>
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<tr>
<td>Closed arms</td>
<td>1257.70 ± 84.78</td>
<td>1103.02 ± 83.81</td>
<td>n.s.</td>
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Data are shown as Mean ± S.E.M n = 12 animals per group.

* Student’s t test p < 0.05.
** Student’s t test p < 0.01.
Figure 1: Increased association of β2AR, β Arrestin-1 and p53 with Mdm2 in the ventral CA1 one month after trauma. No change in the total levels of Mdm2 (top panel) and p53 (bottom panel) in the dorsal or ventral CA1 (A). Mdm2 was immunoprecipitated from ventral (black bars) and dorsal (gray bars) of the CA1. Blots were probed for β2AR, (B), β Arrestin-1 (βArr1) (C), and p53 (D). Insets are representative immunoblots from each group. Graphs represent Mean±S.E.M. of n=6-8 animals per group. *Student's t test p<0.05 vs. Control.
Task 5: The impact of pre-exposure to juvenile stress on the outcome of exposure to UWT –

**Introduction**

Three complementary approaches were engaged to assess long-term expression changes of target molecules in an animal model of posttraumatic stress disorder (PTSD), focusing on subregions of amygdala and hippocampus as key brain structures for emotional behavior and with alterations in PTSD (Hughes & Shin, 2011).

The rat model of PTSD utilizes underwater trauma (UWT) as a brief traumatic experience that is further combined with juvenile stress for including one of the most prominent risk factors, childhood adversity (Heim & Nemeroff, 2001; Horovitz et al., 2012), in our model. The model is further extended by including an odor reminder cue of the initial trauma, providing a platform for studying trauma re-experiencing (Ardi et al., 2014; Ritov & Richter-Levin, 2013).

However, after exposure to a trauma, only a subset of individuals will develop lasting PTSD-associated symptoms – a fact often neglected in animal studies (Goswami et al., 2013). We began to address such individual differences by applying "behavioral profiling", where activity and anxiety-like responses tested four weeks after UWT of individual animals are compared to a control group. That allows for identifying rats with extreme behavioral responses after UWT exposure. Expression of target molecules was then analyzed with respect to the individual behavioral profile, providing an approach to identify the specific molecular factors associated with the observed behavioral phenotype.

**EXPERIMENT 1: Long-term effects of UWT on protein expression levels in the BLA and the dorsal and ventral hippocampus**

In this study we began to investigate long-term changes in protein expression four weeks after UWT, with and without previous juvenile stress, in the dorsal and ventral dentate gyrus (DG) and the Cornu Ammonis (CA)1 region of the hippocampus as well as in the basolateral complex of the amygdala (BLA). Target proteins important for inhibitory and excitatory signaling (Mahan & Ressler, 2012) were selected,
namely the GABA-synthetizing enzyme GAD65 and the alpha 1 and alpha 2 subunits of the GABA A receptor as well as the NMDA receptor subunits NR2a and NR2b.

First, expression changes after exposure to juvenile stress and UWT were assessed, resulting in a complex pattern in both inhibitory and excitatory factors in the different brain regions.

Secondly, since activity and anxiety-like behavior of all animals was assessed before preparation, it was possible to build individual behavioral profiles. Re-analysis of expression data with respect to the individual profiles allowed then to identify key factors of altered anxiety-like behavior in trauma-exposed animals.

**Experiment 1: Methods**

**Animals**

Male Sprague Dawley rats (~22 days old, 30-50 g) were used for the experiments. Animals were housed in groups of ~4, at 22 ± 2°C under 12-h light/dark cycles with water and food available ad libitum. The experiments were approved by the University of Haifa Ethics and Animal Care Committees.

**Behavioral protocols**

Following delivery and an additional acclimation period of five consecutive days rats were randomly assigned to one of the three groups: (1) Control (Control, n=34); (2) UWT stress + odor reminder (UWT(+), n=33); (3) Juvenile and UWT stress exposures + odor reminder (J+U(+), n= 29). Rats assigned to juvenile stress were exposed to different acute stressors over three days at an age of 27-29 days: Day 1: Forced swim (10 min; opaque circular water tank; diameter 0.5 m, height 0.5m, water depth 0.4m, water temperature 22±2°C); Day 2: three 30 min trials of elevated platform (12 x 12cm, height 70cm; ITI (Inter-Trials Interval): 60 min in the home cage); Day 3: 2h restrain (11 x 5 x 4 cm metal mesh restraining box preventing forward-backward movement and limited side-to-side (Tsoory et al., 2007).

Four weeks later, in adulthood (PND 60), animals were subjected to underwater trauma (UWT) stress: following three consecutive days of habituation to a standard cage (2 min per day) rats were exposed to vanilla odor for 30 s inside the cage (following initial 2 min habituation) and then were immediately placed in a water-
filled plastic tank. After 5s of free swimming, they were restraint under water for 45s, using a special metal net (20 x 20 x 15 cm; Wang et al., 2000; Ardi et al., 2014). UWT(+) rats were exposed to the UWT stress but not to juvenile stress. Control rats were exposed to the 3 days of habituation, and in the 4th day they were exposed to the odor only.

Four weeks following the UWT exposure (PND90-91), J+U (+), UWT(+) and Control rats were re-exposed to the odor as described above and then tested in the Open Field (OF), as described before (Avital & Richter-Levin, 2005). Briefly, following odor re-exposure, rats were immediately placed in the corner of the open field (90 x 90 x 50 cm Plexiglas box; dim red-light illumination) and were allowed to explore the arena for 8 min.

24 h later, all rats were re-exposed to the odor again and then tested in the elevated plus maze (EPM) as described before (Pellow et al., 1985). Briefly, following odor re-exposure, rats were immediately placed in the center of the arena (110 x 110 cm, 70 cm above the floor; two opposing open arms and two opposing closed arms with 35 cm high Plexiglas walls, no roof; full light illumination) facing an open arm and were allowed to explore for 8 min.

Behavior during OF and EPM tests was recorded and analyzed by EthoVision XT8 tracking system.

Harvesting of brain tissue
One day following the last behavioral test (PND 92), brains were taken out for biochemical analysis, were snap frozen and punches from areas of interest were harvested in a cryostat apparatus (at -20°C; Leica, Wetzlar, Germany) by taking 1.5mm long punches with a stainless steel puncher (1mm diameter). Frozen tissue samples were collected from each hemisphere covering the rostro-caudal axis of the basolateral amygdala complex (BLA; -1.8mm from Bregma; 23), the dorsal dentate gyrus (dDG) and the dorsal Cornu Ammonis 1 (dCA1; both starting -2.8mm from Bregma). Bilateral ventro-dorsal punches were taken from the ventral DG and CA1 (vDG and vCA1; starting -7.6 mm from Bregma, horizontal orientation).

Protein sample preparation & Western blot
Frozen tissue samples were homogenized in 300µl Urea Lysis Buffer (1mM EDTA, 0.5% Triton X, 6M Urea, 100µM PMSF) with freshly added protease and
phosphotase inhibitor cocktail (complete ultra and PhosStop tablets, Roche Diagnostics, Mannheim, Germany) and incubated at 100°C for 5 min. 10 µg of each sample was loaded for 12 % SDS-polyacrylamide gel for electrophoresis (SDS-PAGE). After semi-dry transfer (nitrocellulose membrane) and blocking of unspecific bindings, incubation with primary antibodies (see Tab. 1) took place overnight at 4°C.

**Table 1: Primary antibodies used for western blots**

<table>
<thead>
<tr>
<th>antibody</th>
<th>dilution</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA A receptor alpha 1 subunit</td>
<td>1:2500</td>
<td>Synaptic systems, Goettingen, Germany</td>
</tr>
<tr>
<td>GABA A receptor alpha 2 subunit</td>
<td>1:2500</td>
<td>Synaptic systems, Goettingen, Germany</td>
</tr>
<tr>
<td>GAD65</td>
<td>1:500</td>
<td>BD Pharmingen, Franklin Lakes, NJ, USA</td>
</tr>
<tr>
<td>NMDA receptor 2A subunit</td>
<td>1:1000</td>
<td>Millipore, Billerica, MA, USA</td>
</tr>
<tr>
<td>NMDA receptor 2B subunit</td>
<td>1:1000</td>
<td>Santa Cruz, Dallas, TE, USA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1:2000</td>
<td>Cell Signaling, Beverly, MA, USA</td>
</tr>
</tbody>
</table>

After incubation with complementary secondary antibody (α rabbit, polyclonal, 1:15 000; α goat, polyclonal, 1:10 000; α mouse, polyclonal, 1:10 000), ECL Plus substrate (Advansta, Menlo Park, CA, USA) enabled chemiluminescence detection and densitometric analysis with Quantity One 1-D Analysis software. Ratios between the optical density of target protein and the control protein GAPDH were calculated for each sample and normalized to a reference brain sample that was loaded on each gel for standardization across gels. The density was then normalized to the mean density of the Control group for each target and area.

**Statistical Analysis**

Differences in the behavioral responses and protein expression levels between the different stress groups was conducted by using one-way ANOVA, followed by LSD post hoc tests. Protein expression data was then re-analyzed with respect to the individual behavioral profiling by using Univariate analysis for effects of group,
profile and their interaction for each brain target and region. For this analysis, four animals classified as affected from the control group were excluded. Paired comparisons were performed for significant interactions to further analyzed within groups and profiles: One way ANOVA followed by LSD post hoc test for group in unaffected animals, t-test for UWT(+) vs. J+U(+) in affected and t-test for affected vs. unaffected in UWT(+) and J+U(+).
Experiment 1: Results

Exposure to Juvenile Stress, UWT and UWT reminder induces distinct expression patterns of selected GABAergic and Glutamatergic factors in the ventral and dorsal hippocampus and in the BLA.

One-way ANOVA followed by post hoc comparisons revealed unique changes in expression patterns four weeks after the exposure to the combined juvenile stress and UWT on expression (Tab.2):

Table 2: Effects of an Exposure to Juvenile Stress, UWT and UWT reminder. Protein expression of GABAergic and glutamatergic factors was assessed in the dentate gyrus (DG) and Cornu Ammonis 1 (CA1) regions of the dorsal and ventral hippocampus and the basolateral complex of the amygdala (BLA) after UWT(+) and J+U(+). Arrows indicate significant up- or downregulation compared to the mean of the Control group (*p < 0.05; ** p < 0.01; *** p < 0.001) and between UWT(+) and J+U(+) (#= p < 0.05; ## p < 0.01).

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Brain region</th>
<th>Main effects</th>
<th>Post hoc comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAD 65</td>
<td>dDG</td>
<td>$F_{(2,93)} = 8.595, p &lt; 0.001$</td>
<td>$\uparrow$ J+U(+) ***, #</td>
</tr>
<tr>
<td></td>
<td>dCA1</td>
<td>$F_{(2,93)} = 1.069, n.s.$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vDG</td>
<td>$F_{(2,92)} = 3.165, p &lt; 0.05$</td>
<td>$\downarrow$ J+U(+) *</td>
</tr>
<tr>
<td></td>
<td>vCA1</td>
<td>$F_{(2,91)} = 4.677, p &lt; 0.05$</td>
<td>$\uparrow$ J+U(+) ##</td>
</tr>
<tr>
<td></td>
<td>BLA</td>
<td>$F_{(2,93)} = 6.367, p &lt; 0.01$</td>
<td>$\uparrow$ J+U(+) **, #</td>
</tr>
<tr>
<td>GABA A alpha1</td>
<td>dDG</td>
<td>$F_{(2,93)} = 2.130, n.s.$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dCA1</td>
<td>$F_{(2,93)} = 11.747, p &lt; 0.001$</td>
<td>$\uparrow$ J+U(+) ***, ##</td>
</tr>
<tr>
<td></td>
<td>vDG</td>
<td>$F_{(2,92)} = 3.368, p &lt; 0.05$</td>
<td>$\uparrow$ J+U(+) *</td>
</tr>
<tr>
<td></td>
<td>vCA1</td>
<td>$F_{(2,93)} = 2.978, p = 0.056$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BLA</td>
<td>$F_{(2,93)} = 5.267, p &lt; 0.01$</td>
<td>$\uparrow$ J+U(+) **, #</td>
</tr>
<tr>
<td>GABA A alpha2</td>
<td>dDG</td>
<td>$F_{(2,93)} = 2.206, n.s.$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dCA1</td>
<td>$F_{(2,93)} = 0.074, n.s.$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vDG</td>
<td>$F_{(2,92)} = 9.075, p &lt; 0.001$</td>
<td>$\uparrow$ UWT(+) * $\uparrow$ J+U(+) ***</td>
</tr>
<tr>
<td></td>
<td>vCA1</td>
<td>$F_{(2,93)} = 1.424, n.s.$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BLA</td>
<td>$F_{(2,91)} = 4.492, p &lt; 0.05$</td>
<td>$\uparrow$ UWT(+) * $\uparrow$ J+U(+) *</td>
</tr>
<tr>
<td>NMDA NR2a</td>
<td>dDG</td>
<td>$F_{(2,93)} = 0.923, n.s.$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dCA1</td>
<td>$F_{(2,93)} = 3.052, p &lt; 0.052$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vDG</td>
<td>$F_{(2,93)} = 7.226, p &lt; 0.001$</td>
<td>$\downarrow$ UWT(+) ** $\downarrow$ J+U(+) **</td>
</tr>
<tr>
<td></td>
<td>vCA1</td>
<td>$F_{(2,92)} = 2.359, n.s.$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BLA</td>
<td>$F_{(2,92)} = 7.430, p &lt; 0.001$</td>
<td>$\downarrow$ UWT(+) ** $\downarrow$ J+U(+) **</td>
</tr>
<tr>
<td>NMDA NR2b</td>
<td>dDG</td>
<td>$F_{(2,93)} = 6.546, p &lt; 0.01$</td>
<td>$\downarrow$ UWT(+) ** $\downarrow$ J+U(+) **</td>
</tr>
<tr>
<td></td>
<td>dCA1</td>
<td>$F_{(2,93)} = 1.521, n.s.$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vDG</td>
<td>$F_{(2,93)} = 3.981, p &lt; 0.05$</td>
<td>$\downarrow$ UWT(+) **</td>
</tr>
</tbody>
</table>
The dorsal and ventral CA1 displayed rather distinct changes following stress exposures: In the dCA1 only GABA A alpha1 was increased after J+U(+) (Fig. 2A), while in the vCA1 the NMDA NR2b subunit was increased after both, UWT(+) and J+UWT(+) (Fig. 2B).
These alterations might indicate a specific functional involvement of these regions. However, taking into account the heterogeneous nature of the trauma-exposed population, we built individual behavioral profiles that allow identifying affected populations within the stress-exposed groups. To further elucidate these findings, a re-analysis of the protein expression data with respect to the behavioral profiling was conducted allowing the isolation of key neural factors that contribute to a specific behavioral phenotype.

**Interaction of behavioral classification and stress exposure reveal key changes in expression patterns**

Re-analysis of the protein expression data with Univariate analysis for each target protein in each brain region revealed significant effects of the behavioral classification (unaffected vs. affected) for the GABA A alpha 1 subunit in the dorsal and ventral CA1 and in the BLA as the only factor (Fig. 3: F(1,87)=5.124, p<0.05; F(1,87)=4.522, p<0.05; F(1,87)=5.087, p<0.05, respectively).
Moreover, interactions between stress exposure and the behavioral profile were analyzed to identify changes that contribute to behavioral alterations in affected animals, but also for identifying protective factors in stress-exposed but unaffected individuals. A significant interaction was only observed for GABA A alpha 1 in the ventral CA1 and for NMDA NR2b in the dorsal CA1 (see Table 2 for summary):

![Figure 3: Effect of behavioral profile on GABA A alpha 1 subunit expression.](image)

While in dCA1 and BLA of affected animals (including rats from J+U (+) and UWT(+); n=21) expression of the GABA A alpha 1 subunit was increased, in the vCA1 such an increase was rather observed in the unaffected animals, suggesting a distinct regional function of this factor. All values are %density of mean Control (Mean±SEM). *, significant difference unaffected vs. affected, p<0.05.

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Brain region</th>
<th>Interaction stress exposure group x behavioral profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAD 65</td>
<td>dDG</td>
<td>F(1,87)= 1.217, n.s.</td>
</tr>
<tr>
<td></td>
<td>dCA1</td>
<td>F(1,87)= 0.912, n.s.</td>
</tr>
<tr>
<td></td>
<td>vDG</td>
<td>F(1,86)= 1.301, n.s.</td>
</tr>
<tr>
<td></td>
<td>vCA1</td>
<td>F(1,86)= 0.437, n.s.</td>
</tr>
<tr>
<td></td>
<td>BLA</td>
<td>F(1,87)= 2.332, n.s.</td>
</tr>
<tr>
<td>GABA A alpha 1</td>
<td>dDG</td>
<td>F(1,87)= 1.581, n.s.</td>
</tr>
<tr>
<td></td>
<td>dCA1</td>
<td>F(1,87)= 2.192, n.s.</td>
</tr>
<tr>
<td></td>
<td>vDG</td>
<td>F(1,87)= 2.051, n.s.</td>
</tr>
<tr>
<td></td>
<td>vCA1</td>
<td>F(1,87)= 4.338, p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>BLA</td>
<td>F(1,87)= 0.173, n.s.</td>
</tr>
<tr>
<td>GABA A alpha 2</td>
<td>dDG</td>
<td>F(1,87)= 2.980, n.s.</td>
</tr>
<tr>
<td></td>
<td>dCA1</td>
<td>F(1,87)= 0.262, n.s.</td>
</tr>
<tr>
<td></td>
<td>vDG</td>
<td>F(1,86)= 1.945, n.s.</td>
</tr>
<tr>
<td></td>
<td>vCA1</td>
<td>F(1,87)= 0.032, n.s.</td>
</tr>
<tr>
<td></td>
<td>BLA</td>
<td>F(1,85)= 0.708, n.s.</td>
</tr>
<tr>
<td>NMDA</td>
<td>dDG</td>
<td>F(1,87)= 0.283, n.s.</td>
</tr>
</tbody>
</table>

Table 3: Interaction between stress exposure and behavioral profile on expression of GABAergic and Glutamatergic factors in DG and CA1 of the dorsal and ventral hippocampus and in the BLA as assessed by Univariate analysis.
<table>
<thead>
<tr>
<th>NR2a</th>
<th>dCA1</th>
<th>F(1,86)= 1.211, n.s.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vDG</td>
<td>F(1,87)= 0.002, n.s.</td>
</tr>
<tr>
<td></td>
<td>vCA1</td>
<td>F(1,86)= 0.056, n.s.</td>
</tr>
<tr>
<td></td>
<td>BLA</td>
<td>F(1,86)= 0.236, n.s.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NMDA NR2b</th>
<th>dDG</th>
<th>F(1,87)= 0.016, n.s.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dCA1</td>
<td>F(1,87)= 4.673, p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>vDG</td>
<td>F(1,87)= 0.019, n.s.</td>
</tr>
<tr>
<td></td>
<td>vCA1</td>
<td>F(1,87)= 0.346, n.s.</td>
</tr>
<tr>
<td></td>
<td>BLA</td>
<td>F(1,86)= 0.891, n.s.</td>
</tr>
</tbody>
</table>

The nature of interaction was revealed by planned comparisons: GABA A alpha 1 in the vCA1 was increased after J+U(+) only in unaffected rats (Fig. 4A; ANOVA for group in unaffected: F(2,68)=7.136, p<0.01), but no such increase was observed in affected animals (T(19)=0.546, n.s.). Accordingly, an increased expression in unaffected vs. affected animals was observed only in J+U(+) rats (T(14.061)= -2.158, p<0.05).

NR2b expression did not differ significantly between stress exposure groups within unaffected animals (Fig. 4B; ANOVA for group in unaffected: F(2,68)=2.527, n.s.), but in affected animals a reduction in expression was observed after UWT(+) compared to J+U(+) (T(15.202)= -3.026, p<0.01). Within UWT(+) rats, this reduction in NR2b expression was specific for affected animals (T(20.858)= -3.450, p<0.01). However, in J+U(+) rats the reduction in NR2B expression was observed rather in unaffected animals (T(27)= 2.519, p<0.05).
Together, these results suggest that the increased expression of GABA A alpha 1 in the ventral CA1 of unaffected animals might serve as a protective factor after a history of stress experience, while the more complex pattern observed for NR2b in the dorsal CA1 may indicate a contribution to developing a pathological behavioral phenotype that is further modulated by previous exposure to stress in juvenility.
EXPERIMENT 2: Long-term effects of combined juvenile stress and UWT on mRNA expression levels in sublayers of the dorsal dentate gyrus (DG) and the BLA

Complementary to experiment 1, we investigated in impact of UWT and additional juvenile stress on the expression of target genes related to inhibitory and excitatory signaling in sublayers of the dentate gyrus. Using laser capture microdissection and quantitative real time PCR, high-resolution gene expression profiles added further insights into molecular long-term alterations within our PTSD rat model. Of special interest was here the dorsal DG as the input station for incoming information into the hippocampal formation. As a highly organized structure, the DG receives inputs from the entorhinal cortex into the outer two-thirds of its molecular layer. The granule cell layer contains the cell bodies of the primary excitatory neurons in the DG, sending their axons through the hilus region towards the CA3 region of the hippocampus (Ascadi & Kali, 2007). Especially hilus and granule cell layer are rich on a diversity of GABAergic interneurons (Houser, 2007), with in part specialized locations and contents of neuropeptides which themselves can act as GABAergic co-transmitters and neuromodulators (Freund & Buzsáki, 1996). These interneurons exert strong feedforward and feedback inhibition, shaping thereby the information flow in the hippocampal formation (Ascadi & Kali, 2007). We now started to analyze the expression of the neuropeptides cholecystokinin (CCK), somatostatin (SST) and neuropeptide Y (NPY) as well as the inhibitory GABA A receptor subunits alpha 1 and 2, GAD65 and GAD67 and the excitatory NMDA receptor subunits NR2A and NR2B in sublayers of the dorsal DG as well as in the BLA, a region that is known to modulate DG plasticity (Vouimba & Richter-Levin, 2005), after UWT. Furthermore, the behavioral profiling was applied based on activity and anxiety like responses in the EPM four weeks after UWT and the expression data was re-analyzed according to the individual performance of the animals.

Experiment 2: Methods

Behavioral protocols
Male Sprague Dawley rats (~22 days old, 30-50 g) were used as described in experiment 1. Following delivery and an additional acclimation period of five
consecutive days rats were randomly assigned to one of the three groups: (1) Control (Control, n=13); (2) UWT stress + odor reminder (UWT(+), n=13); (3) Juvenile and UWT stress exposures + odor reminder (J+U(+), n=13).

Essentially, juvenile stress and underwater trauma stress with odor reminder were conducted as described in experiment 1. However, four weeks after UWT (PND90), rats were re-exposed to the odor as described before and immediately tested in the EPM. In contrasts to experiment 1, animals were sacrificed immediately after the behavioral test in order to avoid effects of EPM exposure on gene expression.

The brains were taken out and snap frozen immediately in powdered dry ice and stored at -80°C until further processing.

Laser capture microdissection (LCM) and RNA isolation

20µm thick coronar sections were cut on a cryostat at the level of the dorsal hippocampus and BLA (-2.3 mm – -4.16 mm from Bregma; Paxinos & Watson, 1998) and thaw mounted on poly-L-lysine coated RNase-free membrane slides (Leica Microsystems, Wetzlar, Germany). After fixation in -20°C cold 70 % ethanol and brief cresyl violet acetate staining under nuclease-minimized conditions, the outer 2/3rd of the molecular layer, the granule cell layer and the hilus of the dorsal DG as well as the BLA were microdissected from 10-14 sections per animal and collected in the cap of a RNAse-free plastic tube using a laser capture microdissection system (Leica Microsystems, Wetzlar, Germany). Sample lysis and subsequent isolation of total RNA via a spin column system was conducted with the RNeasy Micro Plus kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions, including steps for removal of genomic DNA.

Reverse transcription and real time PCR

First-strand synthesis of cDNA was performed with the Sensiscript Reverse Transcription kit (Qiagen, Hilden, Germany), specifically designed for low amounts of RNA, in the presence of 2.5mM dNTPs, 50µM Oligo (dT)18 and 50 µM random decamer first strand primers (Life Technologies, Carlsbad, CA, USA) as well as RNase Inhibitor (SuperaseIN; 20 U/µl; Life Technologies, Carlsbad, CA, USA) for 60 min at 37°C. A 1:5 dilution of cDNA samples was used for determination of target
gene expression levels via quantitative PCR using the ABI Prism Step One real time PCR apparatus (Life Technologies, Carlsbad, CA, USA) and TaqMan® reagents with pre- designed assays for target genes (see Tab. 2-1) and the housekeeping gene glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH; endogenous control, assay ID: Rn_99999916_s1, Life Technologies, Carlsbad, CA, USA). Target and housekeeping genes were labeled with different fluorescent dyes, allowing for quantitative multiplex PCR.

Table 4 TaqMan gene expression assays used (all pre-designed by Life Technologies, Carlsbad, CA, USA).

<table>
<thead>
<tr>
<th>Target gene</th>
<th>assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholecystokinin (CCK)</td>
<td>Rn00563215_m1</td>
</tr>
<tr>
<td>Neuropeptide Y (NPY)</td>
<td>Rn01410145_m1</td>
</tr>
<tr>
<td>somatostatin (SST)</td>
<td>Rn00561967_m1</td>
</tr>
<tr>
<td>GAD65</td>
<td>Rn00561244_m1</td>
</tr>
<tr>
<td>GAD67</td>
<td>Rn00690300_m1</td>
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<tr>
<td>GABA A receptor alpha 1 subunit (Gabra1)</td>
<td>Rn00788315_m1</td>
</tr>
<tr>
<td>GABA A receptor alpha 2 subunit (Gabra2)</td>
<td>Rn01413643_m1</td>
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<tr>
<td>NMDA receptor 2A subunit (Grin2a)</td>
<td>Rn00561341_m1</td>
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<tr>
<td>NMDA receptor 2A subunit (Grin2b)</td>
<td>Rn00680474_m1</td>
</tr>
<tr>
<td>BDNF</td>
<td>Rn02531967_s1</td>
</tr>
</tbody>
</table>

All samples were run in triplicate assays, consisting of 50 cycles of 15s at 95°C and 1 min at 60°C, preceded by a 2 min decontamination step at 50°C with Uracil-N-Glycosidase and initial denaturation at 95°C for 10 min.

For data analysis, the mean cycle threshold (CT) was determined for each triplicate assay and relative quantification of each target gene was conducted with the ddCT method (Livak and Schmittgen, 2001), normalizing each sample to the overall content of cDNA using GAPDH as an internal control (dCT; dCT = dCT (target gene) = (CT (target gene)) - (CT (GAPDH)). Normalization of all ddCT values was done relative to control group with ddCT= dCT(sample) – mean dCT (control group). Transformation to RQ values for a specific target gene and area was done according to RQ=2\(^{-\text{ddCT}}\) with RQ\%(control)=100.
Statistical analysis.

ANOVA for group followed by LSD tests for post-hoc comparison was done for each region and target gene. Expression differences of each target within each region were compared using paired samples T-tests.

Experiment 2: Results

Combined juvenile stress and UWT increases lastingly anxiety-like behavior in the EPM

Four weeks after UWT, activity during the EPM test was reduced in rats that experienced UWT with a previous exposure to juvenile stress, as indicated by total distance covered on the EPM (Fig. 5A; F(2,36)=8.470; p<0.01) and total mobility time (Fig. 5B; F(2,36)=5.446; p<0.05). In this group anxiety-like behavior was reduced as well, as indicated by the anxiety index (open arm/ open + closed arm) for distance (Fig. 5C; F(2,36)=9.680; p<0.001) and time spent in the arms of the maze (Fig. 5D; F(2,36)=11.467; p<0.001).
Combined juvenile stress and UWT increased the expression of CCK four weeks after UWT

One-way ANOVA followed by LSD post hoc comparison examining group effects for each subregion and target gene demonstrated an increase in CCK mRNA expression levels after UWT in combination with juvenile stress only in the granule cell layer of the DG (Fig. 6A; F(2,36)=3.681; p<0.05). UWT(+) alone was not sufficient to induce increased CCK expression.

From all target genes analyzed in the dorsal DG granule cell layer, CCK was the only factor with a long-term alteration in its mRNA expression levels. In the Hilus of the dDG as well as in the BLA no significant expression changes were observed (Fig. 6B, C).
Figure 6: Increased mRNA expression levels of CCK in the dorsal DG granule cell layer after juvenile stress and UWT. (A) Four weeks after UWT mRNA expression levels of CCK were upregulated specifically in the granule cell layer of the DG in rats with previous experience of juvenile stress and underwater trauma (J+U(+)). No expression differences in selected markers genes of interneurons were observed in the Hilus of the dorsal DG (B) or in the BLA (C).

%RQ: relative quantification to control group; all values are mean±SEM, * significant difference between stress groups, p<0.05.
Behavioral profiling reveals individual animals with long-lasting alterations of activity and anxiety-like behavior

Individual behavioral profiles were built by comparing EPM measurements indicating activity and anxiety in the EPM with the control group. For that, first the normal distribution of (1) total distance, (2) total mobility time and of the (3) anxiety indices for distance and (4) for time spent on the arms was determined in the control group, reflecting the behavioral response of a "normal and healthy" population. Then, the 25th percentiles of the distributions were calculated and for each individual animal it was assessed whether it falls in such an extreme response (below 25% or above 75% of the control group's distribution) in each of the four EPM parameters. In order to be classified as "affected", i.e. showing extreme activity and anxiety responses in the EPM, an individual rat had fall into the lower or higher 25% in all of the four parameters (Fig. 7A).

As demonstrated by Pearson's chi-square test, the prevalence of affected animals depends on the previous stress experience ($\chi^2(2)=6.240, p=0.044$), with a combined exposure to juvenile stress and UWT containing the highest portion of emotionally affected animals (Fig. 7B).

Figure 7: Behavioral profiling reveals an increased prevalence of emotionally affected rats after combined exposure to juvenile stress and UWT. (A) The normal distribution of the indicated activity-and anxiety associated parameters measured in the EPM four weeks after UWT was determined for the control group. The 25th percentiles were calculated and for every animal it was assessed whether it reaches more than 75% or less than 25% of the control group in all 4 parameters. Animals that expressed such extreme behavior in all four indicated parameters were classified as “affected”. (B) Pie charts demonstrate that the prevalence of “affected” animals is increased after exposure to combined juvenile stress and UWT (J+U(+)).
Behavioral classification reveals an upregulation of NPY in the granule cell layer of affected animals

The mRNA expression data was re-analyzed with respect to the behavioral profile by comparing unaffected vs. affected animals (over all groups). This revealed a distinct upregulation of NPY in the granule cell layer of dorsal DG (Fig. 8A; T(37)= -1.654; p<0.05), while no expression difference was observed for CCK between emotionally affected animals (T(37)= -0.726; n.s.). In the same sublayer, expression of the GABA-synthesizing enzyme GAD65 was increased in affected animals as well (T(37)= -2.103; p<0.05). In the Hilus, another isoform of this enzyme, GAD67, was upregulated in affected animals (Fig. 8B; T(37)= -2.126; p<0.05). No significant differences for any target were observed in the BLA (Fig. 8C).
Figure 8: Increased mRNA expression levels of NPY in the dorsal DG granule cell layer of emotionally affected rats. (A) Four weeks after UWT mRNA expression levels of NPY were upregulated in individuals classified as “affected” based on their activity and anxiety-like behavior in the EPM in the granule cell layer of the DG. In addition, expression of the GABA-synthesizing enzyme GAD65 was increased as well. (B) The expression of the other isoform of this enzyme, GAD67, was increased in the hilus of affected animals. (C) No expression changes according to the behavioral profile were observed in the BLA.

%RQ: relative quantification to control group; all values are mean±SEM, # significant difference unaffected vs. affected, p<0.05.
Together, a distinct long-lasting expression regulation of CCK and NPY in the granule cell layer of the dorsal DG was observed (see table 5 for overview).

**Table 5: Overview** of effects of stress exposure group, behavioral profile or an interaction between stress exposure and profile effects on mRNA expression of selected target genes in the dorsal DG and the BLA. Arrows indicates significant up-regulation, after combined juvenile stress and UWT (JU) or in animals classified as emotionally affected (aff), respectively. X indicates significant interaction.

<table>
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<tr>
<th></th>
<th>dDG Granule cell layer</th>
<th>dDG Hilus</th>
<th>BLA</th>
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<td></td>
<td>group</td>
<td>profile</td>
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<tr>
<td>GAD65</td>
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<td>GABA A alpha 1</td>
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But while expression changes of NPY appear to be associated with activity and anxiety-related behavior, no such association was observed for the upregulation of CCK. Therefore, CCK function in the DG within our PTSD model might be related to other consequences of traumatic stress apart from emotional dysregulation, for example cognitive dysfunction or inapt trauma-related memory.

The long-term impact of combined juvenile stress and UWT on cognitive function and emotional memory formation will be addressed in future studies.

Accordingly, it will be tested if the prevention of such an upregulation of CCK and NPY expression in the dorsal DG (e.g. by using local viral vector mediated knock down) would extenuate such alterations in cognitive and emotional behavior within our animal model of PTSD.
Experiment 3: Long-term effects of combined juvenile stress and UWT on CCK- and NPY-positive interneurons in the dorsal DG granule cell layer

- Preliminary results -

The screening of mRNA expression changes for selected inhibitory and excitatory target genes in sublayers of the dorsal DG revealed a distinct upregulation of CCK in the granule cell layer four weeks after UWT in animals with a previous exposure to juvenile stress. Indeed, CCK is a powerful modulator of anxiety and memory, with anxiogenic and pro-mnemonic properties (Koks et al., 2000; Sherrin et al., 2009; Sebret et al., 1999).

In the DG, CCK is believed to be mainly expressed in a subgroup of basket cells (Kosaka et al., 1985) and modulate together with the fast-spiking parvalbumin-containing basket cells network activity in the DG (Lee and Soltesz, 2011). Pharmacological studies further revealed that CCK increases glutamatergic transmission in the DG (Sinton, 1988; Gabriel et al., 1996; Deng et al., 2010) as well as long-term potentiation (LTP) as a model of synaptic plasticity (Wen et al., 2014). Moreover, in the previous screening we found an upregulation of NPY mRNA expression levels in animals characterized as emotionally affected by behavioral profiling based on activity and anxiety-related parameters in the EPM. Interestingly, NPY has opposite effects compared to CCK, acting as an anxiolytic agent (Cohen et al., 2012; Serova et al., 2013) and inhibiting glutamate release as well as reducing LTP in the DG (Whittaker et al., 1999). Moreover, NPY can modulate the inputs into CCK-positive basket cells in the DG (Ledri et al., 2011).

To confirm now the expression increase of CCK and NPY in the DG granule cell layer observed in on the mRNA level, we began to assess the impact of combined juvenile stress and UWT on the number of CCK-positive interneurons in this subarea using a fluorescence-immunohistochemical (F-IHC) approach. Likewise, we compared the number of NPY-positive interneurons between emotionally affected and unaffected rats. This allows for a semi-quantitative comparison of CCK/ NPY protein levels and a first assessment of an involvement of a specific interneuron subpopulation within out rat model of PTSD.
**Experiment 3: Methods**

**Behavioral protocols**
Male Sprague Dawley rats (~22 days old, 30-50 g) were used as described in experiment 1. UWT with odor exposure was done as described in experiment 1 (UWT(+), n=5) and a subset of animals also received previous juvenile stress (J+U(+), n=6, see experiment 1). On PND90, four weeks after UWT, those rats were re-exposed to the odor and tested at the OF, followed by an odor re-exposure and testing in the EPM at PND91. On PND92, all animals were perfused with the fixative paraformaldehyde (PFA). In this preliminary experiment, control animals (n=5) were not tested in the EPM and OF, only re-exposed to the context of the UWT and perfused 90 min later at PND90. Experiments with a more stratified behavioral protocol, using controls that undergo the same test battery as the experimental groups are conducted momentarily.

**Perfusion & slice preparation**
All animals were perfused first with 0.9% Saline, followed by 4% PFA (Sigma-Aldrich, St. Louis, MO, USA). Brains were prepared, kept in 4% PFA at 4°C overnight for postfixation, immersed in 30% sucrose and then snap frozen. 30 µm thick coronal sections at the level of dorsal hippocampus were cut at a cryostat and stored free-floating in PBS + 0.02% NaN₃ (Sigma-Aldrich, St. Louis, MO, USA) at 4°C.

**Fluorescence Immunohistochemistry**
After blocking of unspecific binding sites with Backgroundsniper (Biocare Mediacal, Pike Lane, CA, USA; 20 min at room temperature), the free-floating sections were incubated with combinations of primary antibodies (in 3% bovine serum albumin (BSA) + 0.3% TritonX + PBS; over night at 4°C): rabbit α CCK (1:200; Acris, Herford, Germany) + mouse α GAD67 (1:400; Millipore, Billerica, MA, USA) or rabbit α NPY (1:3000; Abcam, Cambridge, UK) + mouse α GAD67 (1:400). After consecutive incubation with the fluorochrome-linked secondary antibodies (Alexa Fluor 555 donkey α rabbit 1:1000 + Alexa Fluor 488 donkey α mouse 1:1000; both Invitrogen, Carlsbad, CA, USA) in 3% BSA + 0.3% TritonX + PBS for 1 h at room
temperature, slices were incubated with 4',6-diamidino-2-phenylindole (DAPI; MP Biomedicals, Santa Ana, CA, USA; 0.1 mg/ml, 5 min at room temperature) for unspecific staining of nuclei. All slices were mounted on glass slides, embedded with Immunomount (Thermo Scientific, Waltham, MA, USA) and stored at 4°C protected from light.

**Analysis & Statistics:**

Digital images of each section were taken with an AxioCam MRc on Zeiss AxioScope A1 (Carl Zeiss, Jena, Germany; excitation 488 and 560 nm; 5X objective). On 2-4 sections per animal (4-8 left and right hippocampi) double-labeled cells were manual counted in the granule cell layer, including the subgranular zone (< 2 cell diameters distant from GC), by an experimenter blind to experimental groups using ZEN lite 2012 software (Carl Zeiss, Jena, Germany). For each animal the average cell number in one hippocampus (unilateral) was calculated. Animals from the different stress groups were compared using Oneway-ANOVA followed by LSD post hoc test while unaffected vs. affected animals were compared with a paired sample T-test.

**Experiment 3: Results**

**The number of CCK-positive interneurons is increased four weeks after UWT, with and without previous exposure to juvenile stress**

The number of CCK-positive interneurons (double-labeled with the marker for GABA-ergic cells, GAD67) was increased after UWT (F(2,13)=30.544; p<0.001). LSD post hoc comparison revealed that this increase occurred after UWT(+) alone (Fig. 9; p<0.001 to control) but that it was not further modulated by additional juvenile stress (p<0.000 to control; n.s. to UWT(+)).

This result confirms the long-lasting increase in CCK expression observed in the mRNA screening in experiment 2. Additionally, it indicates an increased function of CCK-positive interneurons in the DG granule cell layer after UWT alone and with previous juvenile stress exposure.
The number of NPY-positive interneurons does not differ in the dorsal DG granule cell layer of emotionally affected animals

Behavioral profiles for these animals were built as described for experiment 2, based on total distance, total mobility time and the anxiety indices for distance and time measured in the EPM test at PND91. The percentiles (below 25% and above 75%) were calculated based on the normal distribution of the control animals from experiment 1 that were tested in parallel to these animals. Animals were classified as affected when they fell into the 25th percentiles in all four parameters.

Since control animals were not tested in the EPM four weeks after UWT, the unaffected (n=4) and affected groups (n=7) contain only animals from the UWT(+) and J+U(+) groups in this preliminary experiment. The comparison of the number of
NPY-positive interneurons in the dDG granule cell layers showed no difference between unaffected and affected animals (Fig. 10; T(9)=0.168; n.s.).

Figure 10: No difference in the number of NPY-positive interneurons in the dorsal DG granule cell layer between emotionally affected and unaffected animals. (A) Four weeks after UWT the number of NPY-positive interneurons was equal between animals classified as “affected” vs. “unaffected”, based on their activity and anxiety-like behavior in the EPM. Note that in this preliminary experiment only slices from rats that have been exposed to UWT or juvenile stress and UWT, but not from controls, were analyzed (Controls not tested in EPM, no behavioral profiling possible). (B-C) Representative microscopic images illustrate comparable number of NPY-positive interneurons in the granule cell layer, including the subgranular zone. NPY-positive cells are labeled in red, the interneuron marker GAD67 is labeled in green. Values are mean±SEM.

However, in experiment 2 we observed an increase of NPY mRNA level in the same region on affected animals. The lack of increase in cell numbers could therefore indicate that either the increased mRNA is not translated in increased protein levels of NPY or that simply the number of NPY-positive interneurons is constant but the individual interneurons express more NPY. Further experiments are required to first confirm the effects and second investigate the putatively divergent mRNA and protein levels.
Task 7: The model as a drug testing platform -

We are at the initial stages of this part of the project and presented here are preliminary data –

Pharmacological tests of the juvenile stress and underwater trauma model

Experiment 1: Behavioral and molecular effects of Fluoxetine on long-term effects of underwater trauma with or without prior juvenile stress

In this experiment we aimed to study the therapeutic power of fluoxetine on the anxiety levels of rats that underwent Underwater Trauma (UWT) following an odor reminder with or without prior juvenile stress. Fluoxetine (FLX) is a common selective serotonin reuptake inhibitor (SSRI) that is widely used to treat posttraumatic stress disorder (PTSD) patients. Thus the experiment was designed as a chronic treatment following UWT. Rats received fluoxetine daily in their drinking water immediately after the UWT, lasting 4 weeks. After 4 weeks of treatment rats were tested for anxiety-like behavior following the odor reminder. Immediately following the behavioral test, brains were removed for mRNA expression levels assessment. We focused on five brain regions, namely the basolateral complex of the amygdala (BLA) as well as the dentate gyrus (DG) and the Cornu Ammonis (CA)1 region of the dorsal and the ventral hippocampus. Using a quantitative real time PCR, gene expression profile of these brain regions we intended to add further insights into stress-related molecular alterations. Thus we analyzed the expression levels of the mineralocorticoid receptor (MR), which is involved in the onset of the stress response, as well as the glucocorticoid receptor (GR) which terminates the stress response and facilitate recovery. Additionally we analyzed the inhibitory GABA A receptor subunits alpha 1 and 2, and the presynaptic markers of GABAergic activity - GAD65 and GAD67.
**Experiment 1: Methods**

**Animals**
Male Sprague Dawley rats (22 days old, 30-50 g) were used for the experiment. Animals were housed in groups of ~3, at 22 ± 2°C under 12-h light/dark cycles with water and food ad libitum. The experiment was approved by the University of Haifa Ethics and Animal Care Committees.

**Experimental groups**
Following acclimation all rats were randomly assigned to one of the following experimental conditions:
- **Juvenile and UWT stress and FLX treatment [J+U+F]** – Rats were exposed to 'juvenile stress' (PND 27-29) and, in adulthood (~PND 60) to 'UWT stress'. Following the 'UWT stress' they were treated chronically with FLX.
- **Juvenile and UWT stress [J+U]** – Rats were exposed to 'juvenile stress' and, in adulthood to 'UWT stress' (no treatment was given).
- **UWT stress [U]** – Rats were exposed only to 'UWT stress' in adulthood.
- **Juvenile stress [J]** – Rats were exposed only to 'juvenile stress'.
- **FLX treatment [F]** – Rats were treated chronically with FLX in adulthood, but were neither exposed to 'juvenile stress' nor to 'UWT stress'.
- **Control [Control]** – Rats were not exposed to stress nor to FLX treatment.

**Experimental design**
After five days of acclimatization, rats were randomly assigned to the different experimental groups. Rats from group J, J+U and J+U+F were exposed to 'Juvenile- stress' at 27-29 PNDs, comprising of 10 min forced swim (PND 27; in an opaque circular water tank of 0.5m diameter; 0.5m height; 0.4m water depth, water temperature 22±2°C), 3 trials of elevated platform (PND 28; 30 min. trials; 60 min Inter-Trials Interval (ITI) in the home cage; elevated platform of 12x12cm at 70cm above floor level, located in the middle of a room) and restraint for 2h under full light illumination (PND 29; metal mesh restraining box of 11x5x4 cm).
In adulthood (~60 PND), all rats were exposed to the odor delivery cage for 2 min per day on three consecutive days for habituation to the cage. On the 4th day, following 2 min in the box all rats were exposed to vanilla odor for 30 s. Then rats from group U, J+U and J+U+F were immediately exposed to the UWT stress, while rats from Control, F, and J groups were returned to home cage. UWT was conducted by placing the rats in a plastic tank and holding them under water for 45 s using a special metal net (20x20x15cm; adapted from Wang et al., 2000). Immediately following UWT, rats were treated chronically with fluoxetine via drinking water for approximately 4 weeks until the behavioral testing. 3 days prior to drug delivery, 24 h water consumption was determined for each cage. FLX daily dosage of 10 mg/kg/day was diluted in drinking water for J+U+F and F groups. This dosage was selected based on previous studies demonstrating that it produces clinically-relevant plasma concentrations, reduces cortical serotonin turnover in rats, and reduces behavioral indices of depression in the forced swim test. Fresh solutions were prepared twice a week using FLX stock solution (3 mg/ml) (Vetmarket, Petah-Tikva, Israel) that was added to drinking water at the required concentration. FLX concentration was determined according to average daily fluid consumption and body weight that were measured twice a week or once a week, respectively. Amber opaque drinking bottles were used to protect FLX from light degradation. All other rats were receiving regular drinking water. 4 weeks following the UWT exposure, after 2 min habituation, all rats were re exposed to the odor for 30 s in a cage with changed contextual features (colored walls, floor covered with paper tissue instead of bedding) and then were tested in the Elevated Plus Maze for 5 min (EPM; two open arms and two closed arms, with 30cm high Plexiglas walls and no roof, 50 cm above the floor). Behavior in the EPM test was recorded and analyzed by EthoVision XT8 tracking system. Immediately after the EPM test all animals were sacrificed by cervical dislocation, their brains were taken out and immediately snap frozen in powdered dry ice. Brains were stored at -80°C until further processing.

Harvesting of brain tissue
Brains were mounted on the cerebellum in the cryostat apparatus (chamber temperature -20°C) in a coronar orientation. The brain was sliced until the
rostral base of the BLA was reached (-1.8 mm from Bregma; Paxinos & Watson, 1998). With a stainless steel puncher of 1 mm diameter 1.5 mm long tissue punches were taken from each hemisphere covering the rostro-caudal axis of the BLA. The brain was further sliced until the dorsal hippocampus was fully visible (-2.8 mm from Bregma). Here, 1.5 mm long stances of the DG and the CA1 regions were harvested bilaterally. The brain was then dismounted form the cryostat holder and re-attached in a horizontal orientation in order to harvest samples from the ventral hippocampus. The brain was sliced from its ventral part until the ventral tip of the hippocampal formation (-7.6 mm from Bregma). Here, 1.5 mm long punches were taken from the DG and CA1 regions in each hemisphere, covering their ventro-dorsal axis.

All five brain regions were harvested from all rats. All tissue samples were kept frozen during the whole procedure and stored at -80°C until further processing. Frozen tissue samples were homogenized in lysis buffer and subsequent RNA and protein purification via a spin column system was conducted with the RNA/Protein Purification kit (Norgen Biotek Corp., Ontario, Canada), according to manufacturer’s instructions. Proteins were stored in -80°C until possible future processing.

Reverse transcription and real time PCR
Following Genomic DNA Elimination, first-strand synthesis of cDNA was performed with the Quantitec Reverse Transcription kit (Qiagen, Hilden, Germany), according to manufacturer’s instructions. A 1:5 dilution of cDNA samples was used for determination of target gene expression levels via quantitative PCR using the ABI Prism Step One real time PCR apparatus (Life Technologies, Carlsbad, CA, USA) and TaqMan® reagents with predesigned assays for target genes (see Tab. 1-1) and the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; endogenous control, assay ID: Rn_99999916_s1, Life Technologies, Carlsbad, CA, USA). Target and housekeeping genes were labeled with different fluorescent dyes, allowing for quantitative multiplex PCR.
Tab. 1-1 TaqMan gene expression assays used (all predesigned by Life Technologies, Carlsbad, CA, USA).

<table>
<thead>
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<tr>
<td>GAD65</td>
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<td>Nr3c1 (GR)</td>
<td>Rn00561369_m1</td>
</tr>
<tr>
<td>Nr3c2 (MR)</td>
<td>Rn00565562_m1</td>
</tr>
</tbody>
</table>

All samples were run in triplicate assays, consisting of 50 cycles of 15s at 95°C and 1min at 60°C, preceded by a 2min decontamination step at 50°C with Uracil-N-Glycosidase and initial denaturation at 95°C for 10 min.

For data analysis, the mean cycle threshold (CT) was determined for each triplicate assay and relative quantification of each target gene was conducted with the ddCT method (Livak and Schmittgen, 2001), normalizing each sample to the overall content of cDNA using GAPDH as an internal control (dCT; dCT = dCT (target gene) - (CT (target gene)) - (CT (GAPDH))). Normalization of all ddCT values was done relative to control group with ddCT = dCT(sample) - mean dCT (control group). Transformation to RQ values for a specific target gene and area was done according to RQ=2^{-ddCT} with RQ%(control)=100.

**Statistical analysis.**

Behavioral data was analyzed using One-way ANOVA followed by Bonferroni tests for post-hoc comparisons or Independent-sampels Kruskal-Wallis test. Molecular data is still preliminary (only 3 animals per group).
**Experiment 1: Results**

**Behavioral results**
We assessed the levels of activity and anxiety-like behavior using the EPM test after re-exposing the rats to the odor reminder. These results are presented in figure 1-1.

![Graph A](image)
![Graph B](image)
![Graph C](image)
![Graph D](image)

between the groups for number of entries to the open arms \([p<0.001]\). Post hoc analyses revealed a significant reduction in the percent of distance covered as well as in time spent in the open arms for rats that were exposed to juvenile stress \((p<0.05, p<0.01)\) or to juvenile stress followed by UWT, with or without FLX treatment \((p<0.001, p<.001)\), compared to controls. It also revealed a significant reduction in number of entries to the open arms for rats that were exposed to juvenile stress or to juvenile stress followed by UWT with FLX treatment \((p<0.05 \text{ compared to control or } U)\).
One-way ANOVA also demonstrated significant differences between the groups in the general activity as indicated by total distance covered in the maze \[F(5,77)=4.41; \ p=0.001\]. Post hoc analysis revealed a significant reduction only for rats that were exposed to juvenile stress as well as UWT and were treated with FLX \((p<0.01 \text{ compared to control} \text{ and } p<0.05 \text{ control to } U)\).

Clearly there was no beneficial effect of the FLX treatment on the rats' anxiety-like behavior as measured by the EPM test. Thus, in our subsequent analysis of gene expression, we decided to focus only on the non-treated groups.

**Preliminary molecular results**

Preliminary gene expression levels for our target molecules and regions are demonstrated in figure 1-2. Decapitations were made immediately following the behavioral test; hence we assume that gene expression levels represent chronic changes that were resulted out of the stressors in juvenility or in adulthood.
Preliminary results of gene expression levels: A similar trend that was partly significant was found in all molecules. In the vCA1 region gene expression levels were reduced for rats that were exposed to juvenile stress or UWT, but not for both, compared to controls. In the dDG and the BLA regions levels were reduced for all stressed groups compared to control. All values mean ±SEM. N: control-3, J-3, U-3, J+U-3. * Significant difference to Control, p<0.05; ** p<0.01; *** p<0.001. # Significant difference to J+U, p<0.05; ## p<0.01.

Fig 1-2
In the vCA1 region, One-way ANOVA indicated significant differences between the groups in the levels of GR [F(3,8)=9.28; p<0.01], MR [F(3,8)=7.65; p<0.01], GABRA1 [Independent-samples Kruskal-Wallis, p<0.03], GAD65 [F(3,8)=8.52; p<0.01] and GAD67 [F(3,8)=19.13; p=0.001]. Post hoc analyses revealed a similar trend within the different molecules. Gene expression levels are reduced for rats that were exposed to either juvenile or UWT stress, but were the same as controls for rats that were exposed to both (or even higher than controls in the case of GABRA1, excluding GAD 67 for which the level in the J+U is less than controls).

In the BLA and in the dDG regions there seem to be a trend of reduced levels of all the target molecules in all the stressed groups, compared to controls. This trend was found significant using One-way ANOVA in GABRA1 levels in the BLA [F(3,8)=5.72; p<0.05] and in GR levels in the BLA [F(3,8)=10.87; p<0.01] and in the dDG [F(3,8)=11.9; p<0.01].

No clear trend is seen at the moment in other regions.

These results are preliminary and more tissues are being analyzed at the moment. We also plan to further study alterations in protein levels for some of the target molecules according to our results.
Experiment 2: The effects of Diazepam on long-term effects of Underwater trauma with or without prior juvenile stress

In this experiment we aim to study the therapeutic power of diazepam on the anxiety levels of rats that underwent Underwater Trauma (UWT) following an odor reminder with or without prior juvenile stress. We try to use this experiment as a tool to better understand the mechanism behind our behavioral model. Diazepam is a benzodiazepine that used to be in common use for the treatment of PTSD patients. It has an anxiolytic effect since it binds to the benzodiazepine site on the GABA\(_A\) receptor, thus enhancing the effect of GABA. Nowadays diazepam is no longer the treatment of choice since it has debilitating side effects and when used chronically it may result in tolerance and dependence.

The diazepam treatment was designed as an acute treatment 30 min before behavioral testing.

Experiment 2: Methods

Animals
Male Sprague Dawley rats (22 days old, 30-50 g) were used for the experiment. Animals were housed in groups of ~3, at 22 ± 2°C under 12-h light/dark cycles with water and food ad libitum. The experiment was approved by the University of Haifa Ethics and Animal Care Committees.

Experimental groups
Following acclimation all rats were randomly assigned to one of the following experimental conditions.

Juvenile and UWT stress + Diazepam \([J+U+D]\) – Rats were exposed to 'juvenile stress' (PND 27-29) and in adulthood (~PND 60), to 'UWT stress'. They received Diazepam 1mg/kg injected ip 30min before the behavioral testing (~PND 90).
Juvenile and UWT stress exposures + Vehicle [J+U] – Rats were exposed to 'juvenile stress' and in adulthood to 'UWT stress'. They received vehicle injected ip 30min before the behavioral testing.

UWT stress + Vehicle [U] – Rats were exposed to 'UWT stress' in adulthood, but not to 'juvenile stress'. They received vehicle injection 30min before the behavioral testing.

Juvenile stress + Vehicle [J] – Rats were exposed to 'juvenile stress', but not to 'UWT stress' in adulthood. They received vehicle injection 30min before the behavioral testing.

Control + Diazepam [D] – Rats were neither exposed to 'juvenile stress' nor to 'UWT stress' in adulthood. They received Diazepam injection 30min before the behavioral testing.

Control + Vehicle [Control] – Rats were neither exposed to 'juvenile stress' nor to 'UWT stress' in adulthood. They received vehicle injection 30min before the behavioral testing.

Experimental design
The experimental design was similar to the previous experiment but instead of the chronic FLX treatment, treatment groups were treated acutely with diazepam. 30 min before rats were re-exposed to the odor 'reminder' and tested in the EPM (~PND 90), J+U+D and D group received an ip injection of Diazepam 1mg/kg (Vetmarket, Petah-Tikva, Israel). Accordingly, all other rats received an ip injection of the vehicle.

Brains are stored at -80°C for future processing.

Statistical analysis.
Preliminary behavioral data was analyzed using One-way ANOVA or Independent-sampels Kruskal-Wallis test.

Experiment 2: Results
Preliminary behavioral results
Behavioral results of the EPM test are still preliminary and presented in Fig2-1. No significant differences between the groups were found yet. There appear to be a trend of a heightened anxiety-like behavior within all rats that were exposed
to juvenile stress, whether it was followed by UWT in adulthood or not; Unless they were treated with diazepam, which seemingly lowered these anxiety-like behavior. In the general activity measure of total distance covered in the maze the trend shows a possible overall effect of reduced activity caused by the diazepam treatment.

![Graphs A-D]

**Fig 2-1** Preliminary results in the EPM test: No significant differences were found. Graphs A & C indicate a trend of higher anxiety-like behavior in rats that were exposed to juvenile stress, either followed by UWT or not, along with an opposite trend of rats that were exposed to juvenile stress followed by UWT but were treated with diazepam before the test. (A) Time spent in the open arms. (B) The number of entries to the open arms. (C) Distance covered in the open arms in relation to total distance. (D) General distance covered in the maze indicates a trend of an overall effect of activity reduction by the diazepam. All values mean ±SEM. N: control-6, F-6, J-6, U-8, J+U-5, J+U+F-6.
**Future plans**
We began to study also the effect of chronic FLX treatment immediately following the juvenile stress and until the UWT. We would like to examine the possibility that the FLX may be more effective at this time point. Additionally we plan to use another possible pharmacological agent, an exogenous neurosteroid. Endogenous neurosteroids serve as modulators of the GABAergic function in the stress response. Thus we hypothesize that they might have a beneficial effect on the rats exposed to juvenile stress and UWT in our model.

**Key research accomplishments**
Towards the end of the third year of the project, the following can already be indicated as research accomplishments:

- The UWT model, which is an ethological model of a brief but intense traumatic event (Richter-Levin, 1998) was further developed here in a way that is of particular relevance to combat soldiers. It was found to have an impact by itself, but to be a convenient platform for examining the added impact of relevant risk factors. This model has already been adopted in other laboratories, e.g:
  Adamec R, Toth M, Haller J, Halasz J, Blundell J. (2012) A comparison of activation patterns of cells in selected prefrontal cortical and amygdala areas of rats which are more or less anxious in response to predator exposure or submersion stress. Physiol Behav. 105(3):628-38.

- The maladaptive response of PTSD patients to reminder cues of the traumatic events is a hallmark of the disorder. We have incorporated this important component into our model. This has enabled us
a) To improve the sensitivity of the model as a drug testing platform.
b) To better understand variables which contribute to the effectiveness of reminder cues (in order to guide treatment).
c) To use the model to elucidate the neural mechanisms associated with abnormal responses to reminder cues.

- A rat model of high relevance to PTSD was confirmed. The finding that PTSD symptoms in this model last for over four weeks establishes it as a relevant model but also enables utilizing this model for long-term drug treatment at different time points following the exposure to the traumatic event.

- Diagnosis of psychiatric disorders in humans is based on comparing individuals to a normal population. In contrast, animal models tend to analyze averaged group effects instead, thus compromising their translational power. This discrepancy is particularly relevant to posttraumatic stress disorder (PTSD), because only a subset of individuals exposed to a traumatic experience eventually develop the disorder. We have developed a novel approach – Behavioral Profiling – with which we are able to identify exposed-affected from exposed non-affected individuals, in a way similar to the human practice. Beyond achieving improved validity of the model, the identification of affected versus exposed, non-affected individuals should enable more accurate association with pathology-related- but also with resilience-related neural mechanisms.

Reportable outcomes

Published Manuscripts (in which the support of the DOD is indicated):


**Abstracts in meetings:**


Richter-Levin G. (2013) Stress and amygdala modulation of hippocampal plasticity - relevance to PTSD. ISN annual meeting, Cancun, Mexico.


Conclusions

This report is of the third year of a 4 years project. The project is set to examine the impact of two risk factors (Childhood stress and sleep restriction) for the development of PTSD, to establish an effective platform for drug testing and to identify potential novel targets for drug development in PTSD.

We have made excellent progress in establishing childhood stress as a risk factor, and in identifying neural mechanisms associated with this risk factor, that may serve as novel potential targets for developing new drugs. We continue with this line of research into the 4th year.

We would like to state that we strongly believe that the PTSD model we have developed is superior to all animal models of PTSD currently in used for the following reasons:

1. This is the only model that takes into consideration the fact that not all individuals exposed to a traumatic event will eventually develop PTSD.
2. This model takes into consideration individual differences, thus enabling a more accurate association of neuronal alterations with behavioral outcomes.
3. This model looks into long-term effects (four weeks after the exposure to the trauma), thus increasing its relevance to PTSD-related factors.
4. This model includes the impact of reminder cues, much like the human case.

We believe it is ready for a wider utilization towards elucidating neural mechanisms associated with PTSD and with stress resilience. We will be happy to share that knowledge and to help set and train any group interested in this model – for the benefit of promoting the development of effective treatment to PTSD.

We have explored the potential contribution of sleep restriction to developing PTSD. Our results do not support such a role for sleep restriction. Following consultation with our colleagues at the Walter Reed Army Institute of Research (a discussion led by Dr. Thomas Balkin), we have conducted few additional experiments to verify that indeed this is the result. We will finalize this set of experiments during the 4th year, so that we could issue a thorough report.
We have established an effective platform for drug testing. We have started to conduct drug testing, to further validate the procedure and this goal will continue into the 4th year. Several papers were published and additional manuscripts are currently in preparation. It can be stated that progress is good and that the main aims of the project will be achieved as planned.

References


