BEHAVIORAL PROFILING AS A TRANSLATIONAL APPROACH IN AN ANIMAL MODEL OF POSTTRAUMATIC STRESS DISORDER

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Abstract

Diagnosis of psychiatric disorders in humans is based on comparing individuals to the normal population. However, many animal models analyze averaged group effects, thus compromising their translational power. This discrepancy is particularly relevant in posttraumatic stress disorder (PTSD), where only a minority develop the disorder following a traumatic experience.

In our PTSD rat model, we utilize a novel behavioral profiling approach that allows the classification of affected and unaffected individuals in a trauma-exposed population.

Rats were exposed to underwater trauma (UWT) and four weeks later their individual performances in the open field and elevated plus maze were compared to those of the control group, allowing the identification of affected and resilient UWT-exposed rats.

Behavioral profiling revealed that only a subset of the UWT-exposed rats developed long-lasting behavioral symptoms. The proportion of affected rats was further enhanced by pre-exposure to juvenile stress, a well-described risk factor of PTSD.

For a biochemical proof of concept we analyzed the expression levels of the GABAA receptor subunits $\alpha 1$ and $\alpha 2$ in the ventral, dorsal hippocampus and basolateral amygdala. Increased expression, mainly of $\alpha 1$, was observed in ventral but not dorsal hippocampus of exposed animals, which would traditionally be interpreted as being associated with the exposure-resultant psychopathology. However, behavioral profiling revealed that this increased expression was confined to exposed-unaffected individuals, suggesting a resilience-associated expression regulation. The results provide evidence for the importance of employing behavioral profiling in animal

models of PTSD, in order to better understand the neural basis of stress vulnerability and resilience.

Key words: Animal models, behavioral profiling, PTSD, risk factors, resilience, hippocampus, GABA A receptor

Introduction

Psychiatric disorders are highly prevalent (Kessler et al., 2005) and have a considerable contribution to disease burden worldwide (Bebbington, 2001). Despite great advances in neuroscience, limited methods for studying details of physiology and molecular biology in humans hold back our understanding of the pathophysiology of such disorders (Nestler and Hyman, 2010). Therefore, valid animal models of psychiatric disorders are needed. Unfortunately, the validity of current models is rather weak, leading to poor predictability of these animal models for the human condition (Kola, 2008; Markou et al., 2009). A significant compromising factor is the discrepancy in defining the affected population. In humans, the diagnosis of psychiatric disorders is based on comparing behavioral and physiological features of an individual person to an averaged normal population. In animal models however, we base our findings on group averages and thereby neglect heterogeneous responses to the manipulation. This discrepancy is particularly problematic in animal models of Posttraumatic stress disorder (PTSD). While in western populations the life-time prevalence for severely stressful events like accidents, natural disasters, assault, rape or combat situation is as high as 75-80%, only around 7% of this population will suffer from clinical relevant PTSD (Kessler et al., 2005; Olaya et al., 2015). However, such a heterogeneous response to the initial trauma is often disregarded in animal models of PTSD, compromising their translational power (Armario and Nadal, 2013; Goswami et al., 2013). Therefore, several studies began to address such individual responses by pre-defining behavioral cut-off criteria that help to form subgroups of animals with a differential stress response pattern (Cohen et al., 2004; Matar et al., 2013).

In the current study, we developed this approach further by using the control animals as the normal population and defining behavioral cut-off values according to their performance in several measurements. In this way, behavioral profiles for individual animals were built, thus resembling the diagnosis procedure in humans more closely. In our model, we utilized “underwater trauma” (UWT) as a brief traumatizing experience. UWT was previously shown to increase anxiety-like behavior (Cohen et al., 2003; Richter-Levin, 1998) and impair spatial memory as well as hippocampal synaptic plasticity (Wang et al., 2000). This model was further extended by including an exposure to UWT reminders, thus providing a platform for studying traumatic re-experiencing in this paradigm (Ardi et al., 2014; Ritov and Richter-Levin, 2014; Ritov et al., 2014). In addition, we also examined the impact of exposure to juvenile stress. Juvenile stress is a model for childhood adversity, one of the most prominent risk factors for developing anxiety disorders (Heim and Nemeroff, 2001; Pynoos et al., 1999; Sherin and Nemeroff, 2011). Thereby, it served as an epidemiological factor to further validate the behavioral profiling approach, by testing the assumption that pre-exposure to juvenile stress will exacerbate the effects of an exposure to UWT and UWT reminders in adulthood and will increase the portion of the affected population.

Lastly, we began to investigate biological correlates of PTSD-related symptoms by evaluating protein expression changes of GABAergic factors in selected limbic brain areas four weeks after UWT. Accumulative data from animals (Jacobson-Pick et al., 2008; Jacobson-Pick and Richter-Levin, 2012; Tzanoulinou et al., 2014) and humans (Lydiard, 2003; Shin and Liberzon, 2010) suggests an involvement of the GABAergic system in stress-related psychopathologies. Stress exposure is expected to induce an

abundance of expression changes which contribute to stress resilience and vulnerability. Therefore, analyzing expression changes in all stress-exposed animals might mask expression changes associated with resilience or vulnerability. However, comparing alterations in stress-exposed unaffected vs. affected rats according to their individual behavioral profiles, should allow us to identify key factors relevant to trauma resilience and/ or vulnerability, thus further enhancing the validity and predictability of our PTSD animal model.

Material and methods

Animals

Male Sprague-Dawley rats (postnatal day (PND) 22 on arrival, 30-50g, Harlan Laboratories, Jerusalem, Israel), were group housed ($22\pm 2^{\circ}\text{C}$; light-dark cycle: 12/12 hours), with water and food ad libitum. All experiments were performed in accordance to the NIH Guide for care and use of laboratory animals and approved by the University of Haifa ethical committee.

Experimental groups

Following delivery and five days acclimation, rats were randomly assigned to one of these groups: (1) Juvenile and UWT (J+U(-), n= 29), (2) Juvenile and UWT + reminder (J+U(+), n=29), (3) UWT (UWT(-), n=36) , (4) UWT + reminder (UWT(+), n=33) and (5) Control (Control, n=34).

Stress protocols

Juvenile stress (J) exposure: consisted of 3 different stressors applied during 3 consecutive days (PND 27-29, under full light condition) as described before (Horovitz et al., 2012a): 1st day - 10 minutes of forced swim stress (tank diameter 50 cm, water depth 40 cm, water temperature $22 \pm 2^\circ\text{C}$); 2nd day - elevated platform (12 x 12 cm, 70 cm above floor level) for 3 x 30 minutes (1 hour ITI in the home cage); 3rd day - 2 hours restraint.

Underwater trauma (UWT) and odor association: the *odor reminder* was combined with the UWT by habituating the rats to a standard plastic cage with a plastic lid for 4 days, 2 min per day. On the 4th day, following the 2 min habituation trial, J+U(+), UWT(+) and control rats were exposed to vanilla odor for 30 s inside the cage, while J+U(-) and UWT(-) were exposed to the cage for additional 30 s without the odor.

Immediately after the exposure to the odor or the cage, all UWT and J+U groups were exposed to the UWT, by placing the rats in a water-filled plastic tank (diameter 50 cm, height 50 cm, water depth 40 cm, water temperature $22 \pm 2^\circ\text{C}$) and, after 5 s of free swimming, restraining them under water for 45 s using a special metal net (20 x 20 x 15 cm) (Ardi et al., 2014).

Behavioral assessments

Behavioral assessments in the open field and elevated plus maze took place four weeks after UWT.

Odor re-exposure: in order to change the context of the reminder, the cage that was used for the first odor exposure was changed by replacing the type of sawdust and adding covers to the cage walls. Rats were habituated to the testing room for 2 min

and then exposed to the vanilla odor for 30 s inside the cage. Immediately after the odor exposure rats were subjected to the behavioral testing.

Open Field (OF) test was carried out as described before (Avital and Richter-Levin, 2005), by placing the rats in the corner of the open field (90 x 90 x 50 cm; dim red-light illumination), and allowing them to explore the arena freely for 5 min.

Elevated plus maze (EPM) test was carried out 24 hours after the OF test as described before (Pellow et al., 1985), by placing the rats in the center of the maze (110 x 110 cm, 70 cm above the floor; two opposing open arms/ closed arms; full light illumination), facing an open arm and allowing them to explore the maze freely for 5 min.

Behavior during OF and EPM tests was recorded and analyzed by EthoVision XT8 video tracking system (Noldus, Wageningen, Netherlands).

Behavioral Profiling

Profiling animals as “Affected” or “Unaffected” was based on the cut-off behavioral criteria (CBC) analysis approach originally proposed by Cohen et al. (Cohen et al., 2003) and further developed by Horovitz et al. (Horovitz et al., 2012b), with several modifications: two variables that represent activity levels and four variables that represent anxiety-like behaviors were selected from the OF and the EPM (Cymerblit-Sabba et al., 2015). In order to create the behavior profiles, first we referred to the performance of the control group as the behavior of the normal population and determined the distribution of values in the control group. Standard deviations were used in order to calculate the upper and lower “cut-off values” for each chosen parameter. In a second step, the performances of each animal that participated in

the experiment were compared to the distribution curve of the control group (Figure 1). In order to be classified as "affected", an animal must exhibit values that are under or above the lower and upper cut-off values in at least four out of the six measurements.

Harvesting brain tissues

One day following the last behavioral test (PND 92), brains were taken out for biochemical analysis from rats of the J+U(+) (n=29), UWT(+) (n=33) and Control (n=34) groups. Brains were snap frozen and in a cryostat apparatus (at -20°C; Leica, Wetzlar, Germany) frozen tissue punches (diameter: 1 mm; depth 1.5 mm) were collected from each hemisphere covering the rostro-caudal axis of the basolateral amygdala complex (BLA; -1.8 mm from Bregma), the dorsal dentate gyrus (dDG) and the dorsal Cornu Ammonis 1 (dCA1; both starting -2.8 mm from Bregma). Bilateral punches were taken from the ventral DG and CA1 (vDG and vCA1; starting -7.6 mm from Bregma, horizontal orientation) according to Paxinos and Watson brain atlas (George and Watson, 1998).

Protein sample preparation and Western Blot

Frozen tissue samples were homogenized in 300µl Urea Lysis Buffer (1mM EDTA, 0.5% Triton X, 6M Urea, 100µM PMSF; Sigma-Aldrich, St. Louis, MO) with freshly added protease and phosphatase inhibitor cocktail (complete ultra and PhosStop tablets; Roche Diagnostics, Mannheim, Germany). 10 µg of each sample was loaded on a 12% SDS-polyacrylamide gel for electrophoresis (SDS-PAGE). After semi-dry transfer (nitrocellulose membrane) and blocking, membranes were incubated with

primary antibodies over night at 4°C, using rabbit α GABAA α 1 1:2500 and rabbit α GABAA α 2 1:2500 (both Synaptic systems, Göttingen, Germany) and rabbit α GAPDH 1:2000 (Cell Signaling, Beverly, MA, USA) as endogenous control.

After incubation with complementary secondary antibody (α rabbit, polyclonal, 1:15 000), ECL Plus substrate (Advansta, Menlo Park, CA, USA) enabled chemiluminescence detection (Supplementary figure 1) and density of signals was analyzed with the 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

First, differences in the behavioral responses and protein expression levels between the different stress groups was conducted by using one-way ANOVA, followed by bonferroni or LSD post hoc tests. Second, individual behavioral profiling was applied and the distribution of affected vs. unaffected populations was calculated by using Pearson's chi-squared test. 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. 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, and t-test for affected vs. unaffected in J+U(+) group.

Results

Averaged group effects in the Open field and the Elevated plus maze following

stress exposure *Total distance*: measuring activity by distance travelled in the OF and the EPM (Figure 2Aa,b), one-way ANOVA revealed a significant main effect for group ($F_{(4,156)}=2.688$, $p<0.05$ and $F_{(4,156)}=9.577$, $p<0.001$, respectively). Further post hoc comparisons revealed a reduction in total activity in the OF only in J+U(+) compared to UWT(+) group, while in the EPM, both J+U(-) and J+U(+) rats showed hypo activity compared to Control and UWT(+) groups.

Anxiety index (distance): calculating the anxiety index by relative presence in the safe zones of the OF and EPM (Figure 2Ba,b), we found a significant main effect for group ($F_{(4,156)}=5.997$, $p<0.001$ and $F_{(4,156)}=10.744$, $p<0.001$). In the OF, both J+U groups showed elevated anxiety levels compared to UWT(+) group. Adding the reminder further increased anxiety levels in the J+U(+) group compared to control and UWT(-) groups. In the EPM, both J+U groups showed elevated anxiety levels compared to all other groups.

Freezing behavior: measuring freezing behavior as expressed in total freezing time in the OF and the EPM (Figure 2Ca,b), we found a significant main effect for group ($F_{(4,156)}=4.47$, $p<0.01$ and $F_{(4,156)}=8.136$, $p<0.001$). In the OF, the J+U(+) group spent significantly more time freezing compared to all other groups. In the EPM, both J+U groups showed more freezing behavior compared to control and UWT(+) groups. In addition, J+U(+) rats spent more time freezing compared to UWT(-) rats as well.

Overall, these results demonstrate long-lasting hypo-activity and increased anxiety-like behaviors after the exposure to the combination of juvenile stress and UWT. This pattern of results is further aggravated by the exposure to the UWT reminder.

Assessing the prevalence of affected animals in the different groups by using the Behavioral profiling approach

Taking into account the heterogeneous nature of a trauma-exposed population, not all stress-exposed individuals may be lastingly affected by the traumatic experience. Thus, we built individual behavioral profiles that allow identifying affected populations within the different groups.

Distribution of "affected" animals across the different groups: The number of individuals that exhibit values that are under or above the lower or upper cut-off values in at least four out of the six behavioral parameters were compared across the different stress-exposure groups and Pearson χ^2 analysis revealed a significant difference in their distribution (Figure 3; χ^2 likelihood ratio(4)=21.05, $p<0.001$).

Specific comparisons between groups using Pearson χ^2 analysis revealed an increased prevalence of "affected" rats in the J+U(-) group compared to control group (χ^2 likelihood ratio(1)=4.75, $p<0.05$), while in the J+U(+) group the prevalence of "affected" rats was increased compared to all other groups (control: χ^2 (1)=18.39, $p<0.001$; UWT(-): χ^2 (1)=10.857, $p<0.001$; UWT(+): χ^2 (1)=9.28, $p<0.01$; J+U(-): χ^2 (1)=4.48, $p<0.05$).

These results clearly demonstrate the effectiveness of the behavioral profiling approach in translating group averages into an analysis that takes into account the heterogeneous response of individuals inside the different groups.

In order to further validate these findings we analyzed the protein expression levels of the GABAA receptor subunits $\alpha 1$ and $\alpha 2$ in the ventral and dorsal hippocampus and in the BLA. Here, based on the fact that the behavioral profiling clearly highlights the odor reminder as a significant factor in the model that empowers the effect of

the double exposure to juvenile stress and UWT (as demonstrated in the prevalence of affected animals in J+U(+) group), we chose to analyze expression changes only in animals that were exposed to the odor reminder (i.e: control, UWT(+) and J+U(+))

Identifying key neural factors by using the behavioral profiling approach

The analysis of expression levels using group averages demonstrates that numerous expression changes occur between the different stress-exposure groups (Supplementary figure 2): Expression of GABAA α 1 was increased in the J+U(+) group compared to control in all analyzed brain regions and also increased compared to UWT(+) in both the dorsal CA1 and DG and the BLA (One-way ANOVA in dCA1: $F_{(2,93)}=11.75$, $p<0.001$; dDG: $F_{(2,93)}=4.138$, $p<0.05$; vCA1: $F_{(2,93)}=3.544$, $p<0.05$; vDG: $F_{(2,93)}=5.12$, $p<0.01$; BLA: $F_{(2,93)}=5.267$ $p<0.01$). GABAA α 2 expression levels were increased in both, UWT(+) and J+U(+) groups, compared to controls in the vDG and BLA ($F_{(2,92)}=9.206$, $p<0.001$ and $F_{(2,93)}=4.49$, $p<0.05$, respectively).

As it was shown above, behavioral profiling demonstrated a heterogeneous lasting effect of stress-exposure on activity and anxiety-like behavior in individual animals within the different groups. Thus, the mere analysis of group averages might mask expression alterations associated with different behavioral profiles of individuals which in turn might reflect different coping strategies with the traumatic stress. Therefore, we re-analyzed expression levels according to the behavioral profiling conducted before. Here, univariate analysis for each target protein in each brain region was applied assessing the interactions between stress exposure and the behavioral profiles (unaffected vs. affected).

Changes in GABAA $\alpha 1$ and $\alpha 2$ expression according to the behavioral profiling: For the GABAA $\alpha 1$ subunit, a significant interaction was only observed in the vCA1 and the vDG (Figure 4Aa,b; $F_{(2,90)}=12.432$, $p<0.001$ and $F_{(2,90)}=4.513$, $p<0.05$, respectively). Planned comparisons in the vCA1 revealed a specific increase of GABAA $\alpha 1$ expression in unaffected rats of the J+U(+) group (ANOVA between groups of unaffected population $F_{(2,63)}=17.71$, $p<0.001$), while no expression regulation was observed in affected animals ($F_{(2,27)}=1.583$, n.s.). This was further confirmed by a direct comparison of vCA1 $\alpha 1$ levels in unaffected vs. affected J+U(+) rats ($T_{(27)}=4.937$, $p<0.01$). A similar expression pattern was observed in the vDG, with an increased expression of GABAA $\alpha 1$ in unaffected vs. affected animals only in J+U(+) rats ($T_{(27)}=12.86$, $p<0.05$; ANOVA for group in unaffected rats only: $F_{(2,63)}=11.01$, $p<0.001$), but no expression regulation in stress-exposed affected animals ($F_{(2,27)}=0.272$, n.s.).

For the GABAA $\alpha 2$ subunit, a significant interaction was observed in the vCA1 (Figure 4Ba; $F_{(2,90)}=4.267$, $p<0.05$), but not in the vDG (Figure 4Bb; $F_{(2,89)}=1.13$, n.s.). Similar to $\alpha 1$ levels in the vCA1, $\alpha 2$ expression levels were only increased in unaffected J+U(+)-exposed rats (T-test unaffected vs. affected $T_{(27)}=2.528$, $p<0.05$; ANOVA for group in unaffected: $F_{(2,63)}=6.203$, $p<0.01$), while no difference was observed in affected animals ($F_{(2,27)}=0.77$, n.s.).

No interactions were found in the BLA or the dorsal parts of the hippocampus (Supplementary figure 3).

Overall, we report here a specific dissociation in the expression of GABAA $\alpha 1$ and $\alpha 2$ subunits in the ventral but not in the dorsal parts of the hippocampus, with a specific elevation only in unaffected animals with a history of a juvenile stress exposure.

Taken together, these results may indicate a role for GABAA $\alpha 1$ and $\alpha 2$ in the ventral hippocampus in stress resilience.

Discussion

Animal models are essential for understanding the neurobiology of psychiatric disorders (Hyman, 2008; Markou et al., 2009; Nestler and Hyman, 2010). Unfortunately, these models tend to suffer from reduced validity that compromises the ability to translate findings from animal studies to the human condition (McGonigle et al., 2014). Poor predictability of animal models is particularly evident in PTSD research. Most PTSD animal models try to address key factors of PTSD according to the DMS-V (American Psychiatric Association, 2013), such as exposure to a traumatic stressor, assessment of PTSD-like behavioral alterations and consideration of the time criterion (Matar et al., 2013). However, one of the most striking features of PTSD, the heterogeneity of the trauma-exposed population, is often neglected. The fact that only a subset of people will develop PTSD after exposure to a traumatic event (Kessler et al., 2005; Santiago et al., 2013), suggests differential mechanisms that mediate stress resilience and vulnerability in a trauma exposed population. This observation is in stark contrast to the traditional approach in many animal models that consider all trauma-exposed animals as the “affected population”. In order to strengthen the validity of the models and increase our understanding of its neurobiological correlates, testing methods based on assessing the response of individual animals are needed (Armario et al., 2013; Goswami et al., 2013).

Several approaches have been developed to address such individual differences in animals' response to stress. One strategy especially used in genetic-oriented research is to select animals with extreme response patterns and create different inbred lines. The individual variability within one inbred line is thereby reduced and groups with extreme phenotypes can be compared more easily, e.g. mouse or rat lines with high vs. low anxiety levels, which also respond differentially to stressors and display distinct activity of neuronal populations in relevant brain circuits (Diaz-Moran et al., 2013). However, while inbred strategies are valuable for determining the genetic components of different behavioral and physiological responses, a direct comparison of animals with different stress responses would resemble the human condition more closely. One possibility is to dissect the heterogeneous response pattern by using cut-off criteria for different behavior measures to form subgroups of animals (Avrastos et al., 2013; Cohen et al., 2003; Lebow et al., 2012). For example, in the cut-off behavior criteria algorithm developed in the Cohen lab, animals are exposed to predator stress and subsequently classified into extreme vs. minimal behavioral responders based on their behavior in the EPM and acoustic startle response tests (Cohen et al., 2003; Cohen et al., 2004; Matar et al., 2013).

In the current study, we now developed this approach further by creating dynamic behavioral cut-off criteria. Here, instead of predefining restrictive cut-off values (Matar et al., 2013), we analyzed the activity and anxiety-like behaviors of the control group, which served as the 'normal population', resembling more closely the human diagnostic procedure. As employed before by Diaz-Moran et al. (Diaz-Moran et al., 2013), the cut-off values are then defined by using ± 1 standard deviation from

the control group mean in representative behavioral measures of activity and anxiety in the OF and EPM.

In the current study, we used brief restraint under water (underwater trauma), as the traumatic stressor. This procedure is known to acutely and enduringly increase anxiety-like behavior (Ardi et al., 2014; Richter-Levin, 1998; Sood et al. 2013) without any tissue damage (Moore et al., 2012). We could confirm these results by analyzing activity and anxiety-like behaviors in the EPM and OF four weeks later on the group level, thus further supporting the relevance of UWT as an animal model of trauma-related PTSD.

To increase the translational validity of the employed UWT rat model, we then introduced the behavioral profiling approach by comparing the performance of individual animals in the UWT and JS+UWT groups to the distribution of the control population. Animals were classified as “affected” when they fell below or above the cut-off values in at least five out of six selected behavioral parameters that reflect anxiety-like behavior and activity (Cymerblit-Sabba et al., 2015). With this approach, around 20% of the UWT-exposed animals were classified as affected, resembling the prevalence of PTSD in exposed human populations (Kessler et al., 2005, Santiago et al., 2013). Moreover, pre-exposure to juvenile stress, consisting of variable, intense, uncontrollable stress in their post-weaning/ pre-pubertal life phase (Horovitz et al., 2012a) resulted in an increase in the proportion of affected animals. This closely resembles the situation in humans, where childhood adversity is one of the most prominent risk factors for developing PTSD upon exposure to a trauma later in life (Heim and Nemeroff, 2001; Sherin and Nemeroff, 2011). Thus, while comparing group averages between UWT and JS+UWT is sufficient to demonstrate an

exacerbation of UWT-induced long-term alterations in activity and anxiety, behavioral profiling was able to confirm the impact of juvenile stress as a risk factor. A further increase in the proportion of affected animals was evident following the exposure to an odor reminder of UWT. This extends previous findings that show increased anxiety following an exposure to trauma reminders shortly after the traumatic experience (Ardi et al., 2014; Cohen et al., 2009; Ritov et al., 2014). This long-term impact of reminders, as highlighted by the behavioral profiling analysis, matches the time criteria of PTSD as a chronic disorder in humans (American Psychiatric Association, 2013). Furthermore, it is also in agreement with findings in humans, since trauma-associated odors were found to be able to provoke anxiety and fear-related memories in PTSD patients (Dittmar et al., 1996; Vermetten and Bremner, 2003).

Together, the increased proportion of affected animals after exposure to juvenile stress as well as olfactory trauma reminders revealed by using the behavioral profiling approach, demonstrates a direct translation of epidemiological findings from humans in our rat model for PTSD, thus further highlighting the relevance of incorporating the analysis of individual responses into PTSD animal models.

A further expectation from the behavioral profiling approach is that the behavioral categorization would be associated with differences in relevant brain mechanisms between stress-exposed affected and unaffected animals.

Among other factors, reduced GABAA receptor function may contribute to the maladaptation of the GABAergic system that is involved in the development of PTSD-like symptoms: For instance, plasma levels of GABA as the internal agonist for GABAA receptors are lastingly reduced in PTSD patients (Vaiva et al., 2006). In

addition, the sensitivity to modulators of the GABAA receptors like benzodiazepines and neurosteroids is reduced in women suffering from PTSD (Moller et al., 2015). A quantitative lower binding of benzodiazepines to GABAA was also confirmed in the hippocampus of male veterans suffering from PTSD by using Positron emission tomography (PET) with the labeled benzodiazepine antagonist (Geuze et al., 2008). In contrast, genetic studies show a significant interaction of childhood trauma and single nucleotide polymorphisms (SNP) in the *Gabra2* gene coding for the GABAA $\alpha 2$ subunit (Nelson et al., 2009), while a certain SNP may even be protective against the development of addiction co-morbidity after childhood trauma (Enoch et al., 2010). GABA has been indicated in anxiety and emotional memory formation within the hippocampus and amygdala (Makkar et al., 2010; Muller et al., 2014; Nuss, 2015). We therefore analyzed alterations in the expression of GABAA receptor subunits in the ventral and dorsal hippocampus as well as in the BLA.

We focused on the expression of the most abundant subunits of the GABAA receptor, $\alpha 1$ and $\alpha 2$. Expression levels of $\alpha 1$ and $\alpha 2$ subunits in amygdala and hippocampus have been shown previously to be regulated by the stress hormone corticosterone (Orchinik et al., 1995) and by behavioral stressors with relevance to PTSD, such as social isolation stress (Matsumoto et al., 2007) and combined juvenile / adult stress (Jacobson-Pick et al., 2008; Jacobson-Pick and Richter-Levin 2012). By analyzing averaged changes within each stress exposure group, we observed an increase in GABAA $\alpha 1$ and $\alpha 2$ subunit expression in most of the selected areas one month after an exposure to UWT and a history of JS exposure. Thus, the expression changes were observed in the stress-exposure group with the most significant

behavioral changes in the EPM and OF and could therefore be intuitively interpreted as being associated with a pathological maladaptation.

However, re-analyzing the expression data employing the behavioral profiling approach revealed that the increase in GABAA $\alpha 1$ expression in the vDG and vCA1 as well as for $\alpha 2$ in the vCA1 exclusively appears in animals that have been exposed to a history of stress (J+U group), but have been classified as unaffected according to their behavioral phenotype.

These results may suggest a role for GABAA $\alpha 1$ and $\alpha 2$ subunits in resilience to traumatic stress while the lack of such a regulation in affected animals might contribute to the maladaptive PTSD-like phenotype. Importantly, this finding was only revealed when using the behavioral profiling approach and would have been missed by analyzing group averages.

Interestingly, the observed expression regulation of GABAA $\alpha 1$ and $\alpha 2$ was restricted to ventral hippocampal areas and was not observed in the dorsal hippocampus. Indeed, stress and stress hormones such as corticosterone differentially affect long-term plasticity (LTP) in the ventral and dorsal hippocampus: acute stress exposure and activation of mineralo- and glucocorticoid receptors results in increased LTP in ventral hippocampus, while LTP in the dorsal hippocampus is attenuated (Maggio and Segal, 2007; Maggio and Segal, 2009). Moreover, in animals with a history of combined juvenile and adult stress exposure, this facilitation of synaptic plasticity in the ventral hippocampus even persists over weeks (Maggio and Segal, 2011).

In fact, the ventral hippocampus plays an important role in mediating behavioral and endocrine stress responses, emotional memory formation and anxiety (Fanselow and Dong, 2010) due to its intimate connection with other limbic structures such as

amygdala, hypothalamus, nucleus accumbens and prefrontal cortical areas (Hubner et al., 2014; Naber and Witter, 1998; Prasad et al., 2013; van Groen et al., 1990; Varela et al., 2014). Therefore, as postulated in the dynamic routing hypothesis, facilitated neuronal transmission in the ventral hippocampus after stress exposure may lead to a dominance of emotional processing over cognitive processing, which is related to dorsal hippocampal areas (Segal et al., 2010). Based on the findings of the current study, one can speculate that emotional information processing in the ventral hippocampus of stress-exposed unaffected rats may be more effectively controlled due to the increased expression of the inhibitory GABAA $\alpha 1$ and $\alpha 2$ subunits, enabling them to better cope with their history of stress exposure.

Whether such a GABAA $\alpha 1$ and $\alpha 2$ expression pattern in the ventral hippocampus is generally associated with reduced anxiety behavior in the open field and elevated plus maze would need further investigations, such as determining the impact of juvenile stress alone and in relation to reminder exposure. In addition, interventional studies using locally delivered viral vectors or pharmacological manipulations could further validate a causal relationship of ventral hippocampal GABAA levels and anxiety.

Clearly, in a complex disorder such as PTSD GABA is not the only player. The current results might provide an interesting entry point for developing resilience-promoting strategies, but more importantly, these results serve to highlight the significant contribution of the behavioral profiling approach. Thus, by analyzing individual performances, neurobiological correlates of vulnerability and resilience can be detected within our models in a way that is not possible when using the traditional averaged group analysis approach.

Conclusions

Overall, we believe that this work serves as a proof of concept for the behavioral profiling approach as a tool for translating the diagnostic procedure of psychiatric disorders into animal models by addressing stress responses of individual animals. Thereby, the ability to address mechanisms of vulnerability and resilience and to isolate their relevant neurobiological factors correlates is improved, enabling the development of new valid therapeutic strategies in the future.

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Figure Legends

Figure 1: Behavioral Profiling Approach

(A) Representative distribution curve of a given variable in the control group. Standard deviations were used for calculating the upper and lower “cut-off values”. The performances of all animals were compared to the relevant distribution curve of the control group.

(B) Representative example of a behavioral profile of an "affected" animal. In order to be classified as affected, an animal must exhibit values below or above the “cut-off values” in at least four out of the six measurements.

Figure 2: Averaged group effects in the Open field and the Elevated plus maze following stress exposure

(A) Total distance (cm): In the OF **(a)**, J+U(+) rats were less active compared to UWT(+) rats. In the EPM **(b)**, both J+U(-) and J+U(+) groups, were less active in the EPM arena compared to control and UWT(+) groups.

(B) Anxiety index (distance): In the OF **(a)**, increased anxiety-like behavior was observed in both J+U groups compared to UWT(+) group, while in J+U(+) rats anxiety-like behavior was further increased compared to control and UWT(-) groups as well. In the EPM **(b)**, both J+U groups showed higher levels of anxiety-like behavior compared to all other groups.

(C) Freezing behavior: In the OF **(a)**, J+U(+) rats spent more time freezing compared to all other groups. In the EPM **(b)**, both J+U groups showed increased freezing behavior compared to control and UWT(+) rats. J+U(+) rats spent more time freezing compared to UWT(-) rats as well.

All values are mean±SEM. *significant difference from control, $p<0.05$, ** $p<0.01$; ^ different from UWT(-), $p<0.05$, ^^ $p<0.01$; # different from UWT(+), $p<0.05$, ## $p<0.01$, ### $p<0.001$; \$ different from J+U(-), $p<0.05$.

Figure 3: Assessing the prevalence of affected animals in the different groups by using the Behavioral profiling approach

By using the behavioral profiling approach, we detected a significant higher proportion of "affected" animals among J+U(-) rats compared to the control population. Moreover, the proportion of "affected" animals was further increased among J+U(+) rats, compared to all other groups

Values are the % unaffected and affected animals in each group. * different from control, $p<0.05$, *** $p<0.001$; ^^ different from UWT(-), $p<0.001$; ### different from UWT(+), $p<0.001$; \$ different from J+U(-), $p<0.05$.

Figure 4: Identifying key neural factors by using the behavioral profiling approach

(A) For GABAA $\alpha 1$ a significant interaction of stress-exposure group x behavioral profile was observed in the ventral CA1 **(a)** and in the ventral DG **(b)**. An increased expression of GABAA $\alpha 1$ was only observed in unaffected rats of the J+U(+) group, compared to unaffected rats from the control and UWT(+) groups. Direct comparisons also revealed an increase of GABAA $\alpha 1$ levels in unaffected rats compared to affected rats within the J+U(+) group.

(B) A similar expression pattern was observed for GABAA $\alpha 2$ in the ventral CA1 of unaffected rats in the J+U(+) group **(a)**, while no significant interaction was found for GABAA $\alpha 2$ in the ventral dentate gyrus **(b)**.

All values are % density of mean Control (mean $\pm$ SEM). *** different from control, $p<0.001$; # different from UWT(+), $p<0.05$, ## $p<0.01$, ### $p<0.001$; + significant difference unaffected vs. affected rats, $p<0.05$, ++ $p<0.01$.

Figure 1

Figure 2

Figure 3

Figure 4