INTRODUCTION

Adverse experiences in early life can have life-long consequences on the adaptation to further stressful events and have been identified as a prime risk factor in the development of psychopathologies such as depression or anxiety disorders later in life (Heim & Nemeroff, 2001; McLaughlin et al., 2010), and for cognitive decline in ageing (Brunson...
et al., 2005). To investigate the neurobiological correlates of childhood adversity, animal models of early life stress have been developed. We and others utilize juvenile stress (JS), an exposure to variable stressors during the post-weaning/ prepubertal life phase (Albrecht et al., 2017). Juvenile stress shapes the response to additional stressful experience in adulthood (adult stress, AS) such as fear conditioning (Müller et al., 2014; Yee et al., 2012), two-way shuttle avoidance learning (Horovitz et al., 2012) or traumatic stress (Ardi et al., 2016), and lastingly increasing anxiety (Ardi et al., 2016; Gruber et al., 2015; Müller et al., 2014). These behavioral changes have also been associated with altered GABAergic functions in different hippocampal fields (see Albrecht et al., 2017 for review).

In particular, JS combined with an additional challenge in adulthood lastingly increased the protein expression of the GABA-A receptor subunits α2 in lysates of the hippocampus (Jacobson-Pick & Richter-Levin, 2012). A refined analysis utilizing JS in combination with traumatic stress in adulthood revealed that the increased expression of the GABA-A receptor subunits α1 and α2 is restricted to the ventral hippocampus (Ardi et al., 2016, 2019).

JS and its combination with AS (JS/AS) have also consequences on long-term potentiation (LTP) in the hippocampus. But while LTP is decreased in the dorsal CA1, LTP is rather increased in the ventral CA1 area of the hippocampus (Grigoryan et al., 2015; Ivens et al., 2019; Maggio & Segal, 2011). Recently we found that astrocytes and a shift in the glutamate-glutamine cycle critically contribute to the lasting increase in ventral CA1 LTP after JS and its combination with AS. Using layer-specific laser microdissection and qPCR, we recently found a reduced steady-state mRNA expression of the astrocytic enzyme glutamine synthetase specifically in the stratum radiatum (SR) of the ventral CA1 and demonstrated its importance for JS induced increase of LTP (Ivens et al., 2019).

Shifts in the balance of excitation/ inhibition within the CA1 may further be determined by a similar long-term regulation of steady-state mRNA levels of neuronal GABAergic and glutamatergic signaling factors. We therefore assessed mRNA expression changes of GABAergic and glutamatergic factors as a long-term consequence of single JS, AS or combined JS/AS in layers of the dorsal and ventral CA1 using laser microdissection and qPCR. We found a differential expression regulation of the GABA synthesizing enzymes GAD65 and GAD67 as well as of Gabra1 and Gabra2, the mRNA for the GABA A receptor subunits α1 and α2, after single versus combined JS/AS. In contrast, mRNA levels of subunits of the glutamatergic receptors AMPA and NMDA were not altered by stress. Correlational analysis assessed the altered balance between excitatory and inhibitory factors and the association with individual expression levels of glucocorticoid (GR) and mineralcorticoid receptors (MR).

2 | METHODS AND MATERIALS

2.1 | Animals

All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Weizmann Institute. Male Wistar rats, born and raised locally, were housed 4 per cage on a 12 hr light-dark cycle with lights on at 7 a.m., room temperature 22 ± 2°C.

2.2 | Behavioral manipulations

At postnatal days (PND) 27-29 rats were exposed to three different stressful episodes once a day (juvenile stress, JS) as described previously (Maggio & Segal, 2011). Briefly, rats were exposed to 15 min of forced swim (PND 27), 30 min exposure to an elevated platform (PND 28) and 2 hr restraining in a tube (PND 29). In young adulthood at PND 60, animals were exposed to 15 min forced swim again (adult stress, AS). Rats were then left undisturbed in their home cages until brain preparation two weeks later. Next to animals receiving combined juvenile and adult stress (JS/AS, n = 6), additional groups of animals were exposed to only one stressful experience in juvenility (JS, n = 6) or adulthood (AS, n = 6). A control group (n = 6) was handled only.

2.3 | mRNA expression

On PND 74, brains were prepared for expression analysis via laser microdissection and quantitative real-time PCR as described previously (Albrecht et al., 2016; Ivens et al., 2019) by snap-freezing them in liquid nitrogen-cooled methylbutane. After storage at −80°C, 20 µm thick horizontal sections of the dorsal (starting at −4.1 mm from Bregma) and ventral hippocampus (−6.3 until −6.8 mm from Bregma, Paxinos & Watson, 1998) were cut on a cryostat and thaw-mounted on poly-L-lysine coated RNase-free membrane slides. After brief staining nuclease-minimized cresyl violet acetate staining, stratum oriens (SO), stratum pyramidale (SP) and stratum radiatum (SR) of the ventral and dorsal CA1 area were microdissected and collected from 8 to 10 sections per animal using a laser microbeam dissection system (Carl Zeiss, Jena, Germany). After tissue lysis total RNA was isolated with the RNeasy Micro Plus kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions, including gDNA removal steps. First-strand cDNA was synthesized with the Sensiscript Reverse Transcription kit (Qiagen, Hilden, Germany) using 2.5mM dNTPs, 50µM Oligo (dT)18 and 50 µM random decamer first strand
primers (Life Technologies, Darmstadt, Germany) as well as RNase Inhibitor (SuperaseIN; 20U/µl; Life Technologies, Darmstadt, Germany) for 60 min at 37°C. Duplex real time PCR was performed with a 1:5 dilution of cDNA using the ABI Prism Step One real time PCR apparatus (Life Technologies, Darmstadt, Germany) and TaqMan® reagents with predesigned assays for the target genes (see Table 1) and for the housekeeping gene Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH; endogenous control, Life Technologies, Darmstadt, Germany). After 2 min 50°C decontamination with Uracil-N-glycosidase and initial de-naturation at 95°C for 10 min, triplicates for each sample were run in with 50 cycles of 15 s at 95°C and 1 min at 60°C. 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 & Schmittgen, 2001), normalizing each sample to the overall content of cDNA using GAPDH as an internal control (dCT; \( dCT = CT(\text{target gene}) - CT(\text{GAPDH}) \)). The dCT value for each sample was used for statistical analysis. Normal distribution of dCT data across groups from one subarea was assessed by Shapiro–Wilks test. The main effect of JS and AS and their interaction were assessed by 2-way-ANOVA. For direct comparison of JS x AS interactions, all four possible groups (Control, JS, AS, JS/AS) were compared with one-way-ANOVA and LSD post hoc test to dissect such an interaction demonstrated an decreased expression of GAD65 within the CA1 SR (\( F(3,20) = 3.32, p = 0.041 \)) especially after combined JS/AS compared to a slight increase after single JS (\( p = 0.022 \)) or AS (\( p = 0.008 \)). No significant main group differences were observed for in the dorsal CA1 SP (\( F(3,20) = 1.95, p = 0.151 \)).

For GAD67, interactions of JS x AS were revealed by the two-way-ANOVA in the dorsal CA1 SP (\( F(3,20) = 5.899, p = 0.025 \)), but the direct comparison of stress groups failed to reach significance (\( F(3,20) = 2.429, p = 0.095 \)). No interactions or main effects of JS and AS were found in any of the other dorsal and ventral CA1 layers analyzed for this factor.

### Table 1

| Target gene | Alias | Assay ID | Probe exon location (nt) | Amplicon length (nt) |
|-------------|-------|----------|--------------------------|----------------------|
| GAD65       | Gad2  | Rn00561244_m1 | 4 to 5                  | 79                   |
| GAD67       | Gad1  | Rn00566593_m1 | 5 to 6                  | 107                  |
| Gabra1      | GABA A receptor, alpha 1 | Rn00788315_m1 | 6 to 7                  | 75                   |
| Gabra2      | GABA A receptor, alpha 2 | Rn01413643_m1 | 7 to 8                  | 123                  |
| Gria1       | AMPA1 | Rn00709588_m1 | 3 to 4                  | 85                   |
| Gria2       | AMPA2 | Rn00691893_m1 | 3 to 4                  | 65                   |
| Grin1       | NMDAR1| Rn01436038_m1 | 3 to 4                  | 68                   |
| Grin2a      | NMDAR2A| Rn00561340_m1 | 3 to 4                  | 59                   |
| Grin2b      | NMDAR2B| Rn00680477_m1 | 3 to 4                  | 79                   |
| Nr3sc1      | GR    | Rn00561369_m1 | 1 to 2                  | 73                   |
| Nr3sc2      | MR    | Rn00565562_m1 | 4 to 5                  | 79                   |

*Note: Predesigned TaqMan® gene expression assays were used in this study (Life Technologies, Darmstadt, Germany).*

### RESULTS

#### 3.1 | Differential regulation of GAD65, Gabra1 and Gabra2 mRNA levels by single stress versus combined stress in the ventral CA1

A two-way-ANOVA for JS and AS effects revealed significant interactions of both stressors on the expression of GAD65 in dorsal CA1 SP (Figure 1a, middle: \( F(1,20) = 4.851, p = 0.04 \)) and in ventral CA1 SR (Figure 1a, left: \( F(1,20) = 6.621, p = 0.018 \)). A direct comparison of all four stress groups in a one-way-ANOVA followed by LSD post hoc test to dissect such an interaction demonstrated a decreased expression of GAD65 within the CA1 SR (\( F(3,20) = 3.32, p = 0.041 \)) especially after combined JS/AS compared to a slight increase after single JS (\( p = 0.022 \)) or AS (\( p = 0.008 \)). No significant main group differences were observed for in the dorsal CA1 SP (\( F(3,20) = 1.95, p = 0.151 \)).
FIGURE 1  Differential regulation of GAD65, Gabra1 and Gabra2 mRNA levels by single stress versus combined stress in the ventral CA1. (a) In the ventral CA1 SR, GAD65 was upregulated 14 days after single juvenile stress (JS) and stress in adulthood (AS), but downregulated after combined exposure to JS/AS. (b) No impact of JS, AS or its combination was observed on the expression of GAD67. (c) Within the ventral CA1 SR the expression levels of the GABA A receptor alpha 1 subunit (Gabra1) were reduced after combined JS/AS only as well. (d) For the Gabra2 subunit of the same receptor type, the expression increases after single JS and AS versus decrease in combined JS/AS was observed in the ventral CA1 SP, in addition with increased expression after JS in the dorsal CA1 SP. For GAD65 and Gabra1, expression differences between the dorsal and ventral CA1 SO and CA1 SP, respectively, were observed. Values are shown as individual data points and mean dCT ± SEM, the normalized expression to the internal control gene GAPDH, with high dCT values indicating lower expression levels. For illustration of quantitative expression changes refer to the table below graph (relative quantification with Control expression levels set to 100%). * Significant difference between groups, \( p < .05 \), **\( p < .01 \); # significant difference dorsal (DH) versus ventral hippocampus (VH) (2-tailed T-test), \( p < .05 \)

| % RO | Gria1 DH | Gria1 VH | Gria2 DH | Gria2 VH |
|------|---------|---------|---------|---------|
|      | Control | JS      | AS      | JS/AS   |
| DH   | 100     | 142.90  | 112.93  | 118.09  |
| VH   | 100     | 200.22  | 135.53  | 99.78   |

| % RO | Grin1 DH | Grin1 VH | Grin2a DH | Grin2a VH | Grin2b DH | Grin2b VH |
|------|---------|---------|----------|----------|----------|----------|
|      | Control | JS      | AS       | JS/AS    |
| DH   | 100     | 152.69  | 75.29    | 93.97    | 100      | 172.72   | 82.74    | 89.78    |
| VH   | 100     | 147.63  | 109.73   | 73.89    | 100      | 143.39   | 25.34    | 89.82    |

FIGURE 2  mRNA expression of glutamatergic factors remains unchanged by JS/AS in the ventral CA1. (a) No lasting expression changes were observed for the AMPA receptor subunits Gria1 and Gria2 after juvenile stress (JS), adult stress (AS) or combined JS/AS exposure in the CA1 stratum pyramidale (SP) of the ventral and dorsal hippocampus (VH and DH), the layer containing the pyramidal cell somata. (b) A reduced expression of the NMDA receptor subunit Grin2a was observed especially in the ventral CA1 SP after AS, while the other subunits Grin1 and Grin2b remained unaffected by stress. Note the expression differences between DH and VH for all factors analyzed. Values are shown as individual data points and mean dCT ± SEM, the normalized expression to the internal control gene GAPDH, with high dCT values indicating lower expression levels. For illustration of quantitative expression changes refer to the table below graph (relative quantification with Control expression levels set to 100%). * Significant difference between groups, \( p < .05 \), **\( p < .01 \); # significant difference dorsal (DH) versus ventral hippocampus (VH) (2-tailed T-test), \( p < .05 \)

| % RO | Grin1 DH | Grin1 VH | Grin2a DH | Grin2a VH | Grin2b DH | Grin2b VH |
|------|---------|---------|----------|----------|----------|----------|
|      | Control | JS      | AS       | JS/AS    |
| DH   | 100     | 152.69  | 75.29    | 93.97    | 100      | 172.72   | 82.74    | 89.78    |
| VH   | 100     | 147.63  | 109.73   | 73.89    | 100      | 143.39   | 25.34    | 89.82    |

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For Gabra1, interactions of JS x AS were revealed by the two-way ANOVA in the dorsal CA1 SR (Figure 1c, left: $F(1,20) = 7.867, p = .011$), but the direct comparison of stress groups was not significant ($F(3,20) = 2.746, p = .07$). In the ventral CA1 SR two-way-ANOVA indicated a trend for JS x AS interaction (Figure 1c, left: $F(1,20) = 3.268, p = .086$) and the direct stress group comparison revealed a decreased Gabra1 expression ($F(3,20) = 3.29, p = .042$) especially in JS/AS compared to all other groups ($p = .019$ for Control, $p = .013$ for JS, $p = .028$ for AS).

**FIGURE 3** Correlation of mRNA expression levels for GABAergic and glutamatergic factors in the ventral hippocampus. (a) Exemplary correlograms for dCT expression values of GAD65 and the AMPA and NMDA receptor subunits Gria1 and Grin1, respectively. Person's correlation coefficient ($r$) for dCT values of GAD65 in ventral CA1 stratum radiatum (SR) and dCT values of the glutamatergic AMPA receptor subunits Gria1 (GluR1) and Gria2 (GluR2) as well as the NMDA receptor subunits Grin1 (GluN1), Grin2a (GluN2a) and Grin 2b (GluN2b) in ventral CA1 stratum pyramidale (SP) are summarized for each stress exposure group (JS: juvenile stress; AS: adult stress) in the table on the right. Note that after exposure to single stress (JS or AS) expression levels were positively correlated, but this effect was lost after combined exposure to JS/AS, indicating a possible shift in excitatory-inhibitory balance in the ventral hippocampus after such a history of stress exposure (b) Exemplary correlograms for dCT expression values of Gabra1 and the AMPA and NMDA receptor subunits Gria1 and Grin1 and table of correlation coefficients for Gabra1 in ventral CA1 SR and glutamatergic receptor subunits in ventral CA1 SP. (c) Exemplary correlograms for dCT expression values of Gabra2 and the AMPA and NMDA receptor subunits Gria1 and Grin1 and table of correlation coefficients for Gabra2 and glutamatergic receptor subunits in ventral CA1 SP. Note again the shift of correlations from control to JS and AS groups and the lack of correlation exclusively in the JS/AS group. * Significant Pearson's correlation (2-tailed), $p < .05$, **$p < .01$, ***$p < .001$
For Gabra2, no main effects of JS or AS were discovered and interactions of JS x AS could not be assessed due to non-normal distribution of data. When applying a Kruskal–Wallis test for comparing the four stress groups, a significant upregulation of this GABA receptor subunit after single JS was observed in the dorsal CA1 SP (Figure 1d, middle: H(3) = 9.153, p = .027; p = .016 to control; p = .014 to JS/AS). In the ventral CA1 SP, a differential regulation of Gabra2 between single and combined stress is revealed (Figure 1d, middle: H(3) = 8.831, p = .032) with an increased expression after JS (p = .034) and AS (p = .007) compared to a slightly decreased JS/AS.

Thus, a differential long-term expression regulation of GAD65, Gabra1 and Gabra2 by single JS or AS versus combined JS/AS is demonstrated especially in sublayers of the ventral CA1. In addition, we compared the expression profiles between the dorsal and ventral CA1 sublayers and found a significant increase of GAD65 mRNA levels in the dorsal compared to ventral CA1 SO after JS (Figure 1a, right; T(10) = −3.202, p = .009). Expression levels of Gabar1 where increased in the dorsal versus ventral CA1 SP after JS (T(10) = −3.756, p = .004) and after AS (T(10) = −3.773, p = .004), while no significant differences between dorsal and ventral GAD67 and Gabra2 levels where observed.

3.2 mRNA expression of glutamatergic components remains unchanged by JS/AS in the ventral CA1

Expression regulation within the excitatory system was checked by analyzing the mRNA expression levels of AMPA and NMDA receptor subunits in the CA1 SP thus covering the somata of the excitatory neurons.

No main effects of JS, AS and no JS x AS interaction were observed in the dorsal or ventral CA1 SP for the AMPA subunits Gria1 and Gria2 or for the NMDAR subunits Grin1 and Grin2b. For Grin2a, however, a main effect of AS was observed (Figure 2b, middle: U = 2.598, p = .008). The group analysis revealed a reduction of Grin2a mRNA in the ventral CA1 SP after AS (H(3) = 10.607, p = .014) compared to Control (p = .016) and JS (p = .002).

In general, the expression levels of Gria1, Gria2 and Grin1 were higher in the dorsal CA1 than in the ventral CA1 in Control animals (Figure 2: Gria1- T(10) = −2.612, p = .026; Gria2- T(10) = −3.073, p = .012; Grin1- T(10) = −4.519, p = .001) and after JS/AS (Figure 2: Gria1- T(10) = −3.199, p = .01; Gria2- T(10) = −3.358, p = .007; Grin1- T(10) = −4.944, p = .001). Increased dorsal CA1 Grin1 expression levels were also observed in the JS group (Figure 2b: T(10) = −2.703, p = .022).

3.3 Correlation of mRNA expression levels for GABAergic and glutamatergic factors in the ventral CA1

To assess the shifts in the balance between inhibitory and excitatory factors after significant expression alterations for GAD65, Gabra1 and Gabra2 in the ventral CA1, a correlation analysis was performed. Positive correlations of expression levels of GAD65 in the ventral CA1 SR with the glutamatergic factors in the ventral CA1 SP were observed in the control group but also after single JS and AS (Figure 3a). Only for GAD65 x Grin2a a negative correlation was observed, most likely driven by reduced Grin2a mRNA levels after AS. The positive correlation of mRNA levels was lost after combined JS/AS.

For Gabra1 in the ventral CA1 SR no significant correlation with the expression levels of glutamatergic factors was observed within the control group, JS or JS/AS, but for Gabra1 x Gria1 after AS (Figure 3b).

For the correlation of Gabra2 with glutamatergic factors negative values were observed in the control group that switched to mainly significant positive correlations after JS and also AS, while again no significant correlations were observed after JS/AS (Figure 3c).

In summary, this demonstrates that combined exposure to JS/AS, but not to single JS or AS, induces a lasting imbalance of excitatory and inhibitory signaling in the ventral hippocampus especially driven by GAD65.

3.4 GAD65 expression in the ventral CA1 SR is associated with corticosterone receptor expression

Finally, we examined whether GAD65 expression changes may relate to differences in corticosterone-dependent signaling and examined the expression levels of MR and GR the ventral and dorsal CA1 layers. No significant stress-induced changes were observed for the mineralocorticoid receptor (MR) in any of the layers. A significant interaction of JS x AS was observed for the expression of the glucocorticoid receptor (GR) within the dorsal and ventral CA1 SR (Figure 4b, left: dorsal: F(1,20) = 10.912, p = .004; ventral: F(1,20) = 7.646, p = .012). No JS x AS interactions or main effects were determined for the for dorsal and ventral CA1 SP and SO. Within dorsal CA1 SR, the stress group comparison revealed a significant decrease of GR mRNA levels (F(3,20) = 3.882, p = .024) in the JS/AS group compared to the differential increase in the JS (p = .02) and AS (p = .009; p = .045 to controls) group. In the ventral CA1 SR, the group comparison failed to reach significance (F(3,20) = 2.641, p = .077). However, a regression analysis suggests a
FIGURE 4 GAD65 expression in the ventral CA1 Stratum radiatum (SR) is associated with glucocorticoid receptor expression. (a) While mRNA expression levels of the mineralocorticoid receptor (MR) for corticosterone were unchanged in CA1 SR, SP and SO, (b) the expression of the glucocorticoid receptor (GR) was affected in the CA1 SR by a single exposure to either juvenile (JS) or adult stress (AS). Although this regulation was only significant in the dorsal hippocampus (DH), the expression profile of GR in the ventral hippocampal (VH) was comparable to that of GAD65 in the same area, suggesting a possible relation of GAD65 and GR expression levels. (c) Statistically, this was tested by a regression analysis using GAD65 dCT expression values as dependent variable of GR and MR dCTs, demonstrating a dependence of GAD65 expression on GR, but not on MR expression in this area. (D) Further correlation analysis for dCTs of GAD65 and GR in each treatment group revealed a strong association between mRNA expression of both factors after either JS or AS, but not after combined JS/AS. Note also the difference in dorsal versus ventral hippocampal expression levels (a,b) for MR and GR in CA1 SO as well as in CA1 SP and SR for MR. Expression values in (a, b) are shown as individual data points and mean dCT ± SEM, the normalized expression to the internal control gene GAPDH, with high dCT values indicating lower expression levels. For illustration of quantitative expression changes refer to the table below graph (relative quantification with Control expression levels set to 100%). * Significant difference between groups, $p < .05$, ** $p < .01$. Pearson’s correlation coefficients (r) are shown next to (c), with *** indicating significant correlation of GAD65 and GR dCTs, $p < .001$.
significant association of GR and GAD65 expression profiles in the ventral CA1 SR (Figure 4c), that is further supported by a correlation analysis per stress group (Figure 4d).

In addition, differential expression levels of MR between the dorsal and ventral CA1 SO were observed (Figure 4a, right; Control: T(10) = −2.298, p = .044; JS: T(10) = −3.184, p = .01; AS: T(10) = −3.68, p = .004; JS/AS: T(10) = −3.778, p = .004) as well as in CA1 SR (Figure 4a, left; JS/AS (Mann–Whitney U-test): U(10) = 2.082, p = .041) and in CA1 SP (Figure 4a, middle; JS/AS: T(10) = −3.002, p = .013). For GR, increased expression in the dorsal compared to the ventral CA1 SO were observed as well (Figure 4b, right; Control: T(10) = −4.095, p = .002; JS/AS: T(10) = −3.88, p = .006).

4 | DISCUSSION

In the current study, we utilized juvenile stress (JS) as a rat model for childhood adversity, a prominent risk factor for developing anxiety disorders and depression as well as cognitive decline later in life (Brunson et al., 2005; Heim & Nemeroff, 2001; McLaughlin et al., 2010). Following JS, we previously reported increased plasticity in the ventral CA1 in association with decreased neurotransmitter metabolism in astrocytes mediated by the enzyme glutamine synthetase (Ivens et al., 2019). Now, we set out to investigate the long-term expression regulation of neuronal factors of GABAergic and glutamatergic transmission in the ventral and dorsal CA1 following JS and its interaction with a subsequent stress experience in adulthood.

We observed a differential change in the expression of inhibitory factors following a single stress exposure in juvenility or adulthood and a dysregulation after combined JS/AS stress exposure. Particularly, GAD65 was increased after single JS or AS but decreased after JS/AS within the ventral CA1 SR, while a similar pattern emerged for Gabra2 in the ventral CA1 SP. For Gabra1 a reduction was observed after combined JS/AS, but not single JS or AS in the ventral CA1 SR. Such a differential expression change was not observed for the expression of GAD67 and for AMPA and NMDA receptor subunits. However, individual AMPA and NMDA receptor subunit expression levels, with the exception of Grin2a, were correlated with levels of GAD65 in the ventral CA SR and with Gabra1 and with Gabra2 in the ventral CA1 SP after single juvenile and young adult stress exposure. In line with a potential loss of allostatic regulation, most of these correlations were inverted after combined JS/AS. Moreover, GAD65 expression in the CA1 SR was correlated with expression of GR, suggesting a possible regulation by corticosterone.

Several studies demonstrate that hippocampal expression levels of GABAergic factors are indeed regulated by stress and corticosterone in rats and mice, showing a reduced expression of hippocampal Gabra1 after chronic administration of corticosterone (Orchinik et al., 1995) and after a chronic stress (Matsumoto et al., 2007). Combined exposure to JS and mild AS, but not a single stress experience, increased the protein expression levels synthesized from Gabra1 and Gabra2 in whole hippocampus preparations (Jacobson-Pick & Richter-Levin, 2012). Moreover, when analyzing the long-term impact of combined JS and traumatic stress in adulthood on GABA-A α1 and α2 subunit expression, the increased expression was restricted to the ventral CA1 and dentate gyrus subregions of the hippocampus but not to the dorsal. Interestingly, they were especially upregulated in a subgroup of resilient animals that behaved like a non-stressed control group in anxiety tests (Ardi et al., 2016, 2019). A similar resilience-associated regulation pattern was not observed when traumatic stress in adulthood occurred without additional JS (Ardi et al., 2016). Thus, upregulation of GABA-A receptor subunits α1 and α2 after a single stress in the ventral CA1 may rather relate to an adaptive stress response via increased ventral hippocampal inhibition whereas a second stress experience in adulthood appears to abolish this regulation.

A differential expression regulation was observed for GAD65 as well in ventral CA1 SR, with a lasting increase after single JS and AS compared to the rather low expression levels after combined exposure. Expression regulation of GAD65 by acute or chronic stress in stress-relevant brain areas like hypothalamic subnuclei or the hippocampal subareas have been reported before (Bowers et al., 1998; Herman & Larson, 2001). A lasting reduction of GAD65 protein is also observed in the ventral, but not the dorsal hippocampus after chronic mild stress (Elizalde et al., 2010). Mice with a total knock out of GAD65 are available that display a behavioral phenotype of heightened stress susceptibility (Müller et al., 2015). Interestingly, heterozygous GAD65 knock out (GAD65±) show a delayed maturation of the GABAergic system with reduced levels of GABA during juvenility (Stork et al., 2000). Submitting GAD65± mice to fear conditioning as AS, they showed comparable freezing levels to unstressed controls, while their wild-type littermates showed an increase of contextual fear memory generalization when sensitized with a preceding JS (Müller et al., 2014). Thus, upregulation of GABAergic signalling may be beneficial after a single stress exposure, providing allostasis, i.e. the maintenance or neuronal functioning even under heightened stress levels (McEwen, 2017). After a second stress experience in adulthood such a regulation appears to break down.

Importantly, the altered GAD65 expression profile was not compensated by regulation of GAD67 mRNA, which is the critical isoform for GABA synthesis especially during prenatal development (Müller et al., 2015). Previous studies have demonstrated the regulation of GAD67 expression in the hippocampus after acute and chronic stress as well as in...
response to chronic corticosterone administration (Bowers et al., 1998; Gilabert-Juan et al., 2016; Stone et al., 2001). Recently, increased GAD67 in the CA1 has been linked to resilience to traumatic stress in a rat model of PTSD (Skórzewska et al., 2020), raising the possibility that the expression regulation of GAD67 may contribute to stress adaptation dependent on the time line of stress exposure and the stress exposure models use.

In our current study, no long-term expression changes of selected NMDA and AMPA receptor subunits were observed in the SP after JS, AS or JS/AS either, indicating largely unaltered excitatory responsiveness of CA1 principal cells (Nomura et al., 1997). The only exception provides Grin2a, which was upregulated in ventral CA1 after AS. Although stressful experiences and corticosterone can influence the expression of such glutamatergic factors in the hippocampus, at least acutely and in certain time windows (Martisova et al., 2012; Owen & Matthews, 2007; Rosa et al., 2002), the lasting impact of combined JS/AS in our experiments thus is seen predominantly concerning the regulation of GABAergic factors. The expression profiles of the glutamatergic factors in the CA1 SP and of GAD65 in CA1 SR correlated positively in non-stressed controls and this correlation was further strengthened after a single stress experience. Similarly, correlation of Gabra2 and the glutamatergic factors on CA1 SP switched form a negative correlation in controls to a positive correlation after single stress either in juvenility or adulthood. Remarkably, such positive correlations were absent in animals that experienced combined JS/AS, further indicating a disturbance of excitation/inhibition balance in the ventral CA1 following combined JS/AS.

Moreover, regression analysis suggested that GAD65 mRNA expression patterns after JS, AS and JS/AS in the ventral CA1 SR are strongly associated with the expression of GR. Previously it has been shown that corticosterone administration transiently decreases GAD65 mRNA expression levels in CA1 SO and SR (Stone et al., 2001), supporting a possible layer-specific regulation of GAD65 expression driven by corticosterone that should be addressed in further studies. Interestingly, Mikkelsen et al. (2008) suggested that a feedback loop between corticosterone levels and the hippocampal GABAergic system may exist that provide a feedback control on CRH neurons in the paraventricular nucleus of the hypothalamus. Thus, the GABAergic system in the ventral CA1 could contribute to a feedback regulation on the endocrine stress axis. By contrast, although significant regulation of GR was evident in the dorsal CA1, this was not associated with any local change in GAD65 levels, indicating a fundamental difference in the regulation of GAD65 between dorsal and ventral hippocampus.

Moreover, increased GR expression by JS and AS were observed specifically in the dorsal CA1 SR, a layer where cell bodies of different subtypes of interneurons are located, including subtypes of basket cells, Schaffer-collateral-associated cells (SCA) and interneuron-specific interneurons (ISI). Interestingly, the subtype of basket cells with cell bodies located in CA1 SR as well as the SCA interneurons express the neuropeptide cholecystokinin (CCK) as a marker, while ISI neurons express vasoactive intestinal polypeptide (VIP) (Pelkey et al., 2017; Albrecht). A stress-induced modulation of CCK and VIP expression within the CA1 region has been previously studied in chronic stress models, although rather minor changes are observed (see Albrecht et al., 2020, for recent review). However, CCK is a well-described anxiogenic agent and a chemogenic activation of CCK-positive cells in the forebrain increases anxiety (Whissel et al., 2019), while blocking CCK-receptors reduces anxiety and increases stress coping in animals with a history of restraint stress or social defeat (Becker et al., 2008; Wang et al., 2011). In our previous study we found that animals that develop increased anxiety after previous exposure to juvenile stress and underwater trauma in young adulthood show increased activation of CCK-positive interneurons in the ventral DG (Regev-Tsur et al., 2020). It would be interesting to study the activation and also GR-dependent modulation of CCK-positive interneurons and the yet less well studied VIP interneurons in the ventral CA1 after juvenile stress and stress in young adulthood (Albrecht et al., 2020, for review).

Taken together, our data suggest that lasting changes in the mRNA expression of GABAergic factors may contribute to adaptive changes in the ventral hippocampus after juvenile stress. Effects of stress experience in the adult on these factors are highly dependent on such pre-experience and can lead to a loss of allostatic regulation of steady-state mRNA levels, putatively involving local GR. While the dorsal hippocampus shows strong connectivity with higher cortical areas and functional role in cognitive processes and spatial memory, the ventral hippocampus is highly connected with brain areas relevant for emotional control such as amygdala and the hypothalamus and is important for stress response, anxiety and fear memory (Fanselow & Dong, 2010; Maggio & Segal, 2012; Segal et al., 2010). A dysregulation of factors controlling excitation/inhibition balance in the ventral CA1 upon dual stress exposure may thus be critical to the development of behavioral and physiological disturbances including anxiety-like responses, altered emotional memory formation and stress coping deficits of juvenile stressed animals in adulthood (Ardi et al., 2016; Horovitz et al., 2012; Müller et al., 2014; Yee et al., 2012).

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REFERENCES

Albrecht, A., Ivens, S., Papageorgiou, I. E., Çalışkan, G., Saiepour, N., Brück, W., Richter-Levin, G., Heinemann, U., & Stork, O. (2016). Shifts in excitatory/inhibitory balance by juvenile stress: A role for neuron-astrocyte interaction in the dentate gyrus. Glia, 64, 911–922. https://doi.org/10.1002/glia.22970

Albrecht, A., Müller, I., Ardi, Z., Çalışkan, G., Gruber, D., Ivens, S., Segal, M., Behr, J., Heinemann, U., Stork, O., & Richter-Levin, G. (2017). Neurobiological consequences of juvenile stress: A GABAergic perspective on risk and resilience. Neuroscience and Biobehavioral Reviews, 74, 21–43. https://doi.org/10.1016/j.neubiorev.2017.01.005

Albrecht, A., Redavide, E., Regev-Tsur, S., Stork, O., & Richter-Levin, G. (2020). Hippocampal GABAergic interneurons and their co-localized neuropeptides in stress vulnerability and resilience. Neuroscience and Biobehavioral Reviews: S0149–7634(20)30634–5.

Ardi, Z., Albrecht, A., Richter-Levin, A., Saha, R., & Richter-Levin, G. (2016). Behavioral profiling as a translational approach in an animal model of posttraumatic stress disorder. Neurobiology of Diseases, 88, 139–147. https://doi.org/10.1016/j.nbd.2016.01.012

Ardi, Z., Richter-Levin, A., Xu, L., Cao, X., Volkmer, H., Stork, O., & Richter-Levin, G. (2019). The role of the GABAA receptor Alpha 1 subunit in the ventral hippocampus in stress resilience. Scientific Reports, 9, 13513. https://doi.org/10.1038/s41598-019-49824-4

Becker, C., Zeeu, C., Rivat, C., Bluegot, A., Hamon, M., & Benoliel, J. J. (2008). Repeated social defeat-induced depression-like behavioral and biological alterations in rats: Involvement of cholecystokinin. Molecular Psychiatry, 13, 1079–1092. https://doi.org/10.1038/sj.mp.4002007

Bowers, G., Cullinan, W. E., & Herman, J. P. (1998). Region-specific regulation of glutamic acid decarboxylase (GAD) mRNA expression in central stress circuits. Journal of Neuroscience, 18, 5938–5947. https://doi.org/10.1523/JNEUROSCI.18-15-05938.1998

Brunson, K. L., Kramár, E., Lin, B., Chen, Y., Colgin, L. L., Yanagihara, T. K., Lynch, G., & Baram, T. Z. (2005). Mechanisms of late-onset cognitive decline after early-life stress. Journal of Neuroscience, 25, 9328–9338. https://doi.org/10.1523/JNEUROSCI.2281-05.2005

Elizalde, N., García-Garcia, A. L., Totterdell, S., Gendive, N., Venzala, E., Ramirez, M. J., Del Rio, J., & Tordera, R. M. (2010). Sustained stress-induced changes in mice as a model for chronic depression. Psychopharmacology (Berl), 210, 393–406. https://doi.org/10.1007/s00213-010-1835-6

Fanselow, M. S., & Dong, H. W. (2010). Are the dorsal and ventral hippocampus functionally distinct structures? Neuron, 65, 7–19. https://doi.org/10.1016/j.neuron.2009.11.031

Gilbert-Juan, J., Bueno-Fernandez, C., Castillo-Gomez, E., & Nacher, J. (2016). Reduced interneuronal dendritic arborization in CA1 but not in CA3 region of mice subjected to chronic mild stress. Brain Behav., 7, e00534.

Grigoryan, G., Ardi, Z., Albrecht, A., Richter-Levin, G., & Segal, M. (2015). Juvenile stress alters LTP in ventral hippocampal slices: Involvement of noradrenergic mechanisms. Behavioral Brain Research, 278, 559–562. https://doi.org/10.1016/j.bbr.2014.09.047

Gruber, D., Gilling, K. E., Albrecht, A., Bartsch, J. C., Çalışkan, G., Richter-Levin, G., Stork, O., Heinemann, U., & Behr, J. (2015). 5-HT receptor-mediated modulation of granule cell inhibition after juvenile stress recovers after a second exposure to adult stress. Neuroscience, 292, 67–79. https://doi.org/10.1016/j.neuroscience.2015.02.050

Heim, C., & Nemeroff, C. B. (2001). The role of childhood trauma in the neurobiology of mood and anxiety disorders: Preclinical and clinical studies. Biological Psychiatry, 49, 1023–1039. https://doi.org/10.1016/S0006-3223(01)01157-X

Herman, J. P., & Larson, B. R. (2001). Differential regulation of forebrain glutamic acid decarboxylase mRNA expression by aging and stress. Brain Research, 912, 60–66. https://doi.org/10.1016/S0006-8993(01)02641-5

Horovitz, O., Tsoory, M. M., Hall, J., Jacobson-Pick, S., & Richter-Levin, G. (2012). Post-weaning to pre-pubertal (juvenile) stress: A model of induced predisposition to stress-related disorders. Neuroendocrinology, 95, 56–64. https://doi.org/10.1159/000331393

Ivens, S., Çalışkan, G., Papageorgiou, I., Cesetti, T., Malich, A., Kann, O., Heinemann, U., Stork, O., & Albrecht, A. (2019). Persistent increase in ventral hippocampal long-term potentiation by juvenile stress: A role for astrocytic glutamine synthetase. Glia, 67, 2279–2293. https://doi.org/10.1002/glia.23683

Jacobson-Pick, S., & Richter-Levin, G. (2012). Short- and long-term effects of juvenile stressor exposure on the expression of GABAA receptor subunits in rats. Stress, 15, 416–424.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Journal of Cellular and Molecular Medicine, 5, 520–531. https://doi.org/10.1023/A:1011809425686

Martisova, E., Solas, M., Horrillo, I., Ortega, J. E., Meana, J. J., Tordera, R. M., & Ramírez, M. J. (2012). Long lasting effects of early-life stress on glutamate/GABAergic circuitry in the rat hippocampus. Neuropharmacology, 62, 1944–1953. https://doi.org/10.1016/j.neuropharmacology.2011.12.019
