The pathophysiological impact of stress on the dopamine system is dependent on the state of the critical period of vulnerability

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Abstract
Unregulated stress during critical periods of development is proposed to drive deficits consistent with schizophrenia in adults. If accurate, reopening the critical period could make the adult susceptible to pathology. We evaluated the impact of early adolescent and adult stress exposure (combination of daily footshock for 10 days and 3 restraint sessions) on (1) midbrain dopamine (DA) neuron activity, (2) ventral hippocampal (vHipp) pyramidal neuron activity, and (3) the number of parvalbumin (PV) interneurons in the vHipp and their associated perineuronal nets (PNNs). Ventral tegmental area (VTA) DA neuron population activity and vHipp activity was increased 1–2 and 5–6 weeks post-adolescent stress, along with a decrease in the number of PV+, PNN+, PV+/PNN+ cells in the vHipp, which are consistent with the MAM model of schizophrenia. In contrast, adult stress decreased VTA DA neuron population activity only at 1–2 weeks post stress, which is consistent with what has been observed in animal models of depression, without impacting vHipp activity and PV/PNN expression. Administration of valproate (VPA), which can re-instate the critical period of plasticity via histone deacetylase (HDAC) inhibition, caused adult stress to produce changes similar to those induced by adolescent stress, presumably by increasing stress vulnerability to early adolescent levels. Our findings indicate that timing of stress is a critical determinant of the pathology produced in the adult: adolescent stress led to circuit deficits that recapitulates schizophrenia, whereas adult stress induced a depression-like hypodopaminergic state. Reopening the critical period in the adult restores vulnerability to stress-induced pathology resembling schizophrenia.

Introduction
The etiology of most major psychiatric disorders remains undetermined, but likely involves genetic and socio-environmental risk factors and their interaction. Interestingly, these disorders share both socio-environmental, including stressful events [1, 2], and genetic risk factors [3]. For example, around 40% of the genetic variations observed in schizophrenia are also found in depression [4], and depression and schizophrenia tend to run in the same families [5]. However, importantly, these conditions differ in the mean age of onset. Whereas schizophrenia is usually diagnosed during late adolescence/early adulthood, depression is more common in adults with the age of diagnosis reaching a peak near 32 years [6]. These evidences point to an intriguing possibility; i.e., that socio-environmental factors, such as stressful events, may influence the risk of developing these disorders, with the age of exposure a critical determinant of the pathophysiology that arises in the adult.

One system impacted by stress is the parvalbumin (PV)-expressing GABAergic interneurons [7]. During pre-adolescence, PV interneurons are highly plastic in terms of excitatory drive and neuronal activity [8], which also causes them to be particularly susceptible to damage by stressors [7]. However, following adolescence PV interneurons develop perineuronal nets (PNNs), a unique extracellular matrix structure that aggregates around the soma and proximal dendrites of certain neurons. These PNNs attenuate plasticity and protects PV interneurons from stress-induced damage [7, 9].
In humans, a loss of PV interneurons in the anterior limbic hippocampus, a region homologous to the ventral hippocampus (vHipp) in rodents [10], is proposed to lead to a hyperactive state of this region. This hyperactivity was found to drive the increased dopamine (DA) system activity in the ventral tegmental area (VTA) that is associated with the psychotic symptoms of schizophrenia [10, 11]. Thus, our hypothesis is that exposure to stress during adolescence, which is a sensitive period in which the PV interneurons are not completely protected by the PNNs, results in PV loss in the vHipp, thus favoring the development of schizophrenia. In contrast, we propose that if the individual is “protected” during this period when PV interneurons are more vulnerable, but experiences stress later in life (in which PV interneurons have been protected by the PNNs), this could facilitate the development of affective disorders, such as depression. Thus, the age of exposure to stress could determine the possible pathophysiological consequences. If the differential developmental vulnerability of PV interneurons is a causative factor, we further predict that pharmacological tools, such as histone deacetylase (HDAC) inhibitors (i.e., valproate (VPA) and SAHA), which are proposed to reopen the critical period of plasticity in the adult [12, 13], could recreate an adolescent phenotype of stress susceptibility.

Material and methods

Animals

Sixty-one timed-pregnant Sprague–Dawley rats (Envigo) were obtained at GD14 and housed individually in plastic breeding tubs. On postnatal day (PD) 24, litters were weaned and housed two or three per cage. Only male offspring (total of 516) were used in this study. Animals from the same litter were assigned to the different experimental groups with all the animals in the same cage devoted to the same experimental procedure. Animals were housed in a temperature (22 °C)- and humidity (47%)-controlled environment (12-h light/dark cycle; lights on at 7 a.m.) with ad libitum access to food and water. All experiments were conducted according to the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh to ensure that the animals were treated humanely and did not experience needless stress beyond the experimental conditions required for the study.

Stress procedure

Adolescent rats were exposed to the combination of daily inescapable footshock (FS; from PD31–40) and three restraint stress (RS) sessions (PD31, 32, and 40), as described elsewhere [14]. Similarly, changes induced by the combination of FS exposure (from PD65–74) and RS (PD65, PD66, and PD74) in adulthood were also evaluated. Briefly, rats were exposed to one session of FS per day for 10 consecutive days. In each session, animals were placed in a plexiglas chamber fitted with a grid floor comprised of 0.48 cm stainless steel rods spaced 1.6 cm apart and housed within a soundproof box (Med Associates Inc.). Twenty-five scrambled FS (1.0 mA, 2 s) were delivered every 60 ± 20 s using Med-PC IV software (Med Associates Inc.). On the first, second, and last day, immediately after the FS exposure, rats were submitted to RS by placing each rat in a plexiglas cylindrical size-adjusted restraint tube. Cylinders measured 14.0 × 3.9 cm for rats at PD31–32, 20.3 × 5.1 cm for rats at PD40, and 23.0 × 6.3 cm for adult rats (length × diameter), ventilated by holes (1 cm diameter). Each RS session lasted 1 h, and immediately after the end of the RS rats were returned to their home cages. Naive animals were left undisturbed in their home cages.

Locomotor response to amphetamine

Between 1 and 2 or 5 and 6 weeks after the stress, rats were tested in an open-field chamber (Coulbourn Instruments) in which locomotor activity was determined by beam breaks and recorded with TruScan software (Coulbourn Instruments). Basal locomotor activity was recorded for 30 min. After that, rats were injected with saline (1 mL/kg) or d-amphetamine sulfate (0.75 mg/kg, i.p.; Sigma) and their locomotor activity was recorded for another 90 min.

Extracellular recordings of DA neurons in the VTA and in the substantia nigra (SN)

VTA and SN recordings were carried out between 1 and 2 or 5 and 6 weeks post stress. Briefly, rats were anesthetized with chloral hydrate (400 mg/kg, i.p.; Sigma) and mounted on a stereotoxic frame (Kopf). The stereotoxic coordinates for the VTA were 5.0 mm posterior from bregma, 0.5 mm lateral to the midline, and 6.0–8.5 mm ventral from the brain surface in animals between PD47–54 (between 1 and 2 weeks after adolescent stress), and 5.3 mm posterior from bregma, 0.6 mm lateral to the midline, and 6.5–9.0 mm ventral from the brain surface in adult animals (between 5 and 6 weeks after adolescent stress and between 1 and 2 or 5 and 6 weeks after adult stress). The stereotoxic coordinates for the SN were 5.4 mm posterior from bregma, 1.8 mm lateral to the midline, and 6.0–8.5 mm ventral from the brain surface in animals between PD47–54, and 5.5 mm posterior from bregma, 2.2 mm lateral to the midline, and 6.5–9.0 mm ventral from the brain surface in adult animals. Electrodes were lowered through six to nine vertical tracks,
and stored in 25% sucrose. Serial 50 μfi then removed and transcardially perfused with saline followed by 4% PFA in torsions [18, 19]. In brief, rats were deeply anesthetized with Fatal-Plus (0.3–0.5 mL, i.p.; Vortech Pharmaceuticals), and biotinylated Wisteria floribunda agglutinin (WFA; 1:2000 dilution, Vector Labs, catalog # B1355) for 48 h at 4 °C. The sections were then incubated with a mixture of 1% normal goat serum, goat anti-mouse Alexa Fluor 594 (1:500, Abcam, catalog # ab150116), and Alexa Fluor 488 conjugated to streptavidin (1:500, Life Science Technology, catalog # S32354). The sections were also processed with DAPI (1:4000, Thermo Scientific, catalog # 62247) to visualize the border of the subiculum. PNNs were identified by staining for WFA, a lectin that labels selectively residues of glyco-proteins within the PNNs. While this does not show the PNN structure explicitly, nor are PNNs located exclusively around PV neurons [20], counterstaining for PV enable us to tell if the structure is indeed a perisomatic PNN encompassing PV interneurons.

For imaging, focus was set on PV-positive neurons and digital images were obtained using SimplePCI6 software (Hamamatsu Corporation). Under ×10 magnification, the subiculum regions of each rostrocaudal section were imaged by three sequential 909 × 692 μm-images along the mediol-temporal axis, using an OlympusBX51 microscope with a Hamamatsu Orca-ER camera. The border between CA1 and ventral subiculum was defined by an abrupt widening of the pyramidal layer, and border between subiculum and pre-subiculum was defined by a sharp reduction in PV intensity and decrease in cell size visualized by DAPI. For cell count, only the pyramidal cell layers, where most PV interneurons were located, were counted. The exposure time for PV was calibrated such that the majority of the PV-positive cells in the PD31 group were visible and within the dynamic range, and all subsequent images of the remaining age groups were taken at an identical exposure. Similar techniques were applied to PNN and DAPI to identify optimal exposure time. For each acquired images, the exposure was set based on PV-positive cells so that the majority of the PV-positive cells were in focus. The cell count was then performed using ImageJ by two individuals blind to the experimental conditions. To facilitate the identification of cells, images were magnified to 150%, and all PV- or PNN-positive cells in focus that are contained within the subicular boundaries were counted regardless of morphology or fluorescent intensity.

VPA/SAHA treatment

Sodium valproate (VPA; 300 mg/kg, i.p. in saline; MP Biomedicals) was administered to adult (PD60) male rats.
for 15 days, with FS + RS administered for 10 overlapping days at PD65–74. At 300 mg/kg, VPA was found to reopen the critical period of plasticity in the visual cortex and to act as anticonvulsant and mood stabilizer in animal models [13, 21, 22].

In order to limit any impact of VPA-mediated mood stabilization on stress, FS + RS was delivered before 10 a.m., with VPA administered after 5 p.m. on the same day. Also, VPA induces effects via modulation of GABA neurotransmission [23]. Since we propose that VPA acts via HDAC inhibition to reopen a sensitive period of plasticity, a similar treatment protocol was applied to a different pan-HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA; 25 mg/kg, i.p. in 10% DMSO; Cayman Chemical) [12], to control for the acute action of VPA on GABA neurotransmission.

**Statistical analysis**

Data are presented as mean ± SEM. We used t tests for two-sample comparisons and one-way or two-way ANOVA followed by Bonferroni’s post-hoc test for more than two groups. Results of statistical tests with p < 0.05 were considered significant. See supplementary information for more details.

**Results**

**Early adolescent and adult stress induce opposite changes in VTA DA system activity**

We showed previously that the exposure of rats to either three RS sessions or daily FS given at PD31–40 leads to a heightened anxiety state in adult rats, but only the combined stressors leads to a hyperdopaminergic state in terms of increased VTA DA neuron population activity and hyper-locomotor response to amphetamine, with the electrophysiological recordings of VTA DA neurons taking place more than 5 weeks after the end of stress [14]. However, the exposure of adult rats (PD65–74) to the same combination of stressors did not change the VTA DA system activity measured in the same time frame [14]. These findings underscore that adolescence is a period of particular susceptibility to long-lasting changes induced by stress. However, it is well-known that stress impacts individuals at all ages potentially creating age-dependent pathologies. In fact, the exposure of adult rats to chronic mild stress, an animal model for the study of depression, resulted in a decrease in VTA DA neuron population activity that is present between 1–2 weeks after the stress [24]. Based on these findings, we decided to evaluate the impact of the combination of FS and RS during early adolescence (PD31–40) or adulthood (PD65–74) on DA system activity, recording VTA DA neurons between 1 and 2 or 5 and 6 weeks after the stress to examine the immediate actions and persistent effects of the combined stressors (Fig. 1).

Confirming our previous findings [14], early adolescent stress increased VTA DA neuron population activity between 5 and 6 weeks after the stress (naive: n = 6 rats/39 active DA neurons, 0.98 ± 0.11 DA neurons/track; stress: n = 6 rats/57 active DA neurons, 1.58 ± 0.07 DA neurons/track). A similar change was also found between 1 and 2 weeks after early adolescent stress, when animals were still adolescent (PD47–PD54; naive: n = 8 rats/51 active DA neurons, 0.87 ± 0.19 DA neurons/track; stress group: n = 7 rats/66 active DA neurons, 1.59 ± 0.15 DA neurons/track). No change in the average firing rate and burst activity of VTA DA neurons induced by early adolescent stress was found, but this is likely due to a shift in the population of neurons (i.e., activation of previously non-firing neurons at slow rates balancing out increases in already firing neurons) [25]. In addition, the increased VTA DA neuron population activity induced by the early adolescent stress was accompanied by an increased locomotor response to amphetamine (n = 10 rats/group; Fig. 1 and Supplementary Fig. 2). This hyperdopaminergic state is consistent with that observed in animal models for the study of schizophrenia [26].

For the adult stress, the combination of stressors did not change VTA DA neuron population activity when recordings were performed between 5 and 6 weeks after the stress (naive: n = 7 rats/52 active DA neurons, 1.12 ± 0.06 DA neurons/track; stress: n = 7 rats/54 active DA neurons, 1.18 ± 0.09 DA neurons/track). However, a decreased VTA DA neuron population activity was observed when the recordings were done between 1 and 2 weeks post stress (naive: n = 8 rats/61 active DA neurons, 1.02 ± 0.07 active DA neurons/track; stress: n = 10 rats/57 active DA neurons, 0.69 ± 0.06 DA neurons/track), which is consistent with one factor observed consistently in three animal models of depression [24, 27–29]. In addition, the adult stress did not change the locomotor response to amphetamine (n = 8–10 rats/group; Fig. 1 and Supplementary Fig. 2). Furthermore, these changes were confined to the VTA, as recordings from SN DA neurons did not reveal a change in activity (Supplementary Fig. 3).

**Greater PV interneuron susceptibility during critical periods may drive DA system hyperactivity induced by early adolescent stress**

An increased activity of the vHipp is proposed to underlie the DA system overdrive in schizophrenia patients and also in animal models of the disease [30]. In fact, vHipp inactivation reversed the hyperdopaminergic state found in a
A combination of daily footshock (FS; through PD31–40) plus three restraint stress sessions (RS; PD31, 32, and 40). Extracellular recordings of VTA DA neurons and locomotor activity after saline or amphetamine (0.75 mg/kg) administration were evaluated between 1 and 2 weeks postadolescent stress (through PD47–54). B Adolescent stress increased the number of spontaneously active VTA DA neurons with the recordings 1–2 weeks post stress (naive group: n = 8 rats, 0.87 ± 0.19 active DA neurons/track; stress group: n = 7 rats, 1.59 ± 0.15 active DA neurons/track; t13 = 2.92, p = 0.012), but with no change in the firing rate (t13 = 0.79, p > 0.05) and in the burst activity (t13 = 0.82, p > 0.05) of the identified spontaneously active DA neurons in the VTA (naive group: n = 51 active DA neurons; stress group: n = 66 active DA neurons). C Consistent with the increased VTA DA neuron population, an increased locomotor response to amphetamine administration was also observed 1–2 weeks postadolescent stress (n = 10 rats/group; t16 = 2.41, p = 0.03). D Long-term effects on DA system activity were also investigated 5 and 6 weeks postadolescent stress (through PD75–82). G An increased number of spontaneously active VTA DA neurons was still present 5–6 weeks postadolescent stress (naive group: n = 6 rats, 0.98 ± 0.11 active DA neurons/track; stress group: n = 6 rats, 1.58 ± 0.07 active DA neurons/track; t12 = 4.63, p = 0.0009), but with no change in the firing rate (t6 = 0.07, p > 0.05) or in the burst activity (t6 = 1.10, p > 0.05) of active VTA DA neurons (naive group: n = 39 active DA neurons; stress group: n = 57 active DA neurons). H In addition, an increased locomotor response to amphetamine administration was also observed 5–6 weeks postadolescent stress (n = 10 rats/group; t18 = 2.42, p = 0.03). K Similar to the adolescent stress, adult male rats were submitted to a combination of daily footshock (FS; through PD65–74) plus three restraint stress sessions (RS; PD65, 66, and 74). Extracellular recordings of VTA DA neurons and locomotor activity after saline or amphetamine (0.75 mg/kg) administration were evaluated between 1 and 2 weeks postadult stress (through PD81–88). I Contrary to the adolescent stress, adult stress decreased the number of spontaneously active VTA DA neurons 1–2 weeks post-stress (naive group: n = 8 rats, 1.02 ± 0.07 active DA neurons/track; stress group: n = 10 rats, 0.69 ± 0.06 active DA neurons/track; t10 = 3.49, p = 0.003) but with no change in the firing rate (t10 = 1.09, p > 0.05) and in the burst activity (t10 = 0.12, p > 0.05) of the identified spontaneously active DA neurons in the VTA (naive group: n = 61 active DA neurons; stress group: n = 57 active DA neurons), and a previous exposure to adult stress did not exacerbate the locomotor response to amphetamine (n = 8–10 rats/group; t6 = 1.08, p > 0.05). J The impact of the adult stress on DA system activity was also investigated 5 and 6 weeks postadult stress (through PD109–116). Q No difference was found for the number of spontaneously active VTA DA neurons between naive and stressed rats 5–6 weeks post-adult stress (naive group: n = 7 rats, 1.12 ± 0.06 active DA neurons/track; stress group: n = 7 rats, 1.18 ± 0.09 active DA neurons/track; t116 = 0.01, p > 0.05), indicting a recovery of the hypodopaminergic state observed 1–2 weeks after the adult stress. Also, R no change in the firing rate (t116 = 0.08, p > 0.05) or in the burst activity (t116 = 0.42, p > 0.05) of active VTA DA neurons (naive group: n = 52 active DA neurons; stress group: n = 54 active DA neurons) was observed. Finally, T no change was observed in the locomotor response to amphetamine (n = 10 rats/group; t18 = 1.38, p > 0.05). Data are presented as mean ± SEM. *p < 0.05. Data are presented as mean ± SEM. *p < 0.05.
neurodevelopmental disruption model of schizophrenia [17]. This abnormal hippocampal activity is thought to be driven by a functional loss of PV-expressing GABAergic interneurons [11]. Importantly, the maturation of PV interneurons determines the onset and the duration of the critical or sensitive period of plasticity [6], which are developmental epochs with not only maximal neuroplasticity [31], but also heightened vulnerability to stress. Other mechanisms also determine the closure of the critical period, which serves to restrict the influence of sensory experiences on plasticity. For example, molecular brakes, such as the PNNs, which are formed mainly around PV interneurons [20], will also emerge during development to close the critical period [32].

PNNs lock plasticity in place and act like a “shield” protecting PV interneurons from metabolic and oxidative damage [9]. However, the formation of PNNs is not complete until early adulthood [20]. Studies show that PV interneurons are more susceptible to damage in periods with immature PNNs [7]. To understand the longitudinal impact of early adolescent stress, we analyzed the developmental trajectories of the expression of PV and PNNs in the ventral subiculum of the vHipp post-stress.

The expression of PV and PNNs was evaluated in rats at PD31 (correspondent to the first day of the adolescent stress), PD41 (1 day after terminating the stress), PD51 (between 1 and 2 weeks after the stress), and PD75 (5 weeks after the stress; n = 4–6 rats/group; Fig. 2). In naive rats, there is an increase in the number of cells stained for PV across periadolescent development, confirming the previous finding that the number of PV interneurons undergo dramatic increase during this period in the vHipp [18]. In addition, an increase in the number of cells surrounded by PNNs was also found across development, including the number of PV-positive cells surrounded by PNNs (Fig. 2). The combined stressors during adolescence decreased the number of PV-positive cells at PD51 and 75 and also impacted the number of cells surrounded by PNNs at PD51, including the PV-positive cells surrounded by PNNs (Fig. 2). However, unlike the impact of early adolescent stress on the number of PV-positive cells in late adolescence, there was a recovery at PD75 in the number of cells surrounded by PNNs, including the PV-positive cells surrounded by PNNs (Fig. 2). This recovery is consistent with evidence suggesting that PNNs are completely reformed 3 weeks after brain infusion of chondroitinase ABC, an enzyme that rapidly digests PNNs within hours [33]. PV surrounded by PNNs at P75 after early adolescent stress, however, are still low (a trend p-value = 0.06).

Given that early adolescent stress decreased the number of PV-positive cells which could result in changes the inhibitory-excitatory balance, we recorded the activity of pyramidal neurons in the vHipp between 1 and 2 or 5 and 6 weeks after early adolescent stress. An increase in the firing rate of pyramidal neurons in the vHipp was observed when the electrophysiological recordings were performed between 1 and 2 weeks (naïve: n = 37 neurons from ten rats, 0.62 ± 0.06 active neurons/track; stress: n = 36 neurons from nine rats, 0.67 ± 0.06 active neurons/track), as well as between 5 and 6 weeks after the early adolescent stress (naïve: n = 26 neurons from seven rats, 0.62 ± 0.09 active neurons/track; stress group: n = 48 neurons from ten rats, 0.80 ± 0.08 active neurons/track; Fig. 3). Together these findings suggest that early adolescent stress induces a PV neuron loss in the vHipp, which may lead to an increase in the activity of pyramidal neurons in this brain region. This hippocampal hyperactivity is reported to drive DA system hyperactivity [17] analogous to what has been proposed in schizophrenia [11].

We also evaluated the expression of PV and PNNs in adult animals exposed to the combined stress at PD81 and PD109 (i.e., 1 and 5 weeks postadult stress, respectively). In contrast to the early adolescent stress, the adult stress did not change the number of PV-positive cells or cells surrounded by PNNs in the vHipp, including the PV-positive cells surrounded by PNNs (Fig. 5). In addition, no change was found in the firing rate of pyramidal neurons in the vHipp between 1 and 2 weeks (naïve: n = 31 neurons from six rats, 0.86 ± 0.13 active neurons/track; stress: n = 29 neurons from six rats, 0.81 ± 0.13 active neurons/track) or 5 and 6 weeks after the adult stress (naïve: n = 27 neurons from six rats, 0.75 ± 0.15 active neurons/track; stress: n = 26 neurons from six rats, 0.72 ± 0.12 active neurons/track; Fig. 3). Thus, given that PV interneurons seem to be protected by the PNNs at adulthood [9], we believe the decreased VTA DA system activity induced by the adult stress could involve changes in other brain regions. For example, a hypodopaminergic state induced by the chronic mild stress in rats was associated with an overactivity of the basolateral amygdala-ventral pallidum system [24].

**Valproate treatment in the adult recreates stress susceptibility of adolescence**

We posit that unregulated stress responses occurring during the critical/sensitive periods of development leads to PV loss and the emergence of circuit deficits consistent with schizophrenia in the adult. If accurate, one would predict that reopening the critical period in the adult could render it susceptible to a similar stress-induced circuit disruption. Thus, we investigated the impact of the critical period on stress susceptibility by examining whether adults with...
reopened critical periods regain stress-induced PV loss and DA system hyperresponsivity.

Several genetic and pharmacological manipulations have been suggested to allow the reopening of the critical period in the adult brain [32]. Among these is VPA. VPA is a mood stabilizer/anticonvulsant that has been shown to reestablish the critical period of plasticity in several brain regions in the adult [34–36]. We tested if combining VPA (300 mg/kg; i.p.) with adult stress would recreate an adolescent phenotype of stress susceptibility. VPA was administered to adult (PD60) rats for 15 days, with FS + RS administered for 10 overlapping days at PD65–74 (Fig. 4). VPA treatment alone did not impact the VTA DA system, but instead substantially altered the impact of co-administered combined stressors. Thus, in normal, untreated adult rats 10 days of FS + RS leads to decreased VTA DA population activity that is present for 1–2 weeks after treatment. However, if rats are treated with VPA, the net effect of co-administered stressors is a persistent increase in VTA DA neuron population activity 1–2 weeks post stress (naive + saline: n = 8 rats/52 active DA neurons, 0.96 ± 0.04 DA neurons/track; stress + saline: n = 9 rats/32
active DA neurons, 0.60 ± 0.05 DA neurons/track; naive + VPA: n = 10 rats/67 active DA neurons, 0.95 ± 0.11 DA neurons/track; stress + VPA: n = 11 rats/110 active DA neurons, 1.46 ± 0.08 DA neurons/track) and 5–6 weeks post-stress (naive + saline: n = 8 rats/47 active DA neurons, 0.95 ± 0.09 DA neurons/track; stress + VPA: n = 8 rats/73 active DA neurons, 1.36 ± 0.06 DA neurons/track; t14 = 3.59, p = 0.003), analogous to that observed with early adolescent stress. No change in firing rate and burst activity of VTA DA neurons was found (Supplementary Fig. 5). The combination of VPA and adult stress also increased the locomotor response to amphetamine 1–2 and 5–6 weeks post stress (n = 8 rats/group; Fig. 4 and Supplementary Fig. 6). In addition, the combination of VPA treatment and adult stress also increased the activity of pyramidal neurons in the vHipp in 1–2 weeks post-stress (naive + saline: n = 33 neurons from seven rats, 0.79 ± 0.14 active neurons/track; naive + VPA: n = 40 neurons from eight rats, 0.83 ± 0.11 active neurons/track; stress + VPA: n = 33 neurons from seven rats, 0.79 ± 0.12 active neurons/track) and 5–6 post-stress (naive + saline: n = 30 neurons from nine rats, 0.59 ± 0.09 active neurons/track; stress + VPA: n = 30 neurons from nine rats, 0.57 ± 0.09 active neurons/track), with no change induced by the VPA treatment alone (Fig. 4). Also, as previously described the adult stress alone did not change the activity of pyramidal neurons in the vHipp (Fig. 3).

We then tested whether reopening the critical period in the adult impacted PV and PNN expression in the vHipp (n = 4–6 rats/group). No effect of the adult stress or an interaction between stress and the VPA treatment was found. But, surprisingly, there was a treatment effect, suggesting that VPA is decreasing the number of cells stained for PV in the vHipp 1 week after the stress, with no change in the number of cells surrounded by PNNs. There was also a treatment effect for the number of PV-positive cells surrounded by PNNs. These effects of VPA alone could potentially mask the electrophysiological changes in the VTA DA system induced by VPA combined with the adult stress. Interestingly, when we evaluated the expression of these markers 5 weeks post stress, a decreased number of PV-positive cells was observed only in VPA-treated animals exposed to the combined stressors, with no change in the number of cells surrounded by PNNs. There was also a reduction in the number of neurons double stained for PV and PNNs (Fig. 5). Together, these findings indicate that VPA, possibly by reopening the critical period in the adult, restores susceptibility to stress-induced pathology resembling schizophrenia. Furthermore, a greater number of non-PV cells surrounded by PNNs was found 5–6 weeks post stress in VPA-treated rats (Supplementary Fig. 7).

How VPA is achieving these effects is not completely known, since this compound has several putative mechanisms of action. However, it is suggested that VPA reopens the critical period of plasticity in sensory systems possibly through HDAC inhibition [36]. In support to this hypothesis, SAHA (25 mg/kg), a more selective HDAC inhibitor, combined with adult stress also increased VTA DA neuron population activity 5–6 weeks post stress, with no changes in firing rate and burst activity (Supplementary Fig. 8). Also, SAHA by itself did not induce any effect. Together,
combined stressors (to amphetamine was also observed in VPA-treated rats exposed to the significant stress; 0.05 stress = 0.95 ± 0.11 active DA neurons/track; stress with VPA, the net effect of co-administered stressors was an increase in pyramidal (Pyr) neurons in the vHipp. induced DA system hyperresponsivity and increased activity of pyramidal neurons in the vHipp. However, VPA treatment changed the response to stress, but did not induce any effect by itself. VPA combined with adult stress increased the firing rate of pyramidal neurons in the vHipp 1–2 weeks post-stress (naive + saline: n = 33 neurons from seven rats, 0.79 ± 0.14 active neurons/track; naive + VPA: n = 40 neurons from eight rats, 0.83 ± 0.11 active neurons/track; stress + VPA: n = 33 neurons from seven rats, 0.79 ± 0.12 active neurons/track; F2,103 = 3.45, p = 0.035, one-way ANOVA followed by Bonferroni post-hoc test, p < 0.05 stress + VPA vs. all other groups). Then, we tested if changes induced by VPA co-administered with FS + RS on the DA system persisted over time (5–6 weeks). An increase in VTA DA neuron population activity was also present in VPA-treated rats 5–6 weeks after the adult stress (naive + saline: n = 8 rats, 0.95 ± 0.09 active DA neurons/track; stress + VPA: n = 8 rats, 1.36 ± 0.06 active DA neurons/track; t14 = 3.59, p = 0.003). In addition, VPA-treated rats also presented an increased locomotor response after amphetamine administration 5–6 weeks post-adult stress (n = 8 rats/group; t14 = 2.82, p = 0.014). The increased firing rate of pyramidal neurons in the vHipp in VPA-treated animal exposed to adult stress was also present 5–6 weeks post-stress (naive + saline: n = 30 neurons from nine rats, 0.59 ± 0.09 active neurons/track; stress + VPA: n = 30 neurons from nine rats, 0.57 ± 0.09 active neurons/track; t33 = 2.71, p = 0.0088). These changes observed after VPA treatment co-administered with FS + RS at adulthood are analogous to those induced by the adolescent stress. Data are presented as mean ± SEM. *p < 0.05

these findings indicate that HDAC inhibition by itself did not induce short- or long-term effects on the VTA DA system.

Discussion
Stress plays a major role in susceptibility to mental disorders in general, including schizophrenia and depression. In fact, the emergence of schizophrenia, which typically manifests during late adolescence and early adulthood, is often associated with stressful events (i.e., trauma, ethnic minority status, and social disadvantage) and adolescents that are at high risk for schizophrenia experience abnormally high reactivity to stress and are more likely to develop schizophrenia if they have decreased tolerance to stress [1, 37, 38]. For depression, the prevalence of the disease has been directly associated with the number of stressful events occurring before the diagnosis [39]. In contrast to schizophrenia, the onset of depression is more common at adulthood [6]. Since these conditions share several genetic markers and socio-environmental risk factors, such as stressful events, in common, we evaluated if timing of stress would be a critical determinant of the pathology that is present in the adult.

We observed a hyperdopaminergic state in animals exposed to early adolescent stress, indicated by an increased VTA DA neuron population activity and increased...
locomotor response to amphetamine, and hyperactivity in the vHipp, which is also the site of PV loss. The PV loss is thought to lead to vHipp hyperactivity which in turn results in a hyperdopaminergic state. This condition is highly consistent with clinical observations in schizophrenia. In fact, schizophrenia patients show abnormally high amphetamine-induced DA release in associative striatum [40], which can be behaviorally modeled in rodents by the observed increase in amphetamine-induced hyperlocomotion. Schizophrenia patients also show an increase in striatal fluorodopa uptake [41] indicative of increased number of active terminals, which is consistent with the increased number of spontaneously active DA neurons observed in the present study. Furthermore, the observed vHipp pyramidal neuron firing induced by early adolescent stress is analogous to the increased glutamate transmission and metabolic activity in the anterior hippocampus observed in patients [42, 43]. Taken together, the stress exposure during early adolescence appears to induce changes that recapitulate the circuit-level disruptions observed in schizophrenia patients. On the other hand, adult stress decreased VTA DA neuron population activity 1–2 weeks post stress, which seems to be followed by a recovery since no differences were observed 5–6 weeks postadult stress. This recovery is consistent with evidence showing that abnormal behaviors and plastic changes induced by the exposure to stress during adulthood revert to normal if sufficient time is given to the organism to recover [44]. In addition, no change in the

**Fig. 5** Impact of VPA treatment (300 mg/kg; i.p.; PD60–74) combined with adult stress (PD65–74) on histological changes in the vHipp. Cell counting was performed on PV interneurons to assess the effect of critical period reopening on stress vulnerability. Specifically, the ventral subiculum (vSub) was sampled at 1–2 weeks and 5–6 weeks post stress (n = 4–6 rats/group). At each time-point, a two-way ANOVA (treatment and condition as main factors) was performed on PV⁺, PNN⁺, and PV/PNN⁺ cells respectively, to evaluate the effect of stress and VPA co-administration. a For PV⁺ cells, only a treatment effect was detected (F1,16 = 8.798, p = 0.0091) at 1–2 weeks post stress. At 5–6 weeks post stress, however, significant main effects of both condition (F1,16 = 7.674, p = 0.014) and treatment (F1,16 = 12.09; p = 0.0031), as well as a significant interaction (F1,16 = 7.684, p = 0.014), were detected. Post-hoc analysis indicated a decrease in the PV⁺ cell count only in stress + VPA group (Bonferroni post-hoc test, p < 0.05 stress + VPA vs. all other groups), analogous to the long-term stress response in adolescent animals. b Adult PNNs seemed to be stable to stress and VPA treatment, as no main effect nor interaction were detected at either 1–2 weeks or 5–6 weeks post stress. c In terms of PV/PNN⁺ cell count, which are markers of putative mature PV interneurons, treatment effect was detected at both 1–2 weeks (F1,16 = 5.132, p = 0.0377) and 5–6 weeks (F1,16 = 6.823, p = 0.0189), but condition effect was only detectable at 5–6 weeks post stress (F1,16 = 5.998, p = 0.0262). Moreover, a significant interaction was detected only at 5–6 weeks post stress (F1,16 = 5.94, p = 0.0268), when the stress-VPA group displayed fewer PV⁺/PNN⁺ cells (Bonferroni post-hoc test, p < 0.05, vs. all other groups), suggestive of a relatively immature status of the PV neurons. Taken together, the data suggests that adult animals treated with VPA regained adolescent-like vulnerability to stress, evident by reduced number of PV⁺ and PV/PNN⁺ cell count at 5–6 weeks post stress. The effect of stress did not manifest in VPA-treated rats until 5–6 weeks post-stress, a phenomenon potentially attributable to a short-term effect of VPA treatment alone on the expression of PV. d Representative figures illustrating the impact of adult stress and VPA treatment on the expression of PV and PNN in the vSub. Data are presented as mean ± SEM. *p < 0.05 after two-way ANOVA, indicating a treatment effect; **p < 0.05 after two-way ANOVA followed by a Bonferroni post-hoc test, indicating significant changes in VPA-treated stressed rats vs. controls.
vHipp activity and in the content of PV in this brain region was induced by the exposure to the combined stressors during adulthood. The hypodopaminergic state observed after the adult stress is consistent with one measure proposed to be related to anhedonia/amotivation that has been consistently observed in three animal models of depression: cold stress, chronic mild stress, and learned helplessness [24, 27, 29].

What could explain the opposite effects induced by early adolescent and adult stress on VTA DA system activity? Adolescence involves several age-related dynamic changes in physiological processes and social environment [45]. Combined with genetically determined developmental alterations, these processes shape the neurobiological substrates that underlie maturation of the adolescent brain. For example, adolescence is a critical period for the refinement of GABAergic transmission, including the maturation of PV interneurons [8, 18]. PV interneurons are fast-spiking interneurons and, likely due to their high firing rates that cause high metabolic load and generation of reactive oxygen species [9], they are particularly vulnerable to both environmental and oxidative stress [7]. It is thought that PV interneurons are even more susceptible to damage by stressor during critical periods of brain plasticity, such as adolescence, in which the PV interneurons are not completely mature [7, 46]. Furthermore, at this time the PNNs, which stabilize glutamatergic inputs onto the PV interneurons to end the plastic phase and also protects these interneurons from metabolic and oxidative damage [7], are not yet completely formed around the interneurons [20]. Thus, these dynamics of brain maturation make the developing brain highly vulnerable to environmental factors, for example the deleterious effects of stress, that can lead to the development of psychiatric disorders [6]. The loss of PV in the prefrontal cortex and hippocampus is one of the most robust findings in the postmortem brain of schizophrenia patients [47, 48] and it has also been replicated in several genetic, environmental, and pharmacological rodent models used to study the disease [49]. Importantly, an altered PV expression has not been found in other psychiatric disorders, such as depression and bipolar disorder [47].

Our findings suggest that the maturational trajectory of PV interneurons in the vHipp is a marker of vulnerability to stress. While vHipp has been shown to be sensitive to stress throughout life, during adolescence the vHipp shows a particular high susceptibility to negative environments. This is largely attributable to the PV interneurons in the region and the massive excitatory inputs that they receive. The PV interneurons in the vHipp, especially in the ventral subiculum, are known to receive diverse yet potent excitatory inputs from regions, such as CA1, amygdala, and thalamus [50]. These excitatory inputs serve as important regulators to the overall plasticity of PV interneurons through altering excitation/inhibition balance, but also remain a possible source of pathology. In fact, dysregulated excitation can alter calcium dynamics, increase metabolic demand, and possibly generate excessive oxidative stress, leading to cell damage and even cell death [46, 51]. This vulnerability persists until the end of the critical period, marked by the encasement of PNNs on the PV interneurons, protecting them from metabolic and oxidative damage [7]. This is supported by the immunohistochemistry data in the current study. Indeed, in the ventral subiculum, PNN expression continues to increase until after PD51, and such change may drive the maturation of the PV interneurons in the region, as putative mature PV interneurons (i.e., neurons co-labeled by PV and PNNs) also continue to increase in number until late adolescence (i.e., PD51). The relative regional immaturity of the PV interneurons during mid-adolescence may underlie their particular vulnerability to stress.

Overlapping mechanisms underlying the regulation of critical period plasticity has been established from visual system and fear learning system, implicating common mechanisms underlying the closure of the critical period [32]. The same principles have been repeatedly observed across brain regions [9], including the hippocampus [52]. The molecules that are involved in limiting critical period plasticity in adulthood are collectively termed as molecular "brakes". Importantly, lifting these "brakes" has been shown to reopen critical period plasticity in adulthood. Several studies have used chondroitinase ABC, an enzyme which breaks down the PNNs, to reopen critical period plasticity [31]. Interestingly, it was found that dissolving PNNs in the vHipp through the local infusion of chondroitinase ABC increased the firing rate of pyramidal cells in the vHipp, as well as a hyperdopaminergic state indicated by an increased VTA DA neuron population activity and locomotor response to amphetamine [53]. Thus, we decided to use a more subtle and critical period-specific effect of HDAC inhibitor, VPA, which is proposed to reopen the critical period of plasticity in the adult and which in our hands did not change the VTA DA system activity by itself, to test whether the observed age-dependent vulnerability to stress is indeed a critical period. Our results indicate that VPA-treated animals regained adolescent-like stress vulnerability, evident from the findings that stress in VPA-treated animals increased VTA DA neuron population activity, vHipp hyperactivity, and the reduction in numbers of PV-positive neurons. Curiously, exposure to VPA in naïve rats reduces significantly the number of PV+ and PV+/PNN+ stained cells in the vHipp at the 1–2 weeks post treatment. This is similar to what was observed with the genetic deletion of HDAC2 specifically from PV interneurons [54]. We believe that the decrease in PV induced by VPA is likely due to a decrease in synthesis without cell loss, since there is a recovery of PV and PNN and no
correlative changes in vHipp or VTA activity, suggesting that vHipp inhibitory circuits are intact. But with stress the loss seems to be persistent, suggesting actual PV cell loss. Therefore, a decrease of PNN/PV would suggest vulnerability, not pathology.

VPA has multiple mechanisms of action, including the potentiation of GABA neurotransmission to enhance inhibitory function and enduring effects on gene transcription as a pan-HDAC inhibitor [55]. HDAC inhibition is more likely to be of relevance to the observed action of critical period reopening. In fact, in primary visual cortex, enhancement of intercortical inhibition does not trigger plasticity in adults, but HDAC inhibition does [36]. Also, adult prefrontal cortex encodes acoustic preferences established during early life and is rendered malleable again later by VPA by renewing prefrontal neuron recruitment in mice [35]. Interestingly, it has been successfully applied to healthy adult humans learning absolute pitch discrimination [34].

To give further support to the finding that the effect of VPA “recreating an adolescent phenotype” of stress vulnerability in adult animals is indeed due to HDAC inhibition, we utilized another pan-HDAC inhibitor, SAHA. SAHA has no documented action on inhibitory neurotransmission, and when used at the dose enhancing critical period reopening, in the visual cortex, enhancement of intercortical inhibition does not trigger plasticity in adults, but HDAC inhibition does [36]. Also, adult prefrontal cortex encodes acoustic preferences established during early life and is rendered malleable again later by VPA by renewing prefrontal neuron recruitment in mice [35]. Interestingly, it has been successfully applied to healthy adult humans learning absolute pitch discrimination [34].

In conclusion, we found that timing of stress is a critical determinant for the pathology that is present in the adult, potentially due to their distinct impacts on the DA system and its regulators. While early adolescent stress led to behavioral, physiological, and histological changes that recapitulate schizophrenia, adult stress induced a hypodopaminergic state observed in animal models of depression. Furthermore, HDAC inhibitors, which are proposed to reopen the critical period plasticity, restores susceptibility to stress-induced pathology resembling schizophrenia in adults. These findings can have strong implications for the etiology, pathophysiology, and underlying neural circuitry of developmentally related disorders, with a particular emphasis on schizophrenia and depression. Schizophrenia and depression are marked by important sex differences [58, 59] and recent studies have demonstrated sex-specific response of PV neurons to early adolescent stress [60]. Thus, further studies are required to investigate if the findings described here in males would be observed in females as well, and whether the time course of susceptibility correlates with postnatal age or pubertal stage. Finally, understanding how developmental factors can lead to pathological states, identifying timing of susceptibility to stressors, and precisely identifying the neural substrates underlying such susceptibility, can provide a roadmap to prevention, rather than treatment after the insult has been established. Our findings can help to elucidate evidence indicating that ultra-high risk individuals that develop schizophrenia are more vulnerable to depression as adults [61].

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Compliance with ethical standards

Conflict of interest AAG has received funds from Landbeck, Pfizer, Otsuka, Lilly, Roche, Asubio, Abbott, Autofony, Janssen, Alkermes, Neuron, and Takeda. FVG and XZ declare that they have no conflict of interest.

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