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A role for progesterone and $\alpha 4$ -containing GABA_A receptors of hippocampal pyramidal cells in the exacerbated running response of adolescent female mice to repeated food restriction stress

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Abstract

Adolescent females are particularly vulnerable to mental illnesses with comorbidity of anxiety, such as anorexia nervosa (AN). We used an animal model of AN, called activity-based anorexia (ABA), to investigate the neurobiological basis of vulnerability to repeated, food restriction (FR) stress-evoked anxiety. Twenty-one of 23 adolescent female mice responded to the 1st FR with increased wheel running activity (WRA), even during the limited period of food access, thereby capturing AN's symptoms of voluntary FR and over-exercise. Baseline WRA was an excellent predictor of FR-elicited WRA (severity of ABA, SOA), with high baseline-runners responding to FR with minimal SOA (i.e., negative correlation). Nine gained resistance to ABA following the 1st FR. Even though allopregnanolone (3 α -OH-5 α -pregnan-20-one, THP), the metabolite of progesterone (P4), is a well-recognized anxiolytic agent, subcutaneous P4 to these ABA-resistant animals during the 2nd FR was exacerbative, evoking greater WRA than the counterpart resistant group that received oil vehicle, only. Moreover, P4 had no WRA-reducing effect on animals that remained ABA-vulnerable. To explain the sensitizing effect of P4 upon the resistant mice, we examined the relationship between P4 treatment and levels of the $\alpha 4$ subunit of GABA_ARs at spines of pyramidal cells of the hippocampal CA1, a parameter previously shown to correlate with resistance to ABA. $\alpha 4$ levels at spine membrane correlated strongly and negatively with SOA during the 1st ABA (prior to P4 injection), confirming previous findings. $\alpha 4$ expression levels were greater among P4-treated animals that had gained resistance than of vehicle-treated resistant animals or of the vulnerable animals with or without P4. We propose that $\alpha 4$ -GABA_ARs play a protective role by counterbalancing the ABA-induced increase in excitability of CA1 pyramidal neurons, and although exogenous P4's metabolite, THP, enhances $\alpha 4$ expression, especially among those that can gain resistance, it also interferes with $\alpha 4$ -GABA_ARs' protective role by desensitizing $\alpha 4$ -GABA_ARs.

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Keywords

Activity-based anorexia; anxiety; anorexia nervosa; resilience; exercise; allopregnanolone

1.INTRODUCTION

Adolescence is a period of great vulnerability to mental illness (Spear, 2000). The stress response system in the brain and periphery exhibit strong and long-lasting (Andersen, 2003; Romeo, 2010) plasticity during adolescence, perhaps because it is still continuing to develop (McCormick & Mathews, 2007; Romeo, 2005; Romeo et al., 2006). As a consequence, a stressful event during adolescence affects the mental health outcome into adulthood (Andersen, 2003; Romeo, 2010). In puberty and adolescence, there is an interaction of the following factors: an increase of environmental stressors, gonadal hormone fluctuations, and a growth spurt in the hippocampus (Chowdhury et al., 2014; Mannan & O'Shaughnessy, 1991; Palumbo et al., 1995; Romeo et al., 2006; Spear, 2000). Estrogen, progesterone and testosterone are the major peri-pubertal hormones influencing plasticity and signaling in the hippocampus, along with reproductive function (Sheryl S Smith & Woolley, 2004). In adulthood, estrogen has excitatory effects in several brain regions, including the hippocampus, hypothalamus, and amygdala, with the impact of increasing or decreasing anxiety, increasing stress responses and decreasing depression (Harte-Hargrove, MacLusky, & Scharfman, 2013; Isgor, Cecchi, Kabbaj, Akil, & Watson, 2003; Nabekura, Oomura, Minami, Mizuno, & Fukuda, 1986; Walf & Frye, 2006). Among the questions that remain to be answered is the role of progesterone (P4) in altering the stress response of adolescents. We sought to address this gap in knowledge by imposing an animal model of stress called activity-based anorexia (ABA) upon adolescent mice and testing P4's cellular and behavioral effects upon ABA response.

The stressor in the ABA model is food restriction and the response to this stress is a marked increase in voluntary wheel running activity (WRA), causing exacerbated weight loss. WRA is a reliable measure of stress response, because it correlates positively with levels of corticosterone (cort) released after food restriction (Duclos, Bouchet, Vettier, & Richard, 2005; Duclos, Gatti, Bessiere, & Mormede, 2009). The WRA is also robustly correlated to anxiety levels measured during ABA (G. Wable, Min, Chen, & Aoki, 2015). Stress usually afflicts an individual more than once. The change in physiology evoked by repeated stress can also be analyzed in this animal model by imposing a second episode of ABA after recovery from the first. Such repeated stress exposures enable investigation of individual variability in habituation, adaptation or sensitization to the repeated stressor. Indeed, one of the strengths of the ABA model is that there is considerable individual variability in the WRA response to the first as well as the repeated episode of the food restriction stressor. The variability in behavior is not noise, because it is correlated in meaningful ways to variability in biological markers, such as the levels of cort (Duclos et al., 2005; Duclos et al., 2009). The extent of GABAergic innervation as well as expression levels of the $\alpha 4$ subunit of GABA_ARs by pyramidal neurons in the CA1 of dorsal hippocampus is also correlated strongly and negatively with the food restriction-evoked increase of WRA, suggesting that up-regulation of the hippocampal GABAergic system may contribute towards suppression

of WRA and thereby resilience to ABA (Aoki et al., 2014; Chowdhury, Wable, Sabaliauskas, & Aoki, 2013).

P4 plays an important role in regulating anxiety. P4's metabolite, THP (3 α -OH-5 α [[β]-pregnan-20-one), reduces anxiety (C. A. Frye & Paris, 2011) (Bitran, Shiekh, & McLeod, 1995) in late adolescence, adulthood (Canonaco, Tavoraro, & Maggi, 1993; Engin & Treit, 2007; C. A. Frye & A. A. Walf, 2004; Koonce & Frye, 2013; Reddy, O'Malley, & Rogawski, 2005) and pre-pubertally (Hui Shen et al., 2007) through positive modulation of GABA_A receptors (GABA_ARs) (Belelli & Lambert, 2005). These findings, together with our recent observation of the strong correlation between WRA response and anxiety (G. Wable et al., 2015), led us to test the following prediction: P4 treatment could decrease an animal's excessive WRA response to food restriction when imposed at late adolescence, which in turn would minimize weight loss and serve to protect animals from ABA. We chose a dosage of subcutaneous P4 that has been demonstrated to be anxiolytic in female mice of similar age (Cheryl A Frye, Walf, Rhodes, & Harney, 2004).

To identify a possible molecular mechanism underlying the behavioral effect of P4, we measured plasmalemmal and cytoplasmic levels of the α 4 subunit of GABA_ARs in pyramidal neurons of the dorsal hippocampal CA1 of these mice. Our reason for measuring the α 4 subunit level in the CA1 was two-fold: 1), the administration as well as withdrawal of P4 and THP increase the expression of α 4 β δ -GABA_AR in the hippocampus over 2 to 3 days (H. Shen et al., 2007); and 2) ABA was previously shown to increase the expression of α 4 β δ -GABA_ARs in the hippocampal CA1 (Aoki et al., 2012; Aoki et al., 2014). We surmised that, if heightened levels of α 4 β δ -GABA_AR in the hippocampal CA1 contributed to the suppression of food restriction-evoked increase in WRA, thereby reducing ABA vulnerability (Aoki et al., 2014), then P4 could induce the expression of α 4 β δ -GABA_AR, which would also be protective against ABA. However, while P4's metabolite, THP, is, in general, anxiolytic, THP can also be anxiogenic. This is because THP has an additional property of desensitizing α 4 β δ -GABA_AR, when expressed in pyramidal cells of the CA1 (H. Shen et al., 2007). This additional desensitizing property of THP upon α 4 β δ -GABA_ARs expressed by CA1 pyramidal cells (as opposed to the consistently positive modulating effect of THP upon α 4 β δ -GABA_ARs expressed by granule cells of the dentate gyrus, involving the difference in the direction of Cl⁻ flux across the two regions) has the potential consequence of increasing excitability of the CA1 pyramidal neurons and thus of anxiety (H. Shen et al., 2007). Because of this dual potential action of THP, knowledge about the changing level of α 4 subunits in the hippocampal CA1 was needed for assessing the action of P4 that would include the interaction of α 4 β δ -GABA_AR in the animal's response to ABA. Here, we report that, indeed, P4 led to exacerbated WRA during the second ABA, specifically among individuals that had attained resilience during recovery from the 1st ABA and a rise of α 4 β δ -GABA_ARs. These observations are consistent with the idea that up-regulation of α 4 β δ -GABA_AR in the dorsal hippocampal CA1 during recovery from the first ABA contributed towards attainment of resilience to the second ABA, while desensitization of α 4 β δ -GABA_ARs by exogenous P4 contributed to anxiogenesis and exacerbated animals' responses to the second ABA.

2. EXPERIMENTAL PROCEDURES

2.1 General aspects about the animals

All procedures relating to the use of animals were according to the NIH Guide for the Care and Use of Laboratory Animals and also approved by the Institutional Animal Care and Use Committee of New York University.

Altogether, 23 animals were used for this study. All animals were female C57BL6 mice, bred at New York University's animal facility in a 12 hour: 12 hour light: dark cycle (lights on at 7 am). At postnatal day (PND) 25, they were weaned and group-housed with same sex littermates. If any litter contained only a single female mouse, she was not included in the experiment. Food was provided *ad libitum* when the animals were not undergoing the food restriction portion of the paradigm [dry chow (PMI Mouse Diet 5001; 336 kcal per 100 g, 28.507% protein, 57.996% carbohydrates, 13.496% fat) and soft food (Clear H2O DietGel@ 76A; 99.8 kcal per 100 g, 4.7% protein, 17.9% carbohydrates, 1.5% fat, 73.4% moisture)] and water was provided *ad libitum* throughout the experiment.

Non-ovariectomized mice were used for the study because ovariectomy has been shown to alter CNS neurosteroid metabolism following injection of exogenous P4 (Corpechot et al., 1993). Also, P4 is produced by the adrenal gland in stress so ovariectomy does not guarantee depletion of circulating P4 (Romeo, Lee, & McEwen, 2005).

2.2 ABA induction and P4 injection

ABA induction consisted of combining food restriction with access to a running wheel, as described in the ABA protocol established in our laboratory (Chowdhury et al., 2013).

Running wheels were purchased from Med Associates, called Low-Profile Wireless Running Wheel for Mouse (Product #: ENV-044). Wheel-running activity (WRA) was recorded continuously using Med Associates' "Wheel Manager" software (Product #: SOF-860).

At noon on PND36, female mice were placed singly in standard cages with *ad libitum* access to laboratory chow while undergoing acclimation to the wheel (Fig 1). Beginning noon on PND41, mice were given access to food only for the first two hours of the dark cycle, from 7:00 pm to 9:00 pm, while having constant wheel access. At noon on PND44, *ad libitum* food access was restored and the mice were placed singly in a fresh cage with no running wheel. They were allowed to recover from ABA for a week, until PND51. From PND45 to 50, they were handled for five minutes every day, to acclimate them for vaginal lavage and injections. At noon on PND51, they were given access to a running wheel again for a re-acclimation period for four days. Vaginal lavages were collected starting PND53 until the end of the experiment. At noon on PND55, food access was again restricted to the first two hours of the dark cycle. Based on the running activity on this day called 2nd ABA FR1, we divided the mice into counter-balanced groups. One group received progesterone (P4) injections (1.0 mg progesterone in 0.1 cc of sesame oil, progesterone and sesame oil from Sigma-Aldrich, MO, USA, catalog number P3972 and S3547 respectively) and the other group received only oil. Injections were administered subcutaneously on the back of

the mice, at 6 pm, an hour before feeding on 2nd ABA FR2, FR3, and FR4. Throughout the experiment, the mice and food were weighed at 6:40 pm, 20 minutes before the onset of the dark cycle. On the days that the mice were transferred to a fresh cage, i.e. PND36, PND44, PND51 and PND59 (day of perfusion), they were weighed at noon so as to not needlessly disturb them more than once a day. Wheel activity was monitored throughout the periods of wheel access. On PND59, all animals except one that was found dead were euthanized by transcardial perfusion with fixatives, so as to be able to analyze the membranous expression of α 4-containing GABA_ARs in the hippocampus.

2.3 Vaginal cytology to determine estrous stage

These were performed along with weighing of the animal beginning in the re-acclimation phase that preceded the 2nd ABA and continuing through the end of the experiment. A pipette containing 1-2 ml of PBS was used to perform vaginal lavage. The lavage was observed under a microscope at 100 times magnification and estrous stage was determined as described in (McLean, Valenzuela, Fai, & Bennett, 2012).

2.4 Brain preparation and immunocytochemistry

2.4.1 Materials—The primary antibody directed against the α 4 subunit of GABA_A receptor (GABA_AR) was obtained from Santa Cruz Biotechnology (catalog #SC7355, lot J1912). Previous studies demonstrated specificity of this antibody for the α 4-subunit of GABA_AR using three EM-immunocytochemical procedures: reduction of immunoreactivity at the plasma membrane and in the cytoplasm of pyramidal neurons in the CA1 of hippocampus of α 4-KO animals at the transition between prepuberty and puberty onset, which is when immunoreactivity for the α 4 and δ subunits of GABA_AR emerge at the spine plasma membrane of the hippocampal CA1 (Shen et al., 2007). Reduction of immunoreactivity was also verified in the CA1 when applying the antibody solution after preadsorption with the antigen corresponding to amino acids 1-14 of the α 4-subunit and when the primary antibody was omitted from the incubation procedure (Sabaliauskas, Shen, Homanics, Smith, & Aoki, 2012). This antibody has also been shown to recognize a single band at 67kDa by Western blotting (Griffiths & Lovick, 2005; Sanna et al., 2003) and to disappear following preadsorption of the antibody with a peptide corresponding to the target sequence (Sanna et al., 2003).

The secondary antibody was obtained from Electron Microscopic Sciences [Rabbit anti goat IgG Gold ultrasmall gold (0.8 nm diameter of gold conjugated to secondary antibody) catalog #25220, lot 20126/1]. The silver intensification kit used was purchased from KPL (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA). Electron microscopic supplies were purchased from Electron Microscopic Sciences, and all chemicals used for perfusion, tissue storage, and immunostaining were purchased from Sigma Chem (St. Louis, MO, USA).

2.4.2. Electron microscopic immunocytochemistry—Procedures for immunocytochemistry were as described before (Aoki et al., 2012; Aoki et al., 2014). Animals were anesthetized using urethane (i.p. 0.34 g/g body weight) during the hours of 12 noon to 4 pm, then euthanized by transcardial perfusion with fixatives, consisting of 4%

paraformaldehyde buffered with 0.1M phosphate buffer (pH 7.4). Glutaraldehyde-fixation was withheld until after immunocytochemistry, so as to optimize antigen-retention. Brains were postfixed for a minimum of ten days and up to 4 months, then sectioned at a thickness of 50 μm in the coronal plane spanning the dorsal hippocampus, and stored at 4 °C, free-floating in phosphate buffered (0.01M PB, pH 7.6) saline (0.9% sodium chloride) (PBS), containing 0.05% sodium azide to prevent bacterial growth.

The immunocytochemical procedure commenced by incubating the free-floating sections in PBS azide containing 1% bovine serum albumin (Sigma Chem) and 1:100 dilution of the goat primary antibody directed against the $\alpha 4$ subunit of GABA_ARs. The free-floating sections were agitated continuously at room temperature for 3 days, after which time, excess unbound primary antibodies were removed by rinsing in PBS. Sections were then incubated overnight at room temperature in the rabbit secondary antibody, consisting of an anti-goat IgG conjugated to 0.8 nm colloidal gold particles. On the following day, excess unbound secondary antibodies were removed by rinsing in PBS, then postfixed by immersing the sections in PBS containing 2% glutaraldehyde (EMSciences EM grade) for 10 minutes. After rinsing in PBS, sections were stored overnight at 4°C, then processed for silver-intensification of the colloidal gold particles, so as to enlarge the gold particles to sizes detectable by EM. The silver-intensified colloidal gold particles (SIG) ranged in sizes from 10 to 100 nm, even though all procedures were run strictly in parallel. The silver-intensified sections were processed osmium-free (Phend, Rustioni, & Weinberg, 1995), so as to avoid oxidation of the SIG particles. The heavy metals used to generate contrast were uranyl acetate, which also served to improve ultrastructural preservation (Lozsa, 1974; Terzakis, 1968) and Reynold's lead citrate. Vibratome sections were then infiltrated with EMBED-812, flat-embedded between two sheets of Aclar plastic, then ultrathin sectioned at a thickness setting of 70 nm at a cutting plane tangential to the vibratome-cut surface, so as to maximize capture of the EPON-vibratome section surface, where penetration by immunoreagents were maximal.

2.4.3 Electron microscopic quantification of $\alpha 4$ subunits of GABA_A receptors

—200 spines receiving excitatory synapses in stratum radiatum (SR) of area CA1 in the dorsal hippocampus were observed at a direct magnification of 40,000 \times and images captured using a 1.2 megapixel Hamamatsu CCD camera from AMT (Boston, MA) from the JEOL 1200XL electron microscope were used for quantification. Spines were identified using criteria described before (Aoki et al., 2014), which consisted of a prominence of thick postsynaptic density (PSD), lack of mitochondria or microtubules, parallel alignment of the plasma membrane associated with the PSD with that of the axon terminal containing clusters of vesicles. All spines encountered at the surface-most regions of vibratome-cut surfaces were analyzed for the position and level of immunoreactivity to the $\alpha 4$ subunits, strictly in the order of encounter, so as to ensure randomness of sampling, until a minimum of 200 spines were encountered from tissue of each animal.

Dendritic shafts were also sampled per animal, also in SR of CA1 of the dorsal hippocampus. These were observed at a direct magnification of 20,000 \times using the same camera and microscope as stated above, and also counted in the order that they were encountered along the vibratome surface. Dendritic shafts were identified by the presence of

mitochondria, and only those parallel to one another were sampled to ensure that they originated from the pyramidal cell layer.

For both, the spines and dendritic shafts, the location of the SIG in each dendritic shaft and spine was noted (at the plasma membrane or intracellular) (Fig 2) and the number of each type as well as the total number was recorded.

The length of the plasma membrane and area of each dendritic shaft were measured using the segmented line tool and polygon tool, respectively, of NIH's software, Image J (version 1.46r). The entire plasma membrane of dendritic shafts was not always visible. Only the visible plasma membrane was counted in the length measure used for analysis. The number of plasma-membranous SIGs for each animal was divided by the total length of the plasma membrane for that animal to obtain a density value (# SIG/unit plasma membrane length). The total plasma membrane length sampled was equalized across all animals (~ 85 μm). A similar measure was calculated for intracellular SIG and total SIG where the total number of intracellular and total SIGs in dendritic shafts of an animal was divided by the total area of sampled dendritic shafts to obtain the number of SIG per unit area.

2.5 Statistics

All statistical analyses were performed using IBM's SPSS 21 and GraphPad Prism version 6. All data were tested using the D'Agostino-Pearson omnibus test for normality. Pearson's or Spearman R was computed to report correlation between measures that respectively were or were not distributed normally. Two-way ANOVA (analysis of variance) tests were conducted on measures of running wheel activity using the between-subject factors of drug and change in vulnerability to ABA as well as drug and change in severity of ABA defined in the next section. The SIG measures for total immunoreactivity and intracellular immunoreactivity in dendritic shafts were not distributed normally so they were square root transformed. Levene's test failed for plasma membranous immunoreactivity (spine and dendritic shaft, drug \times change in vulnerability and drug \times change in severity of ABA) and total spinous immunoreactivity (drug \times change in severity of ABA). So a square root transform of the target variable was used in these instances. Mean and standard error of mean values reported in Table 6 pertain to the untransformed variable.

3. RESULTS

3.1 Quantification of wheel running activities

The goal of this study was to investigate the role of progesterone (P4) in adolescent females' response to a repeated exposure of a stressor. We used food restriction as the stressor of the ABA induction paradigm and wheel running activity (WRA) as a measure of an animal's response to the stressor. All animals underwent two ABA inductions that were separated by 1 week of recovery: half of them received P4 during the 2nd ABA induction, while the other half received just the oil vehicle (Fig 1).

The baseline WRA to the 1st and 2nd ABA inductions were determined by taking an average of the 24-hour activity of the two days preceding the onset of food restriction. The WRA in the 1st ABA was quantified by taking an average of the 24-hour activity during the three

days when food restriction was imposed. As for the 2nd ABA, either P4 or oil alone was systemically injected on the second, third and fourth day of food restriction but not on the first day. Therefore, WRA of the 2nd ABA was calculated by taking the average of the three days during which animals were food restricted and received either P4 or oil. WRA during the first food restriction day of the 2nd ABA provided another type of “baseline”, for comparing the effect of food restriction with and without P4 within individual animals (Fig 3A).

3.2 The 1st baseline running is an indicator of vulnerability to ABA

Twenty-one out of the 23 animals that underwent two ABAs increased running in response to food restriction during the 1st ABA and 8 out of 9 of the oil vehicle-injected animals increased running in response to food restriction during the 2nd ABA. These animals also exhibited a wide range of WRA during the days preceding and during food restriction. During the 1st Baseline, preceding the 1st ABA, some ran greater than 20 km per day, while others ran less than 5 km per day. Similarly, in response to the food restriction during the 1st ABA, some ran 10 km per day, while others increased their running to 30 km per day. In spite of these great individual differences, WRA during the 1st Baseline correlated positively and strongly with WRA during the 1st ABA ($R = 0.49$, $p = 0.016$ for both groups combined) (Fig 4A-left). Similarly, both the oil-injected and P4-injected groups exhibited a strong positive correlation between the WRA of the 2nd Baseline and WRA of the 2nd ABA ($R = 0.92$, 0.69 and $p < 0.0001$, $= 0.012$ for the oil-injected, and P4-injected groups, respectively) (Fig 4B-left). Therefore, baseline WRA is an indicator of vulnerability to ABA, defined as the likeliness of an animal to develop hyperactivity in response to food restriction.

The animal's response to the 1st ABA is comprised of two portions: one is related to the preference of the animal for wheel running, measured as the 1st baseline. The other response is elicited by the stress of food restriction, which we will call severity of 1st ABA (1st SOA), calculated as the WRA during the 1st ABA minus WRA during the 1st baseline (explained verbally in Table 1 and pictorially in Fig. 3A, right). The 1st baseline WRA was negatively correlated with the 1st SOA. This negative correlation was statistically significant ($R = -.703$, $p = 0.0002$ for both groups combined, in Fig. 4A right). In other words, the more that mice ran during the pre-food restriction baseline phase (towards the right along the x-axis of Fig. 4A), the less that they increased running during the food restriction phase (smaller Y-axis value). This correlation between baseline running and food restriction-evoked running was not seen during the 2nd ABA (severity of 2nd ABA, 2nd SOA, calculated as the WRA during 2nd ABA minus WRA during the 2nd baseline, as defined in Table 1 and pictorially explained in Fig. 3A) for either the oil- ($R = -0.04$, $p = 0.905$) or P4-injected ($R = -.317$, $p = 0.316$) groups. This difference between the 1st and 2nd baseline may have arisen because the 2nd baseline was affected by the stressful experience of the 1st ABA, whereas the behavior in the 1st baseline and 1st ABA carried no history of stress exposure.

3.3 Change in baseline running (change in vulnerability) is a predictor of the change in severity to the 2nd ABA, a relationship altered by P4

Since baseline running in the absence of food restriction predicted vulnerability to ABA, we quantified the change in baseline running by comparing WRA during 2nd baseline, the

period preceding the 2nd ABA, relative to the baseline WRA preceding the 1st ABA (Table 1). Henceforth, we refer to this measure as the “change in vulnerability”. We categorized the mice as ‘more vulnerable’ (henceforth referred simply as ‘vulnerable’), if the measure ‘2nd baseline minus 1st baseline’ running was positive and ‘more resistant’ (henceforth referred to as ‘resistant’ or ‘resilient’) if this value was negative (Fig. 3). High runners in the 1st baseline tended to reduce their baseline running after the 1st ABA, as shown by a negative correlation between 1st baseline WRA and the change in vulnerability ($R = -.66$, $p = .001$ for both groups combined).

Mice classified as ‘vulnerable’ showed higher severity than the animals ‘resistant’ to the 1st ABA (1st SOA) (Fig 3-C, Fig 5A - right, $M_{\text{vul}} = 9.707 \pm 1.054$, $n=13$; $M_{\text{res}} = 3.282 \pm 2.064$, $n=10$). This result suggests that vulnerable mice were affected by the 1st ABA induction and this effect persisted through recovery and up to the 2nd baseline phase.

The change in vulnerability (x-axis of Fig. 4-C) was a good predictor of RWA of 2nd ABA, compared to the 1st ABA (Y-axis of Fig. 4-C) in the oil-injected animals ($R = 0.79$, $p = 0.0034$) (Fig. 4C - left) but not for the P4-injected animals ($R = 0.12$, $p = 0.7$) (Fig 4D - left). We surmised that since P4 was injected during the food restriction phase of the 2nd ABA, P4 may have altered the animal's severity of response to food restriction during the 2nd ABA (2nd SOA), without altering the animal's response to other sources of stress, such as social isolation, that could have influenced the baseline WRA. In order to isolate the effect of food restriction upon WRA, we calculated the **change in SOA**, mathematically expressed as WRA in (2nd ABA minus 2nd baseline) minus (1st ABA minus 1st baseline) (Table 1). A positive value for this measure indicated that the animal had exacerbated its response to the 2nd ABA as compared to the 1st ABA. A negative or zero value indicated that the animal had not exacerbated its response to the 2nd ABA as compared to the 1st. The change in SOA correlated negatively with the change in vulnerability of the P4-injected animals ($R = -0.81$, $p = 0.0013$, Fig. 4D - right), meaning that P4 exacerbated the ABA response especially more for those animals that had undergone a reduction in preference for running, following recovery from the 1st ABA. This relationship was absent for the oil-injected animals ($p > 0.2$) (Fig 4C - right), and the change in SOA observed among the resistant oil-injected individuals (those with negative values along the x-axis) were overall less than the values observed for the P4-injected group. Together, these results indicate that the exacerbated hyperactivity effect upon the resistant individuals was specific to the P4 treatment.

3.4 P4 worsens the severity of response in the 2nd ABA

The specificity of the effect of P4 upon ABA, relative to the pre-food restriction vulnerability, prompted us to search for additional behavioral effects of the drug that depended on vulnerability. WRA of the oil-injected group during the 2nd ABA (average of FR2, FR3 and FR4) was compared to two kinds of behavioral measurements (Table 1): 1) WRA during the 1st ABA, indicating change in response to a repeated stressor; 2) 2nd Baseline WRA, indicating the effect of food restriction in that repeated episode of ABA. WRA of the P4-injected group during the 2nd ABA was compared to three kinds of behavioral measurements: 1) WRA during the 1st ABA, indicating P4's modulation of a change in response to a repeated stressor; 2) 2nd Baseline WRA, indicating P4's modulation

of the effect of food restriction in that repeated episode of ABA; and 3) WRA on FR1 of the 2nd ABA (a day before P4 injection began), which also indicated an effect of P4 upon the animal's response to a repeated stressor.

The oil- and P4-groups showed no differences in the WRA on 2nd ABA FR1 (two-way ANOVA, change in vulnerability X subsequent assignments to the drug groups). We measured the increase of WRA in response to food restriction with and without P4, averaged over FR2, FR3 and FR4 of 2nd ABA (2nd SOA, Table 1). P4-injected mice responded with higher 2nd SOA than did the oil-injected mice ($M_P = 7.75 \pm 0.95$, $n=12$, $M_{oil} = 3.82 \pm 1.35$, $n=11$, Mean difference = 3.93 ± 1.63) (Fig 5A - left). This P4-effect was not likely to be due to a pre-drug difference in stress response, as demonstrated by the two groups' largely overlapping response to food restriction during the 1st ABA (i.e., 1st SOA, Table 1), which was without P4 injection ($M_{Pre-P} = 8.52 \pm 1.68$, $n=12$, $M_{Pre-oil} = 5.15 \pm 1.78$, $n=11$) (Fig. 5-A, graph on right).

Thus, P4 exacerbated the food restriction-evoked hyperactivity of animals to the 2nd ABA.

3.5 P4 removes the resistance acquired during recovery

To evaluate the effect of P4 upon response to repeated stress, we examined the measure of RWA to the 2nd ABA minus the 1st ABA WRA. To follow up a significant interaction between drug and change in vulnerability, we tested simple main effects which revealed response to repeated stress by the oil-injected resistant group (mean = -7.57 ± 2.33) to be significantly less than that of the oil-injected vulnerable group (mean = 2.46 ± 1.76 , $p = 0.003$) (Fig 5-B, left graph). There were no differences between P4-injected resistant and vulnerable groups. These results indicate that P4 removed individual differences in the resistance to repeated stress that was acquired during recovery.

3.6 No acute effect of P4

Mice were not injected on FR1 of the 2nd ABA, so that FR1 WRA could serve as a baseline against which to compare the WRA of the same animals under the influence of P4 injections. On FR2, FR3 and FR4, P4 was injected at 6 pm, an hour before food access. To evaluate the acute (1 to 7 hours) effect of P4 injection on the mice, we computed the WRA during the first half of the dark cycle (7 pm to 1 am) after the first, second and third day's exposure to P4, on FR2, FR3, and FR4 respectively. These values will be referred to as "FR2 minus FR1 (first half of dark cycle)", "FR3 minus FR1 (first half of dark cycle)" and "FR4 minus FR1 (first half of dark cycle)". We found no acute effect of P4 (all $p > 0.13$). All sub-groups of mice, whether injected with P4 or with the oil vehicle alone, ran less in the first half of dark cycle on FR2, FR3 and FR4 than FR1 (data not shown). There were no group differences by two-way ANOVAs.

3.7 Delayed effect of P4: P4 removes the resistance 6 hours after the second injection

Having observed that P4 has no acute effect, we then set out to determine whether P4 has any delayed effect, i.e., during the second half of the dark cycle, from 1 am to 7 am. We computed the measures FR2 minus FR1, FR3 minus FR1 and FR4 minus FR1 (second half of dark cycle, i.e. 1 am to 7 am). The measures FR2 minus FR1 (second half of dark cycle)

(Fig 5-C - left) and FR4 minus FR1 (second half of dark cycle) (data not shown) showed no group difference by a two- way ANOVA (all $p > 0.47$). However, the running activity on FR3 minus FR1 (second half of dark cycle) showed a significant interaction between the factors of drug and change in vulnerability ($F(1,19) = 5.173, p = 0.034$) (Fig 5C - right). Simple main effects showed no significant differences between any 2 sub-groups. These findings indicate that P4 removed the resistance that animals had acquired during the 7 days of recovery, with a delay of 7 to 13 hours after the second injection.

3.8 Sensitization to the 2nd ABA is observed in P4-injected resistant mice

The change in SOA measure compares the food restriction evoked hyperactivity in the 2nd ABA relative to the 1st ABA. It provides a measure of sensitization to repeated ABA. As described previously in the first section under Results and in Table 1, animals with positive values of change in SOA were categorized as ‘sensitized’ or worsened, while animals with negative or zero values were categorized as ‘non-sensitized’.

Resistant animals (P- and Oil-groups combined) exhibited a positive change in SOA ($M_{res} = 2.097 \pm 1.379$), whereas the vulnerable animals exhibited a negative change in SOA ($M_{vul} = -3.454 \pm 1.097$). Simple main effects revealed that the P4-injected resistant animals showed a sensitization reflected in the positive change in SOA (mean = 3.071 ± 2.075) as compared to the P4-injected vulnerable animals (mean = -2.463 ± 1.49) (Fig 5B - right). Oil-injected animals showed little change in SOA, whether vulnerable or resistant (Fig. 5B- right)

3.9 Weight loss

There were no group differences in the weight on baseline, or on any day of the 2nd ABA or at perfusion as a proportion of baseline weight (all $p > 0.07$). On average, the mice lost $18.8 \pm 0.9\%$ of their pre-food restriction weight.

3.10 Open Field

P4 is converted to THP and exert sedating effects at certain doses (Korneyev & Costa, 1996). In order to ensure that P4 was not sedating, OF testing was conducted on FR4 at 6:30 pm, 30 minutes after the injections. Data were collected from 11 P4 – injected and 9 oil - injected mice. The distance travelled per minute in the OF was same between the two groups of mice by a t-test ($t(18) = -1.15, p = 0.276$). A two- way ANOVA also did not show any effect of drug ($F(1, 16) = 2.4, p = 0.14$) or change in vulnerability ($F(1,16) = 1.115, p = 0.3$) or any interaction ($F(1, 16) = 0.007, p = 0.93$). The time spent in the center of the OF was the same between the two groups of mice by a t-test ($t(18) = 0.5, p = 0.62$). A two- way ANOVA also did not show any effect of drug ($F(1, 16) = 0.68, p = 0.42$) or change in vulnerability ($F(1,16) = 2.79, p = 0.11$) or any interaction ($F(1, 16) = 0.35, p = 0.55$). These findings demonstrate that P4 did not affect the level of anxiety of the mice 30 minutes after injection on FR4.

3.11 Estrous stage

Mice are known to run more on proestrus than on other days (Basterfield, Lumley, & Mathers, 2009) and have been shown to remain in diestrus following food restriction (Nelson, Gosden, & Felicio, 1985; Riddle et al., 2013). In order to determine whether the

effects of P4 on WRA were also affected by the estrous stage, we assessed it starting 2 days before the onset of food restriction in the 2nd ABA to the end of the experiment. To examine whether the delayed effect of P4 during the second half of the dark cycle of FR3 in the 2nd ABA could be attributed to estrous stage, we performed Chi-Squared Test of Independence of Categorical Variables. Estrous stage had no impact on how much they ran on FR3 in the second half of the dark phase relative to FR1 (Pearson Chi-Square (2) = 2.79, $p = 0.24$, Likelihood Ratio (2) = 3.55, $p = 0.169$, Linear-by-Linear Association (1) = 0.754, $p = 0.38$). The estrous stage during 2nd baseline, FR1, FR2, FR3 or FR4 was not associated with changes in SOA (all $p > 0.07$).

3.12 $\alpha 4$ -immunoreactivity in the stratum radiatum of the dorsal hippocampal CA1

To identify a possible cellular mechanism underlying the behavioral effect of P4, we examined the subcellular levels and localization of $\alpha 4$ subunits of GABA_ARs in pyramidal neurons of the dorsal hippocampal CA1. Our rationale for examining $\alpha 4$ subunits of GABA_ARs was two-fold: P4's metabolite, THP, is known to regulate the expression as well as the activity of $\alpha 4$ -containing GABA_ARs in this brain (reviewed in (H. Shen et al., 2007)). Moreover, we had previously observed that adolescent female rats elevate levels of $\alpha 4$ and δ subunits of GABA_ARs at spines of the dorsal hippocampus within 4 days of ABA induction (Aoki et al., 2012).

The level of $\alpha 4$ -immunoreactivity was measured in three ways, using silver intensified gold immunolabeling (SIG) as indications of the presence of $\alpha 4$ subunit proteins generating immunoreactivity: at the plasma membrane, in the cytoplasm and plasmalemmal +cytoplasmic (i.e., total) within two distinct subcellular domains of pyramidal neurons - in the spine or in the dendritic shaft (Fig. 2). Immunoreactivity at the plasma membrane is likely to reflect the presence of functional $\alpha 4$ -containing GABA_A receptors ($\alpha 4$ -GABARs) and could directly impact the local membrane potential. Immunoreactivity in the cytoplasm is likely to reflect the presence of $\alpha 4$ -GABA_ARs in the reserve pool that could be inserted into the plasma membrane upon appropriate signaling. The total $\alpha 4$ -immunoreactivity reflects an increase or decrease in the protein level of the subunit, independent of the subcellular trafficking step. For $\alpha 4$ -immunoreactivity at spines, the levels were compared across animals by normalizing to the total number of spines encountered, labeled or unlabeled. For $\alpha 4$ immunoreactivity at dendritic shafts, the levels were compared across animals by normalizing to the total lengths of the plasma membrane or total area of the cytoplasm analyzed, strictly in the order of encounter of dendritic profiles at the surface-most portions of vibratome sections. Tables 2, 3 and 4 and Fig. 6 summarize the correlations between measures of individual animals' WRA and $\alpha 4$ -immunoreactivity on spines and dendritic shafts.

3.12.1 $\alpha 4$ -immunoreactivity at the plasma membrane of spines and dendritic shafts: $\alpha 4$ -containing GABA_ARs may play different roles in the presence and absence of exogenous P4—In the absence of P4 (i.e., the oil-injected group), the level of $\alpha 4$ -immunoreactivity at the plasma membrane of spines (but not dendritic shafts) was negatively correlated with SOA in the 1st ABA (= 1st ABA - 1st baseline) (Fig. 6A-right, Table 2) and with SOA in the 2nd ABA (= 2nd ABA - 2nd baseline), (Fig. 6-B, right; Table

2). This finding replicates an earlier study of adolescent female hippocampal spines following only one ABA (Aoki et al., 2014), indicating that $\alpha 4$ -immunoreactivity contributes towards the animal's resistance to food restriction-evoked hyperactivity.

In the P4-injected group, $\alpha 4$ -immunoreactivity in the plasma membrane of spines as well as at dendritic shafts was more than 100% greater than the values detected among the oil-injected group. This difference was significant (Table 5) and also evident by visual inspection of electron micrographs (Fig. 2). Moreover, this enhancement depended on the animal's pre-P4 behavioral trait: P4 enhanced $\alpha 4$ immunoreactivity preferentially among those with low 1st SOA (Fig. 6A-left) and among those that acquired resistance during recovery (negative y-axis values in Fig. 6C-left). In fact, Fig 6C-left indicates a strong correlation between a change in vulnerability during recovery (y-axis) and $\alpha 4$ -immunoreactivity, indicating that the more that animals gained resistance during recovery (the more negative the y-axis value), the more that they could respond to the subsequent P4 injection with increased $\alpha 4$ -immunoreactivity.

The strongly negative correlation of oil-injected brains' $\alpha 4$ -immunoreactivity with SOA of the 2nd ABA (Fig. 6B-right) was absent among the P4-injected animals (Fig. 6B-left). This indicates that the P4 injection during the 2nd ABA perturbed the relationship between $\alpha 4$ -immunoreactivity and resistance to ABA. Instead, the levels of $\alpha 4$ -immunoreactivity at the plasma membrane of spines as well as dendritic shafts of the P4-injected animals were correlated with the change in SOA (Fig. 6D-left). This correlation was positive, indicating that the higher the $\alpha 4$ -immunoreactivity, the greater was the worsening of SOA.

3.12.2 Cytoplasmic (intracellular) $\alpha 4$ -immunoreactivity in spines and dendritic shafts: P4 administration results in differential synthesis of the $\alpha 4$ subunit—

In the P4-injected animals, the correlation of cytoplasmic $\alpha 4$ -immunoreactivity in spines as well as dendritic shafts with WRA was similar to that seen for the $\alpha 4$ -immunoreactivity at the plasma membrane: SOA in 1st ABA (negative correlation in spines, trend in dendritic shafts), and change in vulnerability (negative correlation for both spines and dendritic shafts) (Table 3). As was suggested for the plasma membrane localization of $\alpha 4$ -immunoreactivity, these correlations indicate differential P4 injection induced synthesis of $\alpha 4$ subunits in animals, depending on their trait behavior reflected in the baseline WRA.

In the oil-injected group, there was a negative correlation between cytoplasmic $\alpha 4$ -immunoreactivity in spines and change in SOA that was not seen at the plasma membrane of spines. This correlation was in a direction opposite to that of the P4-injected group (Table 3) and indicates that the higher the $\alpha 4$ -immunoreactivity, the less that SOA worsened. This pattern is consistent with the relationship between behavior and $\alpha 4$ -immunoreactivity at the plasma membrane (Table 2) and in the cytoplasm (Table 3) that were held constant throughout the 1st and 2nd ABA of oil-injected animals of this study (SOA in 1st ABA and SOA in 2nd ABA) and for previously studied cohorts that received no injection (Aoki et al., 2014): namely that $\alpha 4$ expression confers resistance against ABA through increased synthesis (in the cytoplasm) and trafficking of the $\alpha 4$ -containing GABA_ARs to the plasma membrane. They further support the findings of the plasma membrane $\alpha 4$ -immunoreactivity – namely, that $\alpha 4$ -containing GABA_ARs influence behavior differently in the presence and

absence of exogenous P4. Specifically, higher cytoplasmic $\alpha 4$ -immunoreactivity was associated with more habituation in oil-injected animals but more sensitization in P4-injected animals.

3.12.3 Total (plasmalemmal + cytoplasmic) $\alpha 4$ -immunoreactivity in spines and dendritic shafts—In the P4-injected animals, the total $\alpha 4$ -immunoreactivity in spines and dendritic shafts showed the same relationships to measures of RWA as exhibited by the cytoplasmic $\alpha 4$ -immunoreactivity (not shown). In the oil-injected animals, the total $\alpha 4$ -immunoreactivity in spines (but not in dendritic shafts) showed a negative correlation with 2nd SOA and change in SOA (trend), parallel to those exhibited by the cytoplasmic $\alpha 4$ -immunoreactivity (not shown).

3.13 Impact of change in vulnerability upon P4 effects

Given that the effect of P4 on ABA behavior depended upon whether they were resistant or vulnerable prior to P4 injection, we determined the mean value of $\alpha 4$ -immunoreactivity for each group, categorized according to their behavior and drug treatment (Table 5). We then examined the effect of the change in vulnerability upon $\alpha 4$ -immunoreactivity levels in the oil- and P4-injected mice (Table 4 and Fig. 7).

3.13.1. Spinous $\alpha 4$ -immunoreactivity is higher in resistant animals, especially those injected with P4— $\alpha 4$ -immunoreactivity at spines was higher in the CA1 of resistant animals (oil- or P4-injected) than of the vulnerable animals (2.47 times higher at the plasma membrane, 1.32 times higher at the cytoplasm and 0.78 times higher for total SIG counts, Table 5). Simple main effects to follow up a trend to an interaction revealed that P4- injected resistant animals have higher $\alpha 4$ -immunoreactivity at spines, compared to the values from the P4-injected vulnerable animals (7.27 times higher at the plasma membrane, $p = 0.001$ [Fig. 7A-left]; 5.24 times higher at the cytoplasm, $p=0.001$; 3.15 times higher for total counts, $p=0.008$ [not shown]) (Table 5). These observations indicate that P4 injection had opposite effects, depending on the vulnerability that the animals exhibited prior to P4 injections. As a consequence of the bidirectional action of P4, the oil-injected vulnerable animals also had 2.35 times higher level of total (plasmalemmal plus cytoplasmic) $\alpha 4$ -immunoreactivity at spines than spines of P4-injected vulnerable animals ($p = 0.032$) (Table 5).

There was no difference in $\alpha 4$ -immunoreactivity between the oil-injected resistant and vulnerable animals at the plasma membrane and/or in the cytoplasm of spines.

3.13.2. Dendritic shaft $\alpha 4$ -immunoreactivity is higher in resistant animals, especially those injected with P4— $\alpha 4$ -immunoreactivity at dendritic shafts followed the same pattern observed for the spines. Specifically, $\alpha 4$ -immunoreactivity was higher at dendritic shafts of resistant animals than of vulnerable animals (3.22 times higher at the membrane, 2.35 times higher in the cytoplasm and 2.42 times higher for total SIG counts, Table 5). Simple main effects to follow up a significant interaction revealed that P4-injected resistant animals showed 8.27 times higher $\alpha 4$ -immunoreactivity than P4-injected vulnerable animals (8.27 times higher at the plasma membrane, $p = 0.001$ [Fig. 7A-right]; 13

times higher in the cytoplasm, $p < 0.001$ [not shown]; 13.88 times higher for total SIG counts, $p < 0.001$ [not shown]) (Table 4).

As a consequence of the bidirectional action of P4, the oil-injected vulnerable animals also had higher level of $\alpha 4$ -immunoreactivity than P4-injected vulnerable animals (4.36 times higher at the cytoplasm, $p = 0.016$; 4.59 times higher for total counts, $p = 0.013$). Levene's test for homogeneity of variance was significant for this ANOVA ($p = 0.004$).

These findings indicate that P4 injection increased $\alpha 4$ -immunoreactivity at dendritic shafts of the resistant group and somewhat decreased $\alpha 4$ -immunoreactivity for the vulnerable group.

3.14 Impact of P4 and change in SOA

To examine any possible association between the behavioral sensitization to repeated ABA and $\alpha 4$ -immunoreactivity on the spine or dendritic shaft immunoreactivity, we conducted two-way ANOVA on the measures of immunoreactivity using the two fixed factors of drug and change in SOA.

3.14.1 Spinous $\alpha 4$ -immunoreactivity is higher in P4-injected, behaviorally sensitized animals—A significant interaction between the two factors of drug and change in SOA in their effect on the plasmalemmal immunoreactivity on spines was found (Table 4, Fig 7B-left). The $\alpha 4$ -immunoreactivity of P4-injected sensitized group was 1.67 times higher than that of the oil-injected sensitized group ($p = 0.012$) and 7 times higher than that of the P4-injected non-sensitized group ($p < 0.001$) (Table 5). These findings indicate that among the P4-injected animals, increased $\alpha 4$ -immunoreactivity at the spine plasma membrane was associated with sensitization. Decreased $\alpha 4$ -immunoreactivity among the P4-injected animals was associated with non-sensitization, as suggested by the twice higher $\alpha 4$ -immunoreactivity in oil-injected non-sensitized animals compared to the P4-injected non-sensitized ($p = 0.019$).

Similarly to the plasma membrane component, there was a significant interaction between drug and change in SOA in their effect on cytoplasmic immunoreactivity in spines (Tables 4 and 5). $\alpha 4$ -immunoreactivity of the P4-injected sensitized group was 2.47 times higher than that of the oil-injected sensitized group ($p = 0.005$) and 3.07 times higher than that of the P4-injected non-sensitized group ($p < 0.001$). These findings indicate that among the P4 injected animals, increased cytoplasmic $\alpha 4$ -immunoreactivity in the spine was associated with sensitization. The interaction between drug and change in SOA in their effect was also significant for the total count of $\alpha 4$ -immunoreactivity (at the plasma membrane plus cytoplasm). The P4-injected sensitized group had 3.12 times higher $\alpha 4$ -immunoreactivity than the P4 injected non-sensitized group ($p = 0.001$). Oil-injected non-sensitized group had 2.11 times higher $\alpha 4$ -immunoreactivity than P-injected non-sensitized group ($p = 0.004$).

Together, these findings indicate that P4 has an effect on the relationship between $\alpha 4$ -immunoreactivity in spines and behavior in ABA. Specifically, among the P4-injected animals, increased $\alpha 4$ -immunoreactivity at spines was associated with sensitization and decreased $\alpha 4$ -immunoreactivity with non-sensitization.

3.14.2. Dendritic shaft α 4-immunoreactivity is higher in P4-injected, behaviorally sensitized animals

—Analysis of dendritic shafts revealed a similar pattern as that seen for dendritic spines, in that, among the P4-injected animals, increased α 4-immunoreactivity was associated with sensitization and decreased α 4-immunoreactivity with non-sensitization. The one difference between the spine and dendritic shaft components was the subcellular component exhibiting the greatest interaction between the two factors of drug and change in SOA: it was the plasma membrane for spines and the cytoplasm for dendritic shafts. Specifically, sensitized animals exhibited 2.46 times higher α 4-immunoreactivity at the dendritic shaft plasma membrane than of non-sensitized animals. Analysis of simple main effects to follow up a trend to an interaction revealed sensitized P4-injected group to have 4.11 times higher α 4-immunoreactivity than P4 injected non-sensitized group ($p = 0.001$) (Fig. 7B-right). This is less than the 7-times higher α 4-immunoreactivity ($p < 0.001$) observed for the spine plasma membrane. There was a significant interaction between drug and change in SOA in their effect on cytoplasmic α 4-immunoreactivity in dendritic shafts (Tables 4 and 5). The P4-injected sensitized group had thrice the α 4-immunoreactivity of the oil-injected sensitized group ($p = 0.001$) and 8 times higher than that of the P4 injected non-sensitized group ($p < 0.001$). This latter difference between the sensitized versus non-sensitized group is greater than what was observed for the spine cytoplasm (3x, $p = 0.001$).

There was a significant interaction between drug and change in SOA in their effect on total dendritic shaft α 4-immunoreactivity (Table 4). The P4-injected sensitized group had thrice the α 4-immunoreactivity than the oil-injected sensitized group ($p = 0.012$) and 8.1 times higher than that of the P4 injected non-sensitized group ($p < 0.001$). Oil-injected non-sensitized group also had 1.47 times greater α 4-immunoreactivity than P4 injected non-sensitized ($p = 0.038$).

4. DISCUSSION

The main finding is that P4 reversed the effect of α 4-immunoreactivity with respect to the animal's sensitivity to food restriction stress. In the absence of exogenous P4, elevation of α 4-immunoreactivity was associated with the protective behavior, consisting of the suppression of high WRA to repeated ABA: suppression of high WRA is protective, because high WRA can be lethal to food-restricted adolescent female mice (Chowdhury et al., 2013). In the presence of P4, the higher the α 4-immunoreactivity, the greater was the animal's sensitivity to repeated ABA. We provide a working hypothesis for the reversal of the role of α 4-immunoreactivity evoked by P4.

4.1 Baseline voluntary WRA as predictor of vulnerability

Mice exhibit great variability in their behavioral response to ABA. We have systematically captured this variability by quantifying the change in vulnerability to ABA as well as the changes in severity of response to repeated ABA. The quantitative analysis revealed that WRA during the 1st baseline is a good predictor of an individual's vulnerability to ABA. From analysis of repeated ABA, we demonstrated that changes in the baseline WRA of an animal is also a good predictor of changes in the severity of the animal's response to ABA,

i.e., the change in vulnerability. The stress response of high voluntary runners has not been studied directly by others, but we can compare our results with previous studies on the stress response of high aerobic capacity runners. High aerobic capacity runners are reported to exhibit lower cort responses and are proposed to have genetic similarities with animals selectively bred for high voluntary running (Waters et al., 2008). They also adopt a more active coping strategy to stress than low aerobic capacity runners in the forced swim test (Burghardt et al., 2010). Our data revealing a smaller increase in food restriction-evoked activity during the 1st ABA among animals with high baseline activity (Figure 4A-right) are consistent with Burghardt's result on high aerobic capacity runners, since suppression of hyperactivity during food restriction is a more adaptive coping strategy for animals reared in captivity.

4.2 Relationships between WRA and P4 administration

Prompted by previous findings that P4's metabolite, THP, is anxiolytic (Bitran et al., 1995; C. A. Frye & Paris, 2011; C. A. Frye & A. A. Walf, 2004; Koonce & Frye, 2013) and that the food restriction-evoked hyperactivity correlates positively with anxiety (G. Wable et al., 2015), we tested the hypothesis that P4 ameliorates responses to a second ABA induction. Contrary to our prediction, we found that P4 exacerbates the hyperactivity response (Figure 5A, left) as well as sensitize mice that had acquired resistance during recovery from the first ABA induction (Fig 5B, right). Sensitization to the 2nd ABA was quantified as the change in SOA. Among the oil-injected animals, the change in SOA was strongly and positively correlated to the 1st baseline WRA. In contrast, this correlation was lost by P4 injection. Moreover, for the P4-injected group, a stronger and negative correlation was seen between the change in SOA and change in vulnerability, indicating that acquisition of resistance during the recovery period (change in vulnerability is negative) rendered them to become more sensitized to the 2nd ABA (change in SOA is positive). That the resistant animals demonstrated low baseline WRA just prior to the 2nd ABA indicates that their P4 response during the 2nd ABA is specific to the combination of wheel and food restriction and that behavioral sensitization is a distinct effect of the repeated ABA.

Although the dose of P4 that we used has been shown to be anxiolytic in *ad libitum* fed female mice of similar ages (Cheryl A Frye & Alicia A Walf, 2004; Koonce & Frye, 2013), this was not effective for our food-restricted mice, as revealed by our open field test results. The mechanism of change in anxiety level due to food restriction may be via the rise in cort, leading to increased signaling of principal neurons in the amygdala and increasing its outflow (Joels, Sarabdjitsingh, & Karst, 2012). We have also observed a reduction of α 4-GABA_AR expression within inhibitory interneurons in the basolateral amygdala of ABA-induced rats, which could contribute to increased signaling of the principal neurons there (G. S. Wable et al., 2013). Possibly, the rise of cort prevents the anxiolytic action of P4, which is reported to be due to the interaction of THP and GABA_ARs (Canonaco et al., 1993; Engin & Treit, 2007; C. A. Frye & A. A. Walf, 2004; Koonce & Frye, 2013; Reddy et al., 2005). Pubertal stress is found to alter the hormone response to anxiety testing at 8 weeks of age (Olesen, Ismail, Merchasin, & Blaustein, 2011), supporting an idea that an interaction between the HPA axis and P4 could be at play during ABA induction.

4.3 Sensitization to ABA response in P4-injected resistant animals might be via $\alpha 4$ -containing GABA_ARs

4.3.1. P4 affects $\alpha 4$ -immunoreactivity differently, depending on the animal's behavioral phenotype that preceded P4 injection—P4 injection brought about an increase in $\alpha 4$ -immunoreactivity in resistant animals, the same group that had shown high running activity in the first baseline period. A difference in motivation to run on the wheel is associated with considerable difference in neurobiology and other behaviors in rodents, such as nest building and stress response (reviewed in (Rhodes, Gammie, & Garland, 2005)). Mice that choose to run more show a higher increase in brain derived neurotrophic factor (BDNF) protein in the hippocampus (Johnson, Rhodes, Jeffrey, Garland Jr, & Mitchell, 2003). BDNF is an important regulator of the synthesis (Roberts, Hu, Lund, Brooks-Kayal, & Russek, 2006) and trafficking of $\alpha 4\beta\delta$ -GABA_ARs from the cytoplasm to the plasma membrane (Joshi & Kapur, 2009). P4 increases BDNF expression (Meyer et al., 2013; Singh & Su, 2013) and BDNF may possibly be the mediator of the trait behavior-dependent effect of neurosteroids such as THP on the expression of $\alpha 4$ -containing GABA_ARs. The greater wheel running might result in a higher propensity for mature BDNF expression via enhanced post-translational modification (Sartori et al., 2011). P4 administration would lead to a greater increase in mature BDNF release, which, in turn, would lead to higher $\alpha 4$ subunit expression. The lack of correlation between $\alpha 4$ -immunoreactivity and 1st baseline WRA in the oil-injected animals suggests that P4 contributed to the production of additional BDNF that led to an increase of $\alpha 4$ subunit.

The decrease in $\alpha 4$ -immunoreactivity in P4-injected vulnerable animals could be due to an increase in proBDNF on P4 administration that did not ultimately convert to BDNF because they were low runners in baseline and possibly did not have any enhancement of enzymatic machinery for conversion of pro-BDNF to the mature form. proBDNF is known to have effects that are antagonistic to those of BDNF (Lu, Pang, & Woo, 2005). BDNF enhances and proBDNF represses synthesis and activity of synaptic GABA_AR (Riffault et al., 2014). Thus, it is conceivable that an increase in proBDNF without increased BDNF to balance it might result in a decreased expression of $\alpha 4$ subunits as seen in the P4-injected vulnerable animals. The lack of a decrease of $\alpha 4$ -immunoreactivity in oil-injected vulnerable animals as compared to the oil-injected resistant animals demonstrates that P4 administration contributed to the reduction of $\alpha 4$ -immunoreactivity in the vulnerable animals.

It is known that estrogen influences the effect of exercise on BDNF induction (Berchtold, Kesslak, Pike, Adlard, & Cotman, 2001). Given that P4 interacts with estrogen in several ways to affect signaling, disease state and behavior, including wheel running (Baudry, Bi, & Aguirre, 2013; Rodier III & Segal, 1977), it is important that future studies investigate the role of P4 in BDNF induction by exercise.

Previous studies indicate that changes in the expression of $\alpha 4$ -containing GABA_ARs are observed 48 hours following exposure to THP (allopregnanolone), the metabolite of P4 (H. Shen, Q. H. Gong, M. Yuan, & S. S. Smith, 2005; S. S. Smith, Shen, Gong, & Zhou, 2007), closely matching the timing of behavioral change in vulnerability that we observed (WRA on FR3, after the second P4 injection). Fluctuations in the level of P4 and THP due to the

injection may have been superimposed with the release of these hormones from the adrenal glands due to the stress of food restriction, resulting in the increased expression of the $\alpha 4$ -containing GABA_AR in the P4-injected resistant animals. The sensitizing effect of P4 on behavior might then be mediated by the $\alpha 4$ -containing GABA_AR (as demonstrated by the correlation between $\alpha 4$ -immunoreactivity and change in SOA) due to one or more of the following reasons.

4.3.2. $\alpha 4$ -containing GABA_AR may mediate sensitization of ABA response in P4-injected animals—Among the oil-injected animals, the negative correlation between plasma membrane $\alpha 4$ -immunoreactivity and the 1st as well as the 2nd SOA (Table 2) is consistent with our past results in which we found $\alpha 4$ -immunoreactivity to be associated with less severe response to ABA (Aoki et al., 2014). By comparison, the equally strong and negative correlation between plasma membrane $\alpha 4$ -immunoreactivity and the 1st SOA among the animals that were subsequently assigned to receive P4-injection indicates that both the to-be-oil- and to-be-P4 groups were protected from severe ABA response, at least in part, by increased $\alpha 4$ -GABA_ARs, but that the subsequent P4-injection perturbed the $\alpha 4$ -GABA_AR-mediated protection. What might be the mechanism underlying the P4-perturbation of $\alpha 4$ -GABA_AR-mediated protection against ABA vulnerability?

Higher excitability of the pyramidal neurons of the CA1 dorsal hippocampus is associated with higher anxiety level (Huttunen & Myers, 1986). Although $\alpha 4$ -containing GABA_ARs contribute towards anxiolysis through tonic inhibition of pyramidal neurons, the $\alpha 4$ -containing GABA_AR that are localized to the CA1 hippocampal pyramidal neurons are unusual in that they are desensitized by P4's metabolite, THP, thereby reversing these receptors' and THP's joint role from being anxiolytic to being anxiogenic (Hui Shen et al., 2007). Although relatively less known, the anxiogenic role of $\alpha 4$ -GABA_ARs and THP in the CA1 is solidly substantiated: the unusual property of THP is due to the Cl⁻ flux direction-dependent desensitizing effect it has on the receptor, conferred by arginine 353 of the $\alpha 4$ -subunit. THP desensitizes $\alpha 4\beta\delta$ -GABA_ARs only when expressed in cells with inward Cl⁻ flux. The direction of Cl⁻ flux is inward for $\alpha 4\beta\delta$ -GABA_ARs expressed by CA1 pyramidal neurons, but is outward for $\alpha 4\beta\delta$ -GABA_ARs expressed by the dentate gyrus granule cells. This difference in the Cl⁻ flux across the two brain regions is likely to be the reason THP does not desensitize $\alpha 4\beta\delta$ -GABA_ARs in the dentate gyrus but operates consistently as a positive modulator there. The reason THP is uniquely anxiogenic at puberty (H. Shen et al., 2007) may be because $\alpha 4\beta\delta$ -GABA_AR expression level is low prepubertally and in adulthood in the CA1 but rises at puberty. In contrast, $\alpha 4\beta\delta$ -GABA_AR expression is consistently high throughout life in the dentate gyrus. This latter idea of CA1's $\alpha 4\beta\delta$ -GABA_ARs mediating anxiogenesis at puberty through the action of THP is supported by the finding that global knockout of the δ -subunit eliminates the THP-evoked anxiety at puberty (H. Shen et al., 2007). The current study extends upon this previous finding by demonstrating that CA1 pyramidal neurons can be evoked to continue express heightened levels of $\alpha 4$ -containing GABA_AR beyond puberty (ca PND 32), up to early adulthood (ca PND 59), rendering these neurons to be modulated throughout adolescence by THP, and generating anxiety (H. Shen et al., 2007; Hui Shen et al., 2007). Presumably, desensitization of $\alpha 4$ -containing GABA_AR increases excitability of the CA1 pyramidal neurons, because it

removes the shunting inhibition that counteracts the glutamate receptor-mediated excitatory input to spines (Fig. 8). In support of this idea, we have observed that female adolescent rats express heightened levels of NR2A and NR2B subunits of NMDA receptors within dendritic spines of the hippocampal pyramidal cells in the CA1 following ABA induction (Aoki et al., unpublished observations).

$\alpha 4\beta\delta$ -GABA_ARs are exquisitely sensitive to THP, more so than are the $\alpha 1\beta\gamma$ -GABA_ARs (Belelli & Lambert, 2005). Therefore, the developmental switch of THP action is due to a switch in the subtype of GABA_ARs that predominates the hippocampal CA1: THP's primary action during adulthood and pre-pubertally is upon the predominantly expressed $\alpha 1$ -containing GABA_ARs, which THP potentiates, thereby inducing anxiolysis. In contrast, during puberty onset, THP's primary target becomes the $\alpha 4\beta\delta$ GABA_ARs, which it desensitizes, due to the developmentally regulated rise in the expression of these receptors, thereby inducing anxiogenesis (H. Shen et al., 2007; S. S. Smith, Aoki, & Shen, 2009). In addition to this developmental switch in the GABA_ARs, neurosteroid treatment in adulthood also induces an increase of $\alpha 4$ subunits, concomitant with a reduction of $\alpha 1$ subunit levels in the hippocampal CA1 (Hui Shen, Qi Hua Gong, Maoli Yuan, & Sheryl S Smith, 2005). There are a number of other reports indicating that heightened levels of $\alpha 4$ -GABA_ARs are accompanied by reductions of $\alpha 1$ -GABA_ARs (Brooks-Kayal & Russek, 2012; Kumar, Kralic, O'Buckley, Grobin, & Morrow, 2003; Liang, Cagetti, Olsen, & Spigelman, 2004; Roberts et al., 2006; H. Shen et al., 2005). The P4 treatment upon late adolescent ABA animals could have two combined effects: (1) an increase of $\alpha 4$ -to- $\alpha 1$ ratio; and (2) desensitization of $\alpha 4$ -GABA_ARs, which dominate over the potentiation of $\alpha 1$ -GABA_ARs, due to a subunit switch. This study did not evaluate $\alpha 1$ -immunoreactivity, so the contributions made by reductions of $\alpha 1$ subunits towards excitability of pyramidal cells following repeated exposures to food restriction stress remain undetermined. Also undetermined are the contributions made by other parts of the hippocampus, such as the dentate gyrus, or other brain regions to the behavioral phenotype.

One further limitation of our study is that anxiety testing was conducted only on FR4 and only in the acute phase, i.e. an hour after P4 administration. However, we have found that increased anxiety is correlated with higher RWA in the ABA model and that the change in WRA from baseline to the food-restricted phase is also correlated to the change in anxiety level (G. Wable et al., 2015). The correlation between WRA and anxiety level could be the final link between the expression of $\alpha 4$ -containing GABA_ARs and WRA.

4.4 Implications for mental illness

The model of stress we used, ABA, is currently the most accepted animal model for anorexia nervosa (AN), an eating disorder characterized by self-imposed starvation and often by excessive exercise and anxiety, causing malnutrition and severe weight loss (American Psychiatric Association, 2013). AN has a very high relapse rate (greater than 30 %) and the highest mortality rate among all psychiatric disorders (10 -15 %);(Birmingham, Su, Hlynsky, Goldner, & Gao, 2005; Carter, Blackmore, Sutandar-Pinnock, & Woodside, 2004; Herzog et al., 1999; Strober, Freeman, & Morrell, 1997; Sullivan, 1995).

It has been suggested that P4 could ameliorate the symptoms of AN (Young, 1991). Our data indicate that Young's proposal should be considered cautiously, to the extent that findings from the animal model could be extrapolated to the human disease. This is because we have encountered at least one dose of P4 which, when combined with the individual's history of improved resilience, could exacerbate stress reactivity, while showing no efficacy for those individuals with vulnerability to stress. The lack of literature on the impact of administration of P4 in AN could suggest a lack of efficacy. A recent study about the administration of estrogen with P4 found no impact on eating attitudes or body shape perception in adolescents with AN (Misra et al., 2013). There was a decrease in trait anxiety (but not state anxiety) associated with estrogen replacement, but not with P4 replacement.

Since the level of anxiety and response to ABA in the form of wheel activity are correlated, the impact of P4 on the wheel running activity in late adolescence is important to consider in anxiety disorders as well. Future studies with altered doses of P4 and altered ages of ABA induction are needed to more fully understand the impact of P4 on anxiety, ABA and AN.

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HIGHLIGHTS

- Stress reactivity is predicted by animals' baseline voluntary wheel running.
- Stress resilience is associated with $\alpha 4$ -GABA_AR levels in CA1 pyramidal cells.
- Progesterone exacerbates stress reactivity in resilient mice.
- Progesterone does not reduce hyperactivity in mice that fail to attain resilience.
- Progesterone metabolite may cause the negative effect by desensitizing $\alpha 4$ -GABA_ARs.

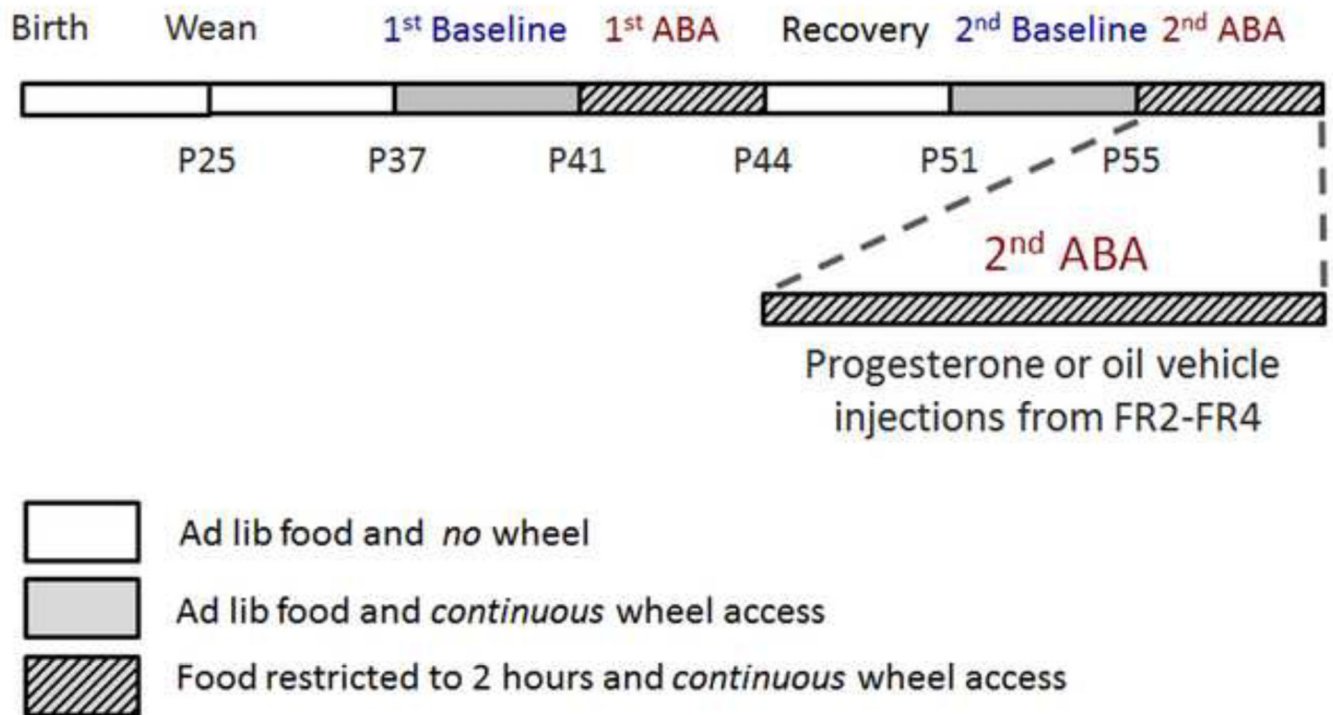


Figure 1. Experimental schedule

Female mice were singly housed with access to a running wheel starting at noon at age P36. After 5 days of acclimation to the wheel (1st Baseline), their food access was restricted to two hours per day (from 7 - 9 PM) for 3 days (1st ABA). At the end of the 1st ABA, they were housed in fresh cages with *ad libitum* food access and no running wheel. Following 7 days of recovery, access to a running wheel was restored. Following 4 days of wheel access with *ad libitum* food access (2nd Baseline), the second induction of ABA (2nd ABA) started, on P55. Mice were divided into two groups on P56, i.e. the second day of food restriction or FR2. The two groups were counterbalanced in terms of running activity on FR1. One group received P4 in oil and the other received only oil. All mice were perfused at noon on P59, at the end of the 2nd ABA, after four days of food restriction.

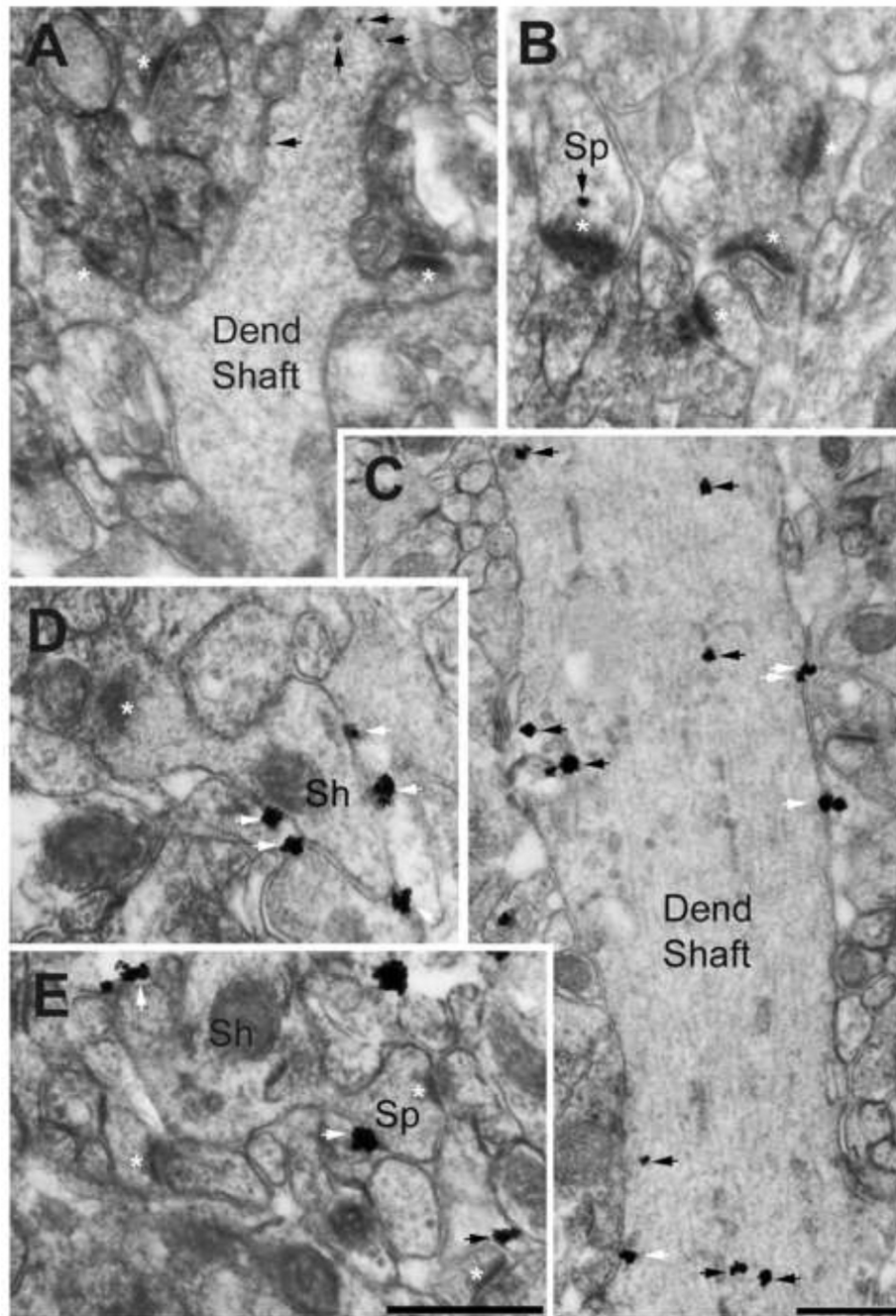


Figure 2. Electron micrographs showing $\alpha 4$ -immunoreactivity in dendritic shafts and spines of the dorsal hippocampal CA1 of adolescent female mice that received P4 injection

Panels A and B were taken from an animal categorized as vulnerable to ABA, while panels C, D and E were taken from an animal categorized as resistant to ABA. Animals had undergone two ABA inductions and were of age PND59. Black arrows point to cytoplasmic location of silver-intensified gold (SIG) particles reflecting $\alpha 4$ -immunoreactivity, while white arrows point to SIG particles located at the extracellular surface of plasma membranes. Note the paucity of SIG particles in the dendritic shaft cytoplasm of the vulnerable animal (Panel A), compared to the dendritic shaft of the resistant animal (Panel

C). Moreover, plasma membrane location of SIG particles is readily apparent in the dendritic shaft of the resistant animal (Panels C and 'Sh' in D), but not of the vulnerable animal. Similarly, SIG is sparse in spines of the vulnerable animal (Sp, white asterisks point to postsynaptic densities, Panel B) but more numerous and located more often at the plasma membrane of the resistant animal (panel E). The black arrow at the lower right corner of panel E points to cytoplasmic labeling in an astrocytic process immediately adjacent to an axo-spinous synapse with a PSD. Calibration bar in E = 500 nm and also applies to panels A, B, and D, all of which were captured at a magnification of 40,000x using AMT Camera system and Hamamatsu's CCD camera. Calibration bar in C = 500 nm, captured at a magnification of 40,000x.

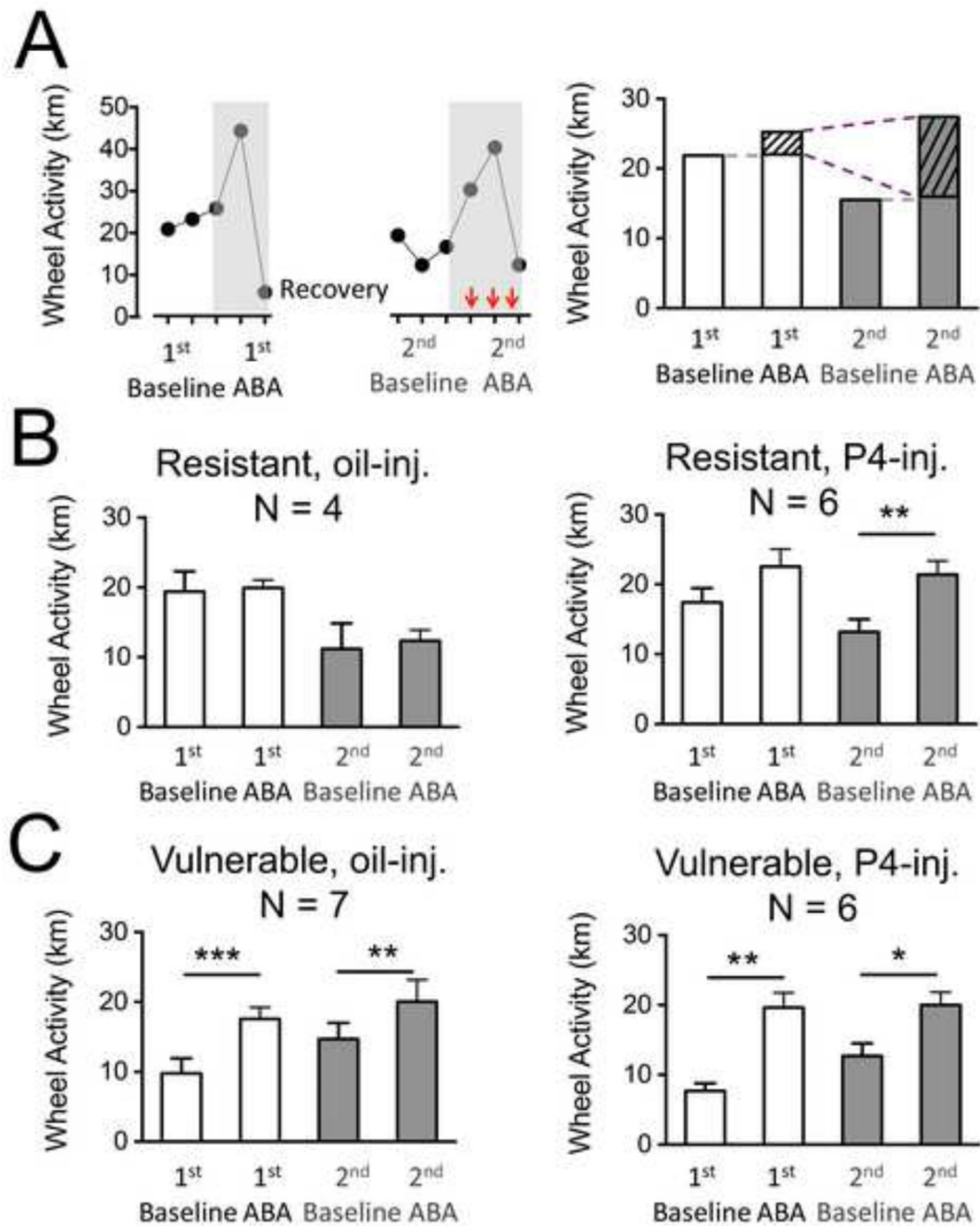


Figure 3. Averaged wheel activity per 24 hours during the four periods of a repeated ABA paradigm

Panel A, left: Daily wheel running activity (WRA) of one animal in the relapse paradigm of ABA. The grey shaded boxes highlight the food restriction periods in the 1st and 2nd ABA. Red (dark gray) arrows indicate the last three days of the 2nd ABA when injections of P4 or oil were given.

Panel A, right: The 1st baseline and 2nd baseline WRA are averaged distances run on the wheel on the two days preceding food restriction in the 1st ABA and 2nd ABA respectively. In this example, the 2nd baseline is less than the 1st baseline, reflecting resistance that is

acquired during recovery. Average distance run on the wheel during the three days of the 1st ABA and the last three days of the 2nd ABA (shown using the vertical red arrows in the left panel) are used as measure of response to ABA. The first day of food restriction (FR1) of the 2nd ABA was not used in the average because the mice did not receive an injection on that day. The dashed gray lines mark the level of baseline activity relative to the ABA activity. The portions of the average activity in the 1st and 2nd ABA marked with slanted lines refer to the measure called severity of ABA (SOA), reflecting the food restriction-evoked component of WRA. The dashed purple lines indicate the comparison of the SOAs of the 1st and 2nd ABAs, used to compute the change in SOA. In this example, the change in SOA is a positive value, indicating sensitization. This is a pattern observed for the P4-injected resistant animals (Fig. 3B-right).

Panels B and C: The wheel activities of all animals in a sub-group are averaged for each period of the experiment. For each animal, the values that are used for the group average are computed as shown in Panel A, right.

Panel B, left: Resistant animals (RWA 2nd baseline < 1st baseline) injected with oil show a non-significant increase in WRA in the ABA period compared to baseline in both episodes of ABA.

Panel B, right: Resistant animals (WRA 2nd baseline < 1st baseline) injected with P4 show a significant increase in WRA in the 2nd ABA period compared to baseline immediately prior to the 2nd ABA.

Panel C, left: Vulnerable animals (WRA 2nd baseline > 1st baseline) injected with oil show a significant increase in WRA in the ABA period compared to baseline in both episodes of ABA.

Panel C, right: Vulnerable animals (WRA 2nd baseline > 1st baseline) injected with P4 show a significant increase in WRA in the ABA period compared to baseline in both episodes of ABA.

* indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$.

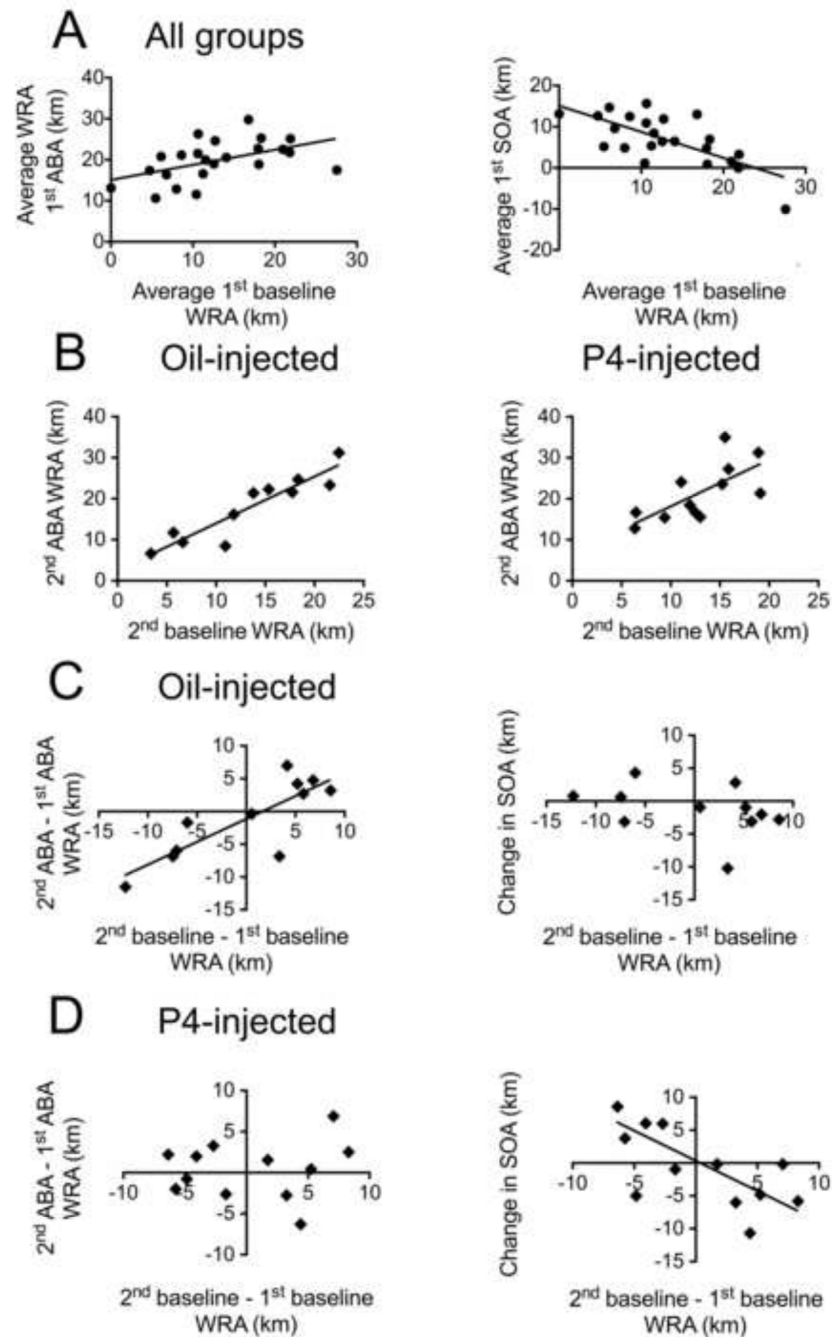


Figure 4. Pre-food restriction baseline WRA is a good predictor of vulnerability to ABA
 The measure of response to ABA induction is WRA during the food restriction period. Vulnerability to ABA is the likeliness that an animal will develop hyperactivity in response to ABA.

Panel A. Baseline WRA for both groups of animals, shown on the x-axis, is the average distance run per 24 hours on the wheel in the two days preceding food restriction in the 1st ABA. Y-axis of left graph shows average distance run per 24 hours on the wheel during the three days of the 1st ABA. The two measures show a positive correlation ($R = 0.49$, $p =$

0.01). Y-axis of right graph shows averaged WRA during the 1st ABA adjusted for the 1st baseline WRA, a measure that reflects food- restriction induced increase in activity (SOA). First SOA is negatively correlated with 1st baseline ($R = -0.66$, $p < 0.0001$).

Panel B. 2nd baseline WRA, shown on the x-axis is a good predictor for WRA during the 2nd ABA for the oil- ($R = 0.92$, $p < 0.0001$) and P4-injected ($R = 0.69$, $p = 0.012$) groups. The first day of food restriction in the 2nd ABA was not included in the average because the mice did not receive an injection on that day.

Panels C and D. Graphs on the left show that the change in vulnerability (plotted on x-axis) is a good predictor for WRA of 2nd ABA compared to the 1st ABA (plotted on y-axis) for the oil-injected ($R = 0.79$, $p = 0.0034$) (Panel C) but not for the P4-injected animals ($R = 0.12$, $p = 0.7$) (Panel D).

Graphs on the right show that the change in vulnerability is a good predictor for the change in SOA (measurement of the worsening of sensitivity to food restriction) of the P4-injected animals ($R = -0.81$, $p = 0.0013$) (Panel D), but not of the oil-injected animals ($R = -0.53$, $p = 0.091$) (Panel C).

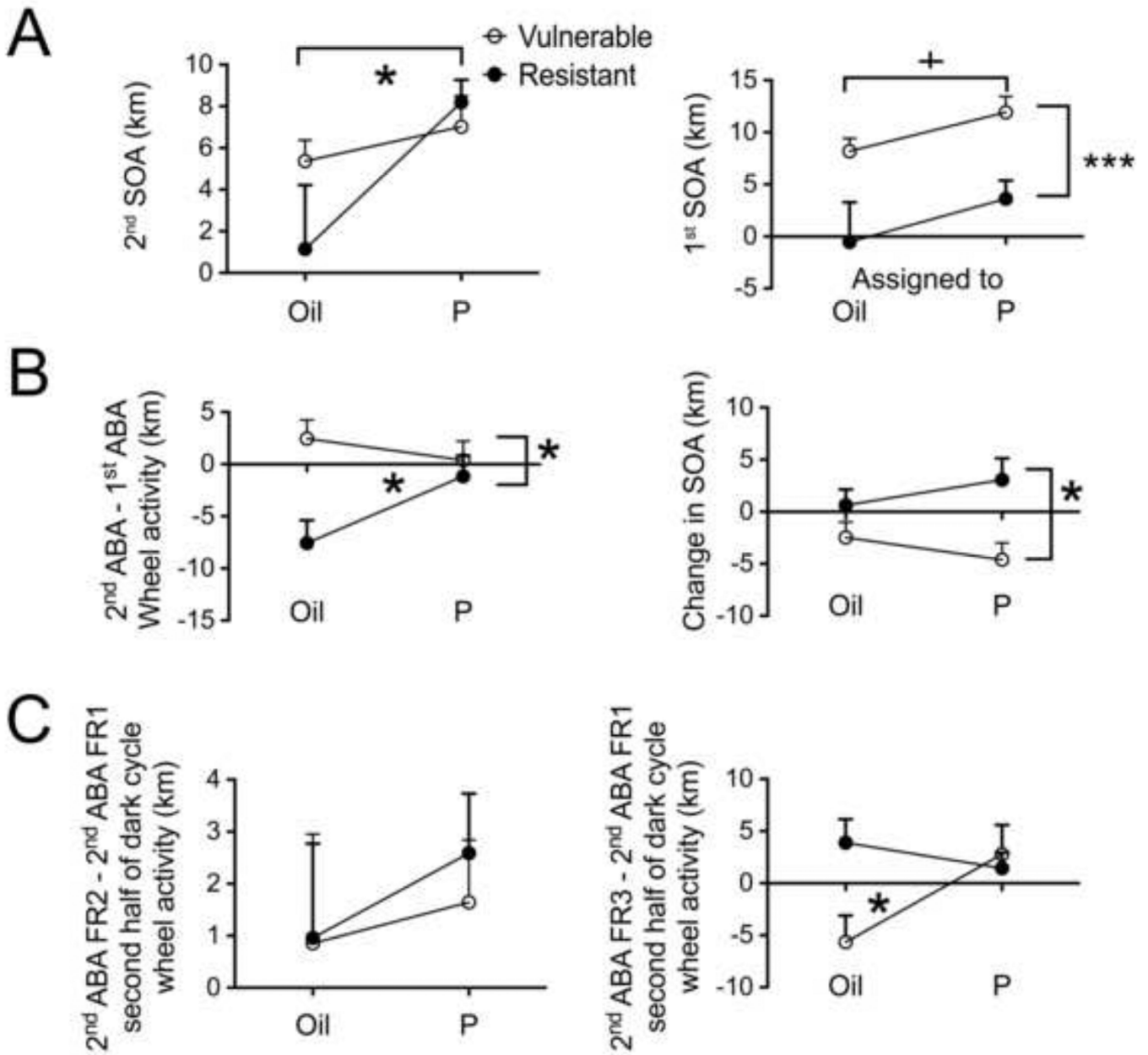


Figure 5. P4 exacerbates the response to the 2nd ABA

Panel A, left graph shows that P4-injected mice responded with higher SOA to the 2nd ABA than did the oil-injected mice (* indicates $p = 0.012$ by two-way ANOVA using drug and change in vulnerability as fixed factors). The y-axis shows the averaged WRA during the 2nd ABA adjusted for the 2nd baseline WRA, a measure that reflects food-restriction induced increase in activity (SOA).

Panel A, right graph shows that animals categorized as vulnerable (P- or oil-injected) based on a positive value for 2nd baseline minus 1st baseline WRA showed a higher SOA to the 1st ABA than the animals categorized as resistant (P-or oil-injected) (***) indicates $p = 0.0003$ by two-way ANOVA using drug and change in vulnerability as fixed factors).

Despite a trend to a difference between the P4-injected (vulnerable plus resistant) and oil-injected (vulnerable plus resistant) mice in their SOA in the 1st ABA (+ indicates $p = 0.058$), post hoc tests showed no difference between the animals that would later receive P4 within the resistant or vulnerable groups or oil injections within the resistant or vulnerable groups. This indicates that there was no pre-existing difference between the animals assigned to the two drug groups within each category of resistant and vulnerable animals.

Panel B, left graph shows response severity in 2nd ABA compared to the 1st ABA. A two-way ANOVA using drug and change in vulnerability as fixed factors shows a main effect of vulnerability and a significant interaction between the factors of vulnerability and drug injection (* indicates $p < 0.05$). P4 injection removes the resistance of mice exhibited in the 2nd baseline period.

Panel B, right graph shows change in SOA. A two-way ANOVA using drug and change in vulnerability as fixed factors shows a main effect of vulnerability, no effect of P4 and no interaction between the factors of vulnerability and drug (* indicates $p < 0.05$). Post hoc tests reveal that the P4-injected resistant animals show a sensitization reflected in the change in SOA as compared to the P4-injected vulnerable animals.

Panel C, left graph shows the change in WRA during the second half of dark cycle (between 7 and 13 hours after P4 injection) from the first day of food restriction (FR1) to the second day of food restriction (FR2) in the 2nd ABA. There are no differences between either the vulnerable and resistant groups or the P4-injected and oil-injected groups.

Panel C, right graph shows the change in WRA during the second half of dark cycle (between 7 and 13 hours after P4 injection) from the first day of food restriction (FR1) to the third day of food restriction (FR3) in the 2nd ABA. There is a significant interaction between the vulnerability and drug groups indicated by * ($p < 0.05$), suggesting that P4 removes the resistance of animals causing them to responding severely, with a delay of 7 hours, specifically after the second injection.

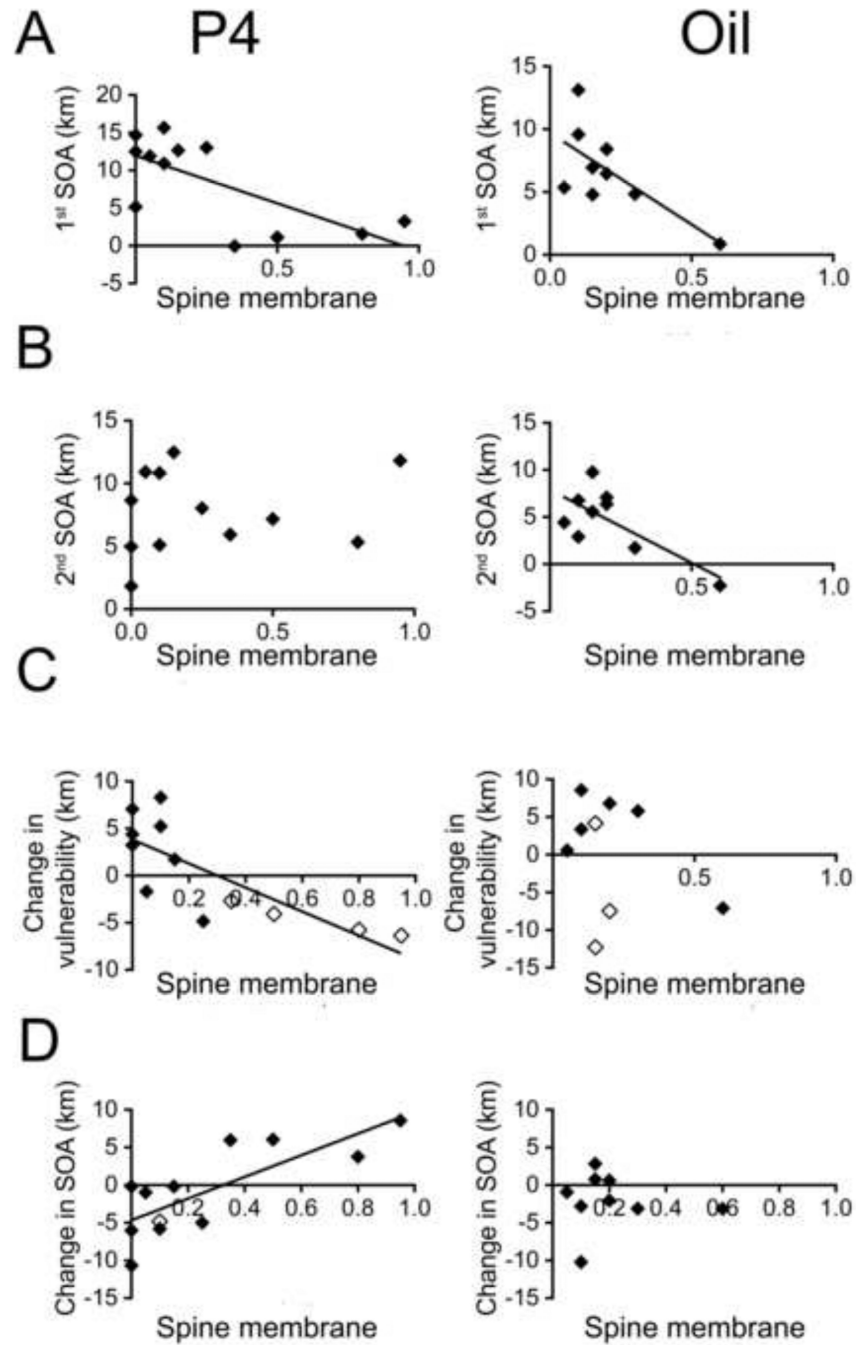


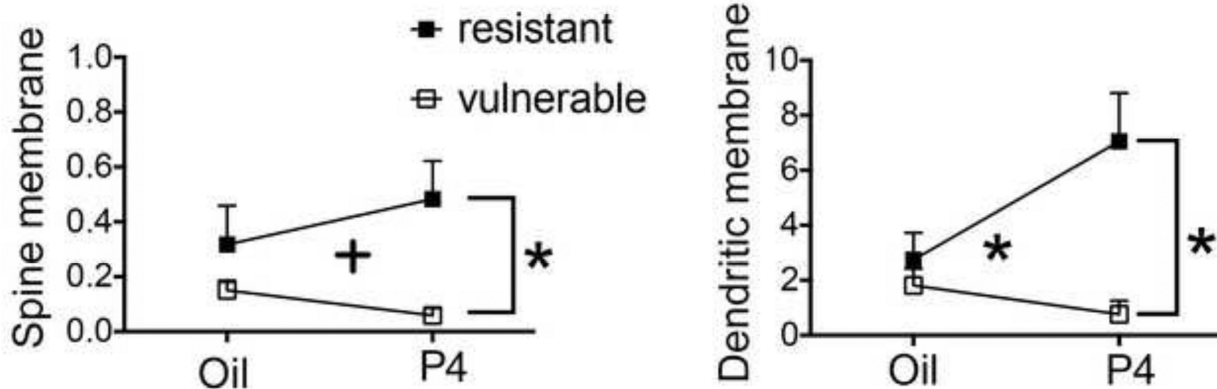
Figure 6. Relations of WRA with $\alpha 4$ -immunoreactivity in spines of mice injected with P4 or oil Graphs on the left show correlation between $\alpha 4$ -immunoreactivity (on the x-axis) and WRA (on the y-axis) for progesteroneP4-injected mice while graphs on the right show corresponding relationships for the oil-injected animals.

Panel A. $\alpha 4$ -immunoreactivity at the spine plasma membrane ('Spine membrane' in the x-axes), seen as silver-intensified gold (SIG) particles, is significantly correlated with the severity of 1st ABA for the P4- injected ($R = -0.69$, $p = 0.011$, left graph) as well as oil-injected ($R = -0.69$, $p = 0.037$, right graph) animals.

Panel B. α 4-immunoreactivity at the spine plasma membrane is significantly correlated with the severity of 2nd ABA for the oil- injected ($R = -0.72$, $p = 0.027$) but not the P4-injected animals.

Panel C. α 4-immunoreactivity at the spine plasma membrane is significantly correlated with the change in vulnerability for the P4-injected animals ($R = -0.785$, $p = 0.003$) but not for the oil-injected animals. Open diamonds indicate animals that showed behavioral sensitization to the 2nd ABA and filled diamonds indicate animals that did not show sensitization. **Panel D.** α 4-immunoreactivity at the spine membrane is significantly correlated with the change in SOA for the P4-injected animals ($R = 0.781$, $p = 0.003$) but not in the oil-injected animals.

A



B

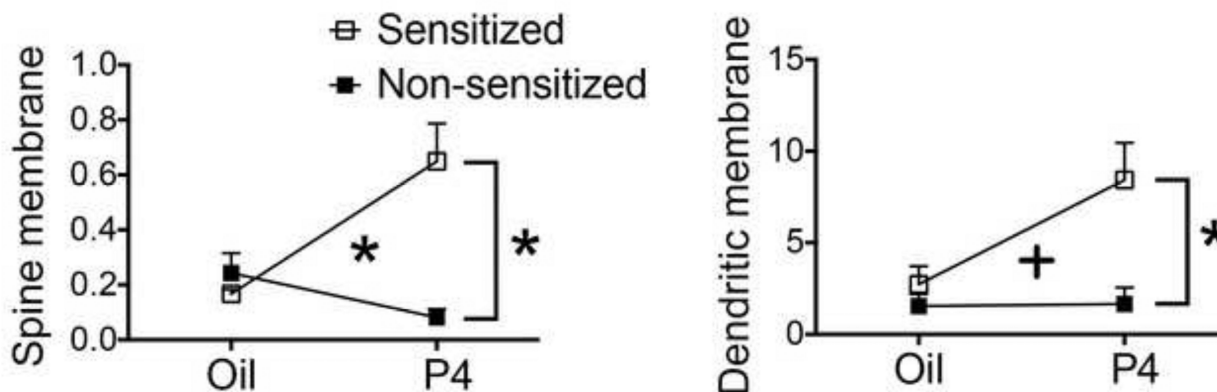


Figure 7. Effect of change in vulnerability and P4 on $\alpha 4$ -immunoreactivity
 Panels A - left and A-right show mean values and standard errors of mean of $\alpha 4$ -immunoreactivity along the plasma membrane in spines (left graphs) and dendritic shafts (right graphs) of vulnerable and resistant groups as well as P4- and oil-injected groups. Two-way ANOVAs using vulnerability and drug as fixed factors show a significant effect of vulnerability and interaction between the fixed factors for all measures.
 Panels B left and right show mean values and standard errors of the mean of $\alpha 4$ -immunoreactivity along the plasma membrane in spines (left graph) and dendritic shafts (right graph) of sensitized and non-sensitized groups as well as progesterone-injected and oil-injected groups. Two-way ANOVAs using change in SOA and drug as fixed factors show a significant effect of change in SOA and interaction between the fixed factors for all measures. ‘Spine membrane’ refers to immunoreactivity at the plasma membrane of spines.
 * indicates $p < 0.5$ and + indicates $p < 0.1$.

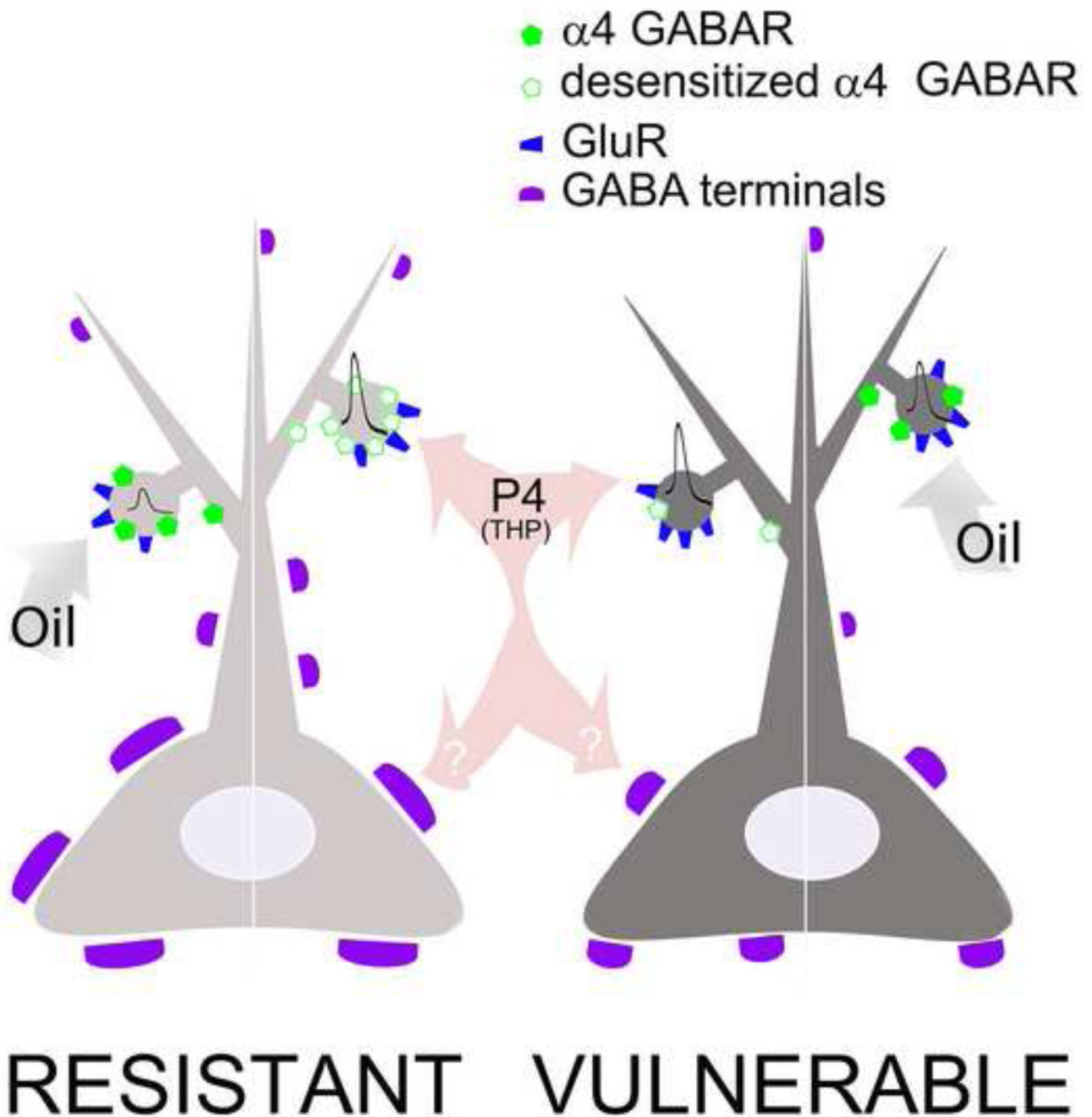


Figure 8. Proposed mechanism for P4-induced behavioral sensitization of resistant animals
 P4 injection exacerbated WRA response to the 2nd ABA induction. $\alpha 4$ -immunoreactivity was associated with resistance measured before the 2nd ABA, yet also with behavioral sensitization in P4-injected mice. We propose a mechanism to explain these dual associations of $\alpha 4$ -GABA_ARs. Assumptions for the model are: 1) the injected P4 is readily converted to THP; 2) exposure to P4's metabolite, THP, causes insertion of $\alpha 4$ -containing GABA_ARs into the membrane, along with up-regulation of its expression during the first 48 hours (Hui Shen et al., 2005); 3) A certain proportion of $\alpha 4$ -GABA_ARs in the dorsal

hippocampus of female adolescents are desensitized by the stress hormone THP and this desensitization is associated with increased anxiety (Shen et al., 2007) ; 4) anxiety is positively correlated with WRA (Wable et al., 2015). Our proposal is that $\alpha 4$ -GABA_ARs play a protective role by counterbalancing the ABA-induced increased excitability of CA1 pyramidal neurons following food restriction, and although exogenous P4 enhances $\alpha 4$ expression, especially among those that can gain resistance, it also interferes with $\alpha 4$ -GABA_ARs' protective role by desensitizing $\alpha 4$ -GABA_ARs.

The green polygons symbolize $\alpha 4$ -GABA_ARs; while the unfilled green polygons symbolize desensitized $\alpha 4$ -GABA_ARs. Blue symbols depict glutamatergic receptors. Animals that up-regulated $\alpha 4$ -GABA_ARs in response to the 1st ABA became resistant (neuron to the left, with many $\alpha 4$ -GABA_ARs on spines), while animals that failed to up-regulate $\alpha 4$ -GABA_ARs remained vulnerable to ABA, as they were during the 1st ABA induction (neuron to the right, with fewer $\alpha 4$ -GABA_ARs). P4 injection during the 2nd ABA increased $\alpha 4$ -GABA_ARs expression more than by oil alone (compare the right versus left halves of the neuron belonging to the resistant animal). P4 injection upon vulnerable animals led to reduction of $\alpha 4$ -GABA_ARs, relative to the expression level observed by neurons of vulnerable animal injected with oil vehicle, only (compare the left and right halves of the vulnerable animal's neuron). Exogenous P4 also desensitized $\alpha 4$ -GABA_ARs. This desensitization is likely to have unmasked the glutamatergic receptors (GluR, blue symbols on spines) that would have been counterbalanced by $\alpha 4$ -GABA_ARs.

Sensitization occurred for the P4-injected resistant animals (right half of the left neuron), because desensitization of $\alpha 4$ -GABA_ARs by THP unmasked more of the glutamatergic receptors, thereby rendering the neurons hyper-excitable, as indicated by the amplitude of the unitary excitatory postsynaptic current (epsc) drawn within its spine. Oil-treated resistant animals were not sensitized, because $\alpha 4$ -GABA_ARs continued to mask glutamatergic receptors (left half of the left neuron, note the small amplitude of the epsc). Neurons of the vulnerable animals were hyper-excitable, with or without P4 treatment, because the $\alpha 4$ -GABA_ARs were never sufficient in number to counterbalance the glutamatergic receptors (note the intermediate to high amplitudes of the unitary epscs). Since the extent of GABAergic innervation onto CA1 pyramidal neurons contributes to the animals' resistance to ABA induction (magenta bars surrounding dendrites and somata) (Chowdhury et al., 2013), it would be worthwhile to test whether P4 and or its metabolite, THP, alters GABAergic innervation upon these neurons (question mark in the arrow). Elevation of $\alpha 4$ -GABA_ARs is associated with a concomitant down-regulation of synaptic, i.e., $\alpha 1$ -containing GABA_ARs (Hui Shen et al., 2005), another factor that may contribute towards P4-mediated sensitization.

Table 1

Measures and comparisons of wheel running activity

Measure	Computation
Severity of ABA (SOA) in 1 st ABA	Average of wheel running activity (WRA) during food restriction period (three days of 1 st ABA) minus WRA in 1 st baseline period
Severity of ABA (SOA) in 2 nd ABA	Average of wheel running activity (WRA) during food restriction period (2 nd , 3 rd and 4 th days of 2 nd ABA) minus WRA in 2 nd baseline period
Vulnerability to ABA	WRA during period preceding food restriction
Change in vulnerability	WRA in 2 nd baseline minus WRA in 1 st baseline
➤ Resistant :	value of measure is negative
➤ Vulnerable :	value of measure is positive
Change in SOA	WRA in (2 nd ABA minus 2 nd baseline) minus (1 st ABA minus 1 st baseline)
➤ Non sensitized :	value of measure is negative
➤ Sensitized :	value of measure is positive
Comparison of WRA in 2nd ABA	Use
With SOA 1 st ABA	Response to a repeated stressor
With WRA in 2 nd baseline	Effect of food restriction in late adolescence
With WRA of first day of 2 nd ABA	Effect of P4 injection as no injection is given on first day

Table 2

Correlation between $\alpha 4$ levels at the plasma membrane of spines and dendritic shafts with measures of WRA

Group	1 st Baseline	2 nd Baseline	Change in vulnerability	WRA 1 st ABA	SDA in 1 st ABA	WRA 2 nd ABA	SOA in 2 nd ABA	WRA in 2 nd ABA minus 1 st ABA	Change in SOA
Oil injected	Spinous plasma membrane SIG level	0.40	0.10	-0.24	-0.67	-0.21	-0.67	-0.25	-0.01
		Pearson Correlation Sig. (2-tailed)	0.79	0.50	0.03	0.57	0.03	0.49	0.97
P4 injected	Spinous plasma membrane SIG level	0.77	0.18	-0.79	-0.70	0.30	0.17	0.11	0.78
		Pearson Correlation Sig. (2-tailed)	0.58	0.00	0.01	0.35	0.60	0.72	0.00
Oil-injected	Shaft plasma membrane SIG density	0.36	-0.03	-0.33	-0.10	0.06	0.21	-0.18	0.28
		Pearson Correlation Sig. (2-tailed)	0.92	0.36	0.78	0.87	0.55	0.61	0.46
P4-injected	Shaft plasma membrane SIG density	0.59	-0.09	-0.79	-0.63	-0.10	-0.02	-0.11	0.61
		Pearson Correlation Sig. (2-tailed)	0.79	0.00	0.03	0.76	0.95	0.74	0.03

Pearson correlations between $\alpha 4$ -immunoreactivity at the plasma membrane of spines and dendritic shafts of pyramidal neurons in the CA1 of the dorsal hippocampus in mice subjected to two episodes of ABA were computed with the following measures of running wheel activity in ABA: 1st baseline, 2nd baseline, change in vulnerability, WRA in 1st ABA, WRA in 2nd ABA, SOA in 1st ABA, SOA in 2nd ABA, WRA in 2nd ABA minus WRA in 1st ABA and change in SOA. For significant correlations with $p < 0.05$, the R and p values are bolded and in red (gray).

Correlation between cytoplasmic α 4-immunoreactivity in spines and dendritic shafts with WRA

Table 3

Group		1 st Baseline	2 nd Baseline	Change in vulnerability	WRA 1 st ABA	SOA in 1 st ABA	WRA 2 nd ABA	SOA in 2 nd ABA	WRA in 2 nd ABA minus 1 st ABA	Change in SOA
Oil-injected	Spinous cytoplasmic SIG level	Pearson Correlation Sig. (2-tailed)	-0.52	-0.34	0.13	-0.68	-0.52	-0.63	-0.22	-0.70
			0.27	0.34	0.77	0.03	0.13	0.05	0.55	0.02
P4-injected	Spinous cytoplasmic SIG level	Pearson Correlation Sig. (2-tailed)	0.85	0.21	-0.86	0.26	0.22	0.02	-0.10	0.68
			0.00	0.51	0.00	0.41	0.49	0.93	0.75	0.02
Oil-injected	Shaft cytoplasmic SIG density	Spearman Correlation Sig. (2-tailed)	0.26	0.16	0.03	0.04	0.02	0.19	0.16	0.19
			0.47	0.65	0.94	0.92	0.97	0.61	0.65	0.61
P4 injected	Shaft cytoplasmic SIG level	Spearman Correlation Sig. (2-tailed)	0.57	-0.12	-0.82	0.23	0.15	0.30	0.08	0.73
			0.06	0.72	0.00	0.45	0.65	0.34	0.82	0.01

Correlations between cytoplasmic α 4-immunoreactivity in spines and dendritic shafts of pyramidal neurons in the CA1 of the dorsal hippocampus in mice subjected to two episodes of ABA were computed with the following measures of running wheel activity in ABA: 1st baseline, 2nd baseline, change in vulnerability, WRA in 1st ABA, WRA in 2nd ABA, SOA in 1st ABA, SOA in 2nd ABA, WRA in 2nd ABA minus WRA in 1st ABA and change in SOA. For significant correlations with $p < 0.05$, the R and p values are bolded and in red (gray).

Summary of two-way ANOVAs performed on the measures of α 4-immunoreactivity in spines and dendritic shafts of mice injected with P4 or oil

Table 4

ANOVA	Effect	Spine			Shaft		
		Membrane	Cytoplasm	Total	Membrane	Cytoplasm	Total
	Drug	F 0.443 p 0.514	0.280 0.603	1.477 0.240	0.117 0.737	0.000 0.998	0.005 0.942
Drug \times change in vulnerability	Change in vulnerability	F 11.139 p 0.004	6.511 0.020	4.862 0.041	12.211 0.003	9.515 0.006	10.209 0.005
	Drug \times change in	F 3.579 p 0.075	8.271 0.010	2.735 0.116	5.287 0.034	10.897 0.004	11.263 0.004
Drug \times change in SOA	Drug	F 0.742 p 0.400	2.965 0.102	0.122 0.731	1.362 0.258	1.238 0.280	1.106 0.307
	Change in SOA	F 9.853 p 0.006	4.165 0.056	3.855 0.065	9.582 0.006	17.830 0.001	17.852 0.001
Drug \times change in	Drug \times change in	F 14.180 p 0.001	13.137 0.002	11.379 0.003	4.136 0.057	12.640 0.002	12.668 0.002

The two factors used in the two-way ANOVAs are drug and either change in vulnerability or change in SOA. The significant effects are bolded and in red (dark gray). The marginally significant effects are bolded and italicized. Note that Levene's test for homogeneity of variance was significant ($p = 0.02$) for the ANOVA on α 4-immunoreactivity in the cytoplasm of dendritic shafts despite a square root transformation.

Mean and standard error of mean values for $\alpha 4$ – immunoreactivity for mice injected with P4 or oil

Table 5

Group	Sub-group	N	Spine			Shaft		
			Membrane	Cytoplasm	Total	Membrane	Cytoplasm	Total
P4 injected	Resistant	6	0.48 ± 0.14	1.1 ± 0.2	0.97 ± 0.18	7.05 ± 1.75	9.8 ± 2.98	10.12 ± 3.02
	Vulnerable	6	0.058 ± 0.027	0.17 ± 0.066	0.23 ± 0.17	0.76 ± 0.48	0.7 ± 0.21	0.68 ± 0.24
	Sensitized	4	0.65 ± 0.14	1.28 ± 0.23	1.21 ± 0.13	8.43 ± 2.03	12.97 ± 3.48	13.29 ± 3.5
	Non-sensitized	8	0.081 ± 0.031	0.31 ± 0.11	0.29 ± 0.072	1.65 ± 0.9	1.44 ± 0.58	1.46 ± 0.62
Oil injected	Resistant	3	0.32 ± 0.14	0.52 ± 0.15	0.88 ± 0.32	2.74 ± 0.98	3.89 ± 1.73	3.99 ± 1.75
	Vulnerable	7	0.18 ± 0.042	0.57 ± 0.16	0.78 ± 0.16	1.81 ± 0.61	3.75 ± 0.56	3.8 ± 0.57
	Sensitized	3	0.17 ± 0.017	0.37 ± 0.044	0.57 ± 0.073	2.73 ± 0.99	4.34 ± 1.33	4.43 ± 1.36
	Non-sensitized	7	0.24 ± 0.073	0.63 ± 0.16	0.91 ± 0.21	1.82 ± 0.61	3.56 ± 0.68	3.61 ± 0.68
Resistant	All	9	0.43 ± 0.1	0.9 ± 0.17	0.94 ± 0.14	5.62 ± 1.37	7.87 ± 2.22	8.07 ± 2.26
Vulnerable	All	13	0.12 ± 0.03	0.39 ± 0.1	0.53 ± 0.13	1.33 ± 0.41	2.35 ± 0.53	2.36 ± 0.54
Sensitized	All	7	0.44 ± 0.12	0.89 ± 0.22	0.94 ± 0.15	5.99 ± 1.62	9.27 ± 2.6	9.49 ± 2.64
Non-sensitized	All	15	0.16 ± 0.042	0.46 ± 0.1	0.58 ± 0.13	1.73 ± 0.54	2.43 ± 0.51	2.46 ± 0.53

Measures for $\alpha 4$ – immunoreactivity on spines are computed as the number of SIGs found per 10 spines, whether on the plasma membrane, in the cytoplasm or overall in the spine (at the plasma membrane + cytoplasmic).

Measures for $\alpha 4$ – immunoreactivity on dendritic shafts were computed as follows. The number of membranous SIGs for each animal was divided by the total length of the plasma membrane sampled for that animal to obtain a density value (# SIG/100 μm of plasma membrane length). The total plasma membrane length sampled was equalized across all animals (~ 85 μm). A similar measure was calculated for cytoplasmic SIG and total (at the plasma membrane + cytoplasmic) SIG where the total number of cytoplasmic and total SIGs in the dendritic shafts of an animal was divided by the total area of sampled dendritic shafts to obtain the number of SIG per unit μm^2 .

Red italics indicate that among the P4-injected animals, values corresponding to the resistant and sensitized animals are higher than those corresponding to the vulnerable and non-sensitized animals, respectively.

Bolded and italicized values indicate a significant difference within the P4-injected animals as described for the red italicized values with an additional significant difference between the P4-injected and corresponding oil-injected group.

Bolded values corresponding to oil-injected animals indicate these are significantly higher than the corresponding P4-injected animals.

Bolded values corresponding to overall resistant and sensitized group indicates these are significantly higher than the vulnerable or non-sensitized group as applicable.