Enlargement of Axo-Somatic Contacts Formed by GAD-Immunoreactive Axon Terminals onto Layer V Pyramidal Neurons in the Medial Prefrontal Cortex of Adolescent Female Mice Is Associated with Suppression of Food Restriction-Evoked Hyperactivity and Resilience to Activity-Based Anorexia

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Abstract

Many, but not all, adolescent female mice that are exposed to a running wheel while food restricted (FR) become excessive wheel runners, choosing to run even during the hours of food availability, to the point of death. This phenomenon is called activity-based anorexia (ABA). We used electron microscopic immunocytochemistry to ask whether individual differences in ABA resilience may correlate with the lengths of axo-somatic contacts made by GABAergic axon terminals onto layer 5 pyramidal neurons (L5P) in the prefrontal cortex. Contact lengths were, on average, 40% greater for the ABA-induced mice, relative to controls. Correspondingly, the proportion of L5P perikaryal plasma membrane contacted by GABAergic terminals was 45% greater for the ABA mice. Contact lengths in the anterior cingulate cortex correlated negatively and strongly with the overall wheel activity after FR ($R = -0.87, P < 0.01$), whereas those in the prelimbic cortex correlated negatively with wheel running specifically during the hours of food availability of the FR days ($R = -0.84, P < 0.05$). These negative correlations support the idea that increases in the glutamic acid decarboxylase (GAD) terminal contact lengths onto L5P contribute toward ABA resilience through suppression of wheel running, a behavior that is intrinsically rewarding and helpful for foraging but maladaptive within a cage.

Key words: anxiety, cingulate cortex, electron microscopic immunocytochemistry, exercise, prelimbic cortex

Introduction

Adolescence is a period of peak physical health and mental capacity, though brain maturation is still incomplete. Brain maturation during adolescence is susceptible to environmental influences (Andersen et al. 2000; Chowdhury et al. 2014) and many mental illnesses emerge for the first time during adolescence (Spear 2000; Romeo 2010). One mental illness that is particularly prevalent among adolescent females is anorexia nervosa (AN), features of which include refusal to maintain normal body weight, fear of weight gain, and dysmorphia (American Psychiatric Association 2013). Once succumbed to the condition of AN, relapse is prevalent (30–50%) (Birmingham et al. 2005) and mortality is extremely high (10–20%) (Sullivan 1995; Zipfel et al. 2000; Birmingham et al. 2005; Bulik et al. 2007), even surpassing depression. Yet, there is at present no accepted pharmacological treatment for this condition. Nearly all adolescent
females experiment with dieting, while only 1% of them succumb to the condition of AN (Hudson et al. 2007). What is the basis for the individual differences in vulnerability to AN? This was the question of our study strived to answer through the use of a mouse model of AN, namely activity-based anorexia (ABA).

ABA is a condition whereby severe voluntary hyperactivity and weight loss is induced simply by restricting food access while providing ad libitum access to a running wheel (Gutierrez 2013). ABA induction captures 3 hallmarks of AN (American Psychiatric Association 2013): Severe weight loss (Gutierrez 2013), over-exercise (Epling et al. 1983; Davis et al. 1997, 1999; Hebebrand and Bulik 2011), anxiety (Kaye et al. 2004, Perdereau et al. 2008, Delavla et al. 2010; Thornton et al. 2011; Wable et al. 2015). Importantly, although ABA is induced by the initial imposition of food restriction (FR), once animals have begun to lose body weight, their voluntary wheel activity becomes so excessive that animals choose wheel running over eating, even during the limited hours of food access. In this way, ABA-induced animals exhibit one additional striking hallmark of the human condition of AN—namely, voluntary FR. When applied to adolescent C57BL6 female mice, this animal model also reveals individual differences in vulnerability, with approximately half of the animals exhibiting severe hyperactivity and weight loss that would be lethal unless removed from the ABA-inducing environment, while approximately a quarter of the population exhibits resilience, due to minimal hyperactivity and weight loss (Chowdhury et al. 2013). Having identified a cohort with variable degrees of vulnerability to ABA induction, our next goal has been to identify the cellular bases for the individual differences in ABA vulnerability.

It has recently been demonstrated convincingly that wheel running is not an abnormal behavior induced by rearing in captivity but is highly rewarding, even to mice in wilderness (Meijer and Robbers 2014). A number of ideas have been proposed to explain why restricted food access evokes heightened activity on the wheel. One idea is that wheel running is in response to hypothermia, since FR-evoked hyperactivity can be reversed through high ambient temperature (Gutierrez et al. 2006). Another is that wheel running is anxiolytic (Hare et al. 2012), thus being perpetuated by individuals that are experiencing anxiety due to the stress of FR. Wheel running may also be an attempt at food foraging [discussed by Gutierrez (2013)]. However, when occurring within a closed environment, wheel running is detrimental, because it exacerbates weight loss (Chowdhury et al. 2013) without any opportunity of increasing food access. Individual differences in ABA vulnerability could arise, at least in part, from differences in the animals’ neural circuitry underlying the decision to suppress wheel running, after undergoing an experience where wheel running is not suitable for survival. This idea is based on our observation that the resilient animals are those that reduce wheel running, thereby minimizing body weight loss and extending survival, while those animals that exhibit the greatest ABA vulnerability, based on the severity of weight loss, are the ones that continue to respond to FR with excessive running on the wheel (Chowdhury et al. 2013). In search for differences in neural circuitry underlying the decision to suppress wheel running, we have turned our attention to the prefrontal cortex (PFC).

Our rationale for analyzing the PFC stems from human data (Kaye et al. 2009). Among individuals with AN, presentations of visual stimuli depicting physical activity result in an exaggerated response of the PFC. The authors suggest that visual stimuli in the form of physical activity may be more rewarding, thereby placing increased demand on the inhibitory control system of AN patients when challenged with a Go/NoGo task (Kullmann et al. 2014). Another study reported that AN patients’ performance of a PFC cognitive task, the Wisconsin Card Sorting Test, was significantly poorer than that of healthy controls (Sato et al. 2013). There is also an intriguing case study, indicating that symptoms of AN were relieved in a male after glioma in the frontal lobe was removed surgically (Goddard et al. 2013). Not only is activation of the PFC of individuals with AN consistently altered, but also in the rat model of ABA, the PFC has been shown to be hypometabolic by micro-position emission tomography (PET; van Kuyck et al. 2007).

These studies suggest that altered activity of the PFC may contribute to the behavioral phenotype of AN among humans and of ABA among rodents, thereby ultimately affecting individuals’ decisions to exercise or to eat.

Excitability of the PFC is determined, in part, by the intracortical circuitry comprised of excitatory pyramidal neurons and inhibitory interneurons. Of them, the layer V pyramidal neurons contribute strongly to the excitatory outflow from the PFC to descending cortical and subcortical regions mediating motor output (Berendse et al. 1992; Gabbott et al. 2005; Riga et al. 2014). Furthermore, while excitability of the layer V pyramidal neurons in the PFC is controlled by the balance of their excitatory and inhibitory inputs, individual gamma-aminobutyric acid (GABA)ergic axo-somatic inhibitory synapses are more influential than the GABAergic axo-dendritic synapses or the numerous but more distally located excitatory axo-spinous synapses. Moreover, parvalbumin (PV) neurons in the PFC, which exert the axo-somatic inhibition, have been shown to fire as animals make decisions to leave a reward (Kvitsiani et al. 2013). This indicates that GABAergic axo-somatic inhibition of pyramidal neurons within the PFC could play a particularly central role in an animal’s choice-related behaviors, such as to eat food or to run on the wheel. Thus, among the many types of synapses that exist within the PFC, we chose to assess the extent of axo-somatic inhibitory contacts formed by GABAergic axon terminals onto layer V pyramidal neurons.

We chose to take an electron microscopic approach for 2 reasons: First, we wished to be able to study a well-defined single class of neurons targeted by GABAergic innervation, in case environmentally and experientially evoked changes in GABAergic contacts differed depending on the postsynaptic neuronal type (pyramidal vs. interneuronal; layer II/III vs. layer V vs. layer VI pyramidal cells). Electron microscopy (EM) allows for differentiation of cell bodies belonging to pyramidal cells versus interneurons (Peters and Jones 1984; White and Keller 1989). Moreover, we wished to be able to use the resolving capability of EM for differentiating those contacts that are direct versus others that are indirect, due to the interposition of thin (<30 nm, below the limit of resolution of light microscopes) astrocytic processes in between GABAergic axon terminals and perikaryal plasma membranes that can reduce the activation of GABAergic receptors [review in Zhou and Danbolt (2013)].

The source of PFC tissue for this analysis was derived, in part, from an earlier published study that examined GABAergic innervation of pyramidal cells in the hippocampus of adolescent female C57BL6 mice exhibiting variable degrees of hyperactivity following 2 ABA inductions during adolescence, spaced 1 week apart (Chowdhury et al. 2013).

Materials and Methods

Animals and ABA Induction

Fourteen female mice, 7 ABA animals, and 7 controls, from 4 litters, were used in this study. All animals used in this study were bred in New York University’s animal facility. All but one of the
PFC tissue used for this study came from brains used for another anatomical study on the hippocampus (Chowdhury et al. 2013). Two breeding pairs were wild-type C57BL/6J mice obtained from Jackson Laboratories. Two other breeding pairs were also obtained from Jackson Laboratories (Bar Harbor, ME, USA) on a C57BL/6J background but crossed with BALB/c strains: CB6-Tg (Gad1-EGFP)G42Zjh/J (G42), which express green fluorescent protein (GFP) in PV-containing interneurons (Chattopadhyaya et al. 2004). GFP label was not utilized as a marker for the electron microscopic study described here. Details of these animals with GFP expression are described in a previous publication from this laboratory: in short, these mice exhibited behavioral and glutam- 
ic acid decarboxylase (GAD) expression phenotypes that were no different from wild types (Chowdhury et al. 2013).

After weaning at postnatal day 25 (P25), the animals were

The animals' estrous cycles were not monitored, because cycles are already known to become disrupted by FR (Nelson 1985; Dixon et al. 2003) and because pubertal animals are too immature to be cycling regularly (Hodes and Shors 2005). We also reasoned that vaginal smears, needed for assessing the estrous cycle, is a stressful procedure that could exacerbate or mask the effect of stress associated with FR.

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 (A3317-01).

Preparation of Brains

ABA animals were euthanized at the end of the second ABA, at ages P59 or P60, by anesthetizing them with urethane (i.p. 34%; 0.15 mL/20 g body weight) during the hours of 12 PM to 4 PM, then transcardially perfusing them with a buffer containing 4% paraformaldehyde. Glutaraldehyde fixation was withheld until after immunocytochemistry, so as to optimize antigen retention. Brains were also collected from CON mice at ages P59 or P60.

The GAD Immunocytochemistry Procedure

Details of the GAD immunocytochemical procedure are similar to the steps described in our previous publication (Chowdhury et al. 2013). Tissues from all animals were processed for immunocytochemistry and EM synchronously, so as to minimize variabilities arising from differences in reagent qualities, incubation time, ambient temperature, etc. The rabbit anti-GAD antibody used in this study (Lot 2069924 of Millipore, Bellerica, MA, USA; catalog number AB11; hereafter referred to as anti-GAD) recognizes both the GAD65 and GAD67 isoforms. Multiple vibratome sections from each animal’s brain, prepared in the coronal plane at a thickness of 40 μm and containing 3 regions [cingulate cortex, area 1 (Cg1), prelimbic cortex (PrL), and medial orbital cortex (MO)] of the PFC (Paxinos and Franklin 2001; Fig. 1), were incubated overnight, at room temperature, under constant agitation, in a buffer consisting of 0.01 M phosphate buffer/0.9% sodium chloride (PBS), adjusted to pH 7.4, together with 1% bovine serum albumin (BSA, w/v, from Sigma Chem., St Louis, MO, USA), 0.05% sodium azide (w/v, from Sigma Chem.), and the GAD antibody at a dilution of 1:400. At the end of the incubation period, sections were rinsed in PBS over a 30-min period, and then incubated for 1 h at room temperature under constant agitation in the PBS/BSA/azide buffer containing biotinylated goat anti-rabbit IgG (Vector, Inc., Burlingam, CA, USA) at a dilution of 1:200. At the end of the incubation period, the sections were rinsed in PBS over a 30-min period and then incubated in PBS containing the avidin-biotinylated horseradish peroxidase complex (HRP) (ABC, from Vector’s ABC Elite kit) for 30 min at room temperature, under constant agitation. The sections were then rinsed in PBS and reacted with the HRP substrate, consisting of 3,3’- diaminobenzidine hydrochloride (DAB, 10 mg tablets from Sigma Chem.) per 44 mL of PBS buffer and hydrogen peroxide (4 μL of 30% hydrogen peroxide per 44 mL of DAB solution). The HRP reaction was begun by adding hydrogen peroxide, and terminated at the end of 11 min by rinsing repeatedly in PBS. Vibratome sections underwent post-fixation using 2% glutaraldehyde for 16 min (EM Sciences, Martifield, PA, USA) in PBS.

Tissue Processing for EM

Following the immunocytochemical procedure, the vibratome sections were processed by a conventional electron microscopic procedure, consisting of post-fixation by immersion in 1% osmium tetroxide/1.0 M phosphate buffer for 1 h, then dehydrated using graded concentrations of alcohol up to 70%, and then post-fixed overnight using 1% uranyl acetate (Terzakis 1968; Lozsa 1974) dissolved in 70% ethanol. On the following day, the sections were further dehydrated using 90% ethanol, followed by 100% ethanol, then were rinsed in acetone, and infiltrated in Epon 812 (EM Sciences), which was cured by heating the tissue at 60 °C, while sandwiched between 2 sheets of Aclar plastic, with lead weights placed on top of the Aclar sheets, so as to ensure flatness of the EPON-embedded sections. These flat-embedded vibratome sections were re-embedded in BEEM® capsules (EM Sciences) filled with EPON 812, then ultrathin-sectioned on a plane tangential to the vibratome sections. The ultrathin sections were collected onto formvar-coated, 400 mesh thin-bar nickel grids (EM Sciences). Ultrathin sections were counterstained with Reynolds’ lead citrate before viewing.
The Sampling Procedure

Two steps were taken to maintain random, unbiased sampling. One, the electron microscopist sampling the ultrathin section was blinded to the animal’s ante mortem behavioral characteristics and treatments. Two, for each cortical area of each animal, somatic profiles were analyzed, strictly in the order that they were encountered from the surface-most portions of vibratome sections spanning layer V. Cell bodies of pyramidal neurons were identified as being positioned in layer V, if they were located midway between pial surface and the white matter. Care was taken to never re-sample an area, so as to avoid analysis of overlapping sectors of perikaryal plasma membrane.

The 3 PFC regions of interest that were sampled and analyzed separately were Cg1, PrL, and MO. The anatomical landmarks of white matter, sulci, overall shape of the coronal section, cell density, and all other features shown in Figure 1A were used to determine the AP levels and boundaries of the subregions of the PFC. We sampled the neuropil surrounding cell bodies of pyramidal neurons located in layer V of the PFC.

Statistical analysis did not reveal any difference in the proportion of perikaryal plasma membrane contacted by GAD terminals of or of the individual lengths of contact formed by GAD terminals onto cell bodies of layer V pyramidal neurons across the AP levels. There is also no behavioral literature pointing to differences in function across the AP levels. Therefore, data from all AP levels were combined for each subregion of the PFC (Fig. 1A) of each animal.

Sampling of the axon terminals contacting cell bodies of layer V pyramidal neurons in prefrontal areas was designed to...
optimize immunodetection. To achieve this, for each region of interest, multiple ultrathin sections revealing the surface-most portions of vibratome sections for that region were chosen. Five to 10 somata were sampled from each subregion of the PFC of each animal. Since there were 3 subregions of the PFC analyzed for each animal, the total number of somata sampled per animal was 15–30. The number of ultrathin sections collected from any one animal’s brain section depended on the natural curvature that the vibratome section took on, even while cured under lead weights. Usually, 5–10 ultrathin sections needed to be collected from each vibratome section, so as to reach the target of sampling from the 3 subregions of the PFC.

Quantification of Axo-Somatic Contact Sizes Made by GAD Axon Terminals and the Proportion of Perikaryal Plasma Membranes Contacted by GAD Axon Terminals

Somatic profiles were identified using standard morphological criteria (Peters et al. 1991). Somatic profiles were further identified to be of pyramidal cells and not GABAergic, based on the absence of deep indentations along the nuclear membranes, homogeneous distribution of chromatin within the nucleoplasm, and the absence of GAD-immunoreactivity in the cytoplasm (Peters and Jones 1984; White and Keller 1989). Contacts formed by GAD-immunoreactive axon terminals (hereafter referred to as “GAD terminal contacts,” for short) were considered to be synaptic when they exhibited >5 vesicles within the profile, a parallel alignment between the plasma membrane of the axon terminal and of the soma and thin or complete absence of postsynaptic density. Essentially, these are the features associated with symmetric synapses (Peters and Jones 1984). Typically, symmetric synaptic junctions were flanked by plasma membranes lacking the pre- to-postsynaptic parallel alignment but with the 2 plasma membranes still juxtaposed directly against one another. These non-junctional flanking regions were included in the quantification of GAD terminal contact lengths (see below for further details).

Digital electron microscopic images were captured from ultrathin sections by using a 1.2-megapixel Hamamatsu CCD camera from AMT (Boston, MA, USA) from the JEOL 1200 XL electron microscope (JEOL Ltd, Tokyo, Japan). The extent of contact formed by GAD terminals upon layer V pyramidal cells of the Cg1, PrL, and MO regions of the medial PFC (Fig. 1A) was quantified using the segmented line tool of NIH’s software, Image J (version 1.46), to measure plasma membrane lengths of the neuronal profiles within digital images of the ultrathin sections. After determining the total perikaryal plasma membrane length of a profile identified to be belonging to a pyramidal neuron, the proportion (in units of percent) of the plasma membrane contacted by GAD terminals was determined by retracing those portions of the plasma membrane directly opposite to the HRP-DAB-labeled axon terminals. Portions of GAD-immunoreactive axon terminals lacking the parallel alignment to the plasma membrane of the cell bodies but still directly juxtaposed were included in our measurements of GAD terminal contacts (Figs 1–3). So as to optimize consistency in discriminating immunolabeled from unlabeled or background labeling, axon terminals containing faint HRP-DAB-like density in the cytoplasm that was less than the electron density of mitochondria were categorized as below threshold for definitively positive immunoreactivity and not included in the quantification (Fig. 1C). Conversely, some of the profiles contacting cell bodies were so densely filled with HRP reaction products that vesicles could not be visualized easily. However, inspection of the same profiles recurring in adjacent ultrathin sections more removed from the surface of vibratome section surfaces verified that these did contain vesicles (Fig. 3), thereby indicating that these intensely darkened profiles were GAD-immunoreactive axon terminals that could be included in the quantification.

The size of GAD terminals forming contacts with layer V pyramidal neurons’ cell bodies was assessed in 2 ways: The length of contact, using the measurement described above, and cross-sectional areas of GAD terminal cytoplasm at the site forming direct contacts with the cell bodies (Fig. 1B). GAD terminal area was measured using Image J’s polygon tool.

The group mean values of the proportion of layer V pyramidal cells’ perikaryal plasma membrane contacted by GAD terminals, plasma membrane lengths contacted by GAD terminals, and the cytoplasmic areas of the GAD terminals at the site of axo-somatic contact were compared across the ABA versus CON groups. The group mean values were compared for each of the 3 PFC subregions separately and after pooling across the areas.

Statistical Analyses

Statistical analysis was done by SPSS statistical software 22.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). Data were verified to be of normal distribution by using the Kolmogorov–Smirnov, the Lilliefors, and Shapiro–Wilk’s W tests. All variables were found to be normally distributed. For comparison of 2 groups, Student’s t-test was used to determine significance of the difference. For comparison of 3 groups, an one-way repeated-measures ANOVA was conducted, followed by Bonferroni’s post hoc analysis. Pearson correlation was conducted between behavioral activities and GAD-immunoreactive measurements. P-values of <0.05 were considered statistically significant.

Results

Variability in Wheel Activity during the First and Second ABA

Seven female C57BL/6J mice, derived from 3 litters, were subjected to 2 sessions of ABA inductions. The ABA inductions were separated by a week of recovery, consisting of ad libitum food access for 24 h and no wheel access. Both ABA inductions occurred during adolescence (P36–P60).

All 7 mice exhibited hyperactivity within 24 h after the onset of FR, relative to the pre-FR baseline activity, and all but one were hyperactive within the first 6 h of FR. The average of the 7 animals’ wheel activity (in km/day) during the 3 days of FR of the first ABA induction increased by 68%, relative to the baseline activity that preceded FR and this increase was significant (18.7 ± 1.68 km/day during baseline; F2,12 = 5.08, P < 0.05, by one-way repeated-measures ANOVA; P < 0.05, Bonferroni’s post hoc test, comparing first ABA with baseline).

Notably, although all animals exhibited FR-evoked hyperactivity, their degrees of hyperactivity were variable. Two of the 7 animals exhibited only a minimal increase in activity following the first ABA (<3.5 km/day increase, Animals 3 and 4), whereas the remaining animals exhibited an increase in activity by at least 7 km/day (Animals 5–8 and 11; Fig. 4A). The time that animals dwelled on the wheel correlated strongly with the distance that animals ran on the wheel (r = 0.9926, P < 0.0001; Fig. 4B), indicating that individual differences in wheel-running distance arose mostly from the individual differences in the “preference” of using the running wheel, rather than the speed with which they ran on the wheel.
Animals restored their body weight within the first 48 h of the recovery period, as was reported earlier (Chowdhury et al. 2013). Greater variability in wheel activity was observed following exposure to the second ABA induction. One of the animals (Animal 3) exhibited strong resistance to the second ABA exposure, in that its activity level after FR was less than the level measured during the initial acclimation to the wheel. Two animals exhibited only a minimal increase in activity following the second ABA (≤5 km/day increase, relative to the running during the baseline; Animals 4, 6, and 7). The remaining animals exhibited an increase in activity by at least 7 km/day (Animals 5, 8, and 11; Fig. 4A). These individual differences were apparent within the first 12 h of the second ABA. Owing to this individual variability in the FR-evoked hyperactivity, there was no statistically significant difference in the group mean average of wheel running during the second exposure to ABA, relative to the pre-FR baseline activity (17.81 ± 2.49 km/day during second ABA; 11.14 ± 1.55 km/day during baseline; \( P = 0.296 \)).

Thus, wheel analysis confirmed that all 7 adolescent female mice exhibited FR-evoked hyperactivity but that the extent of this hyperactivity was variable, especially during the second ABA induction. Each animal’s average hyperactivity evoked during the first and second ABA inductions relative to the baseline activity was ranked and shown in Figure 4A. Using this measure to rank ABA resilience, Animals 3, 4, and 6 were the most resilient among the 7 mice.

As was reported earlier by others (Gelegen et al. 2008; Gutierrez 2013) and us (Chowdhury et al. 2013), a more detailed analysis

Figure 2. GAD axon terminals forming direct contacts onto a cell body of the PrL of the PFC of an ABA animal are shown. Same criteria used for the CON cell bodies were applied to verify that this cell body belongs to a pyramidal neuron (E). Five white asterisks (near contact 3, in between contacts 4 and 5, to the right of contact 1, and 2 in between contacts 7 and 8) are GAD axons that are near but not directly contacting the perikaryal plasma membrane. The 8 direct GAD terminal contacts onto the cell body, indicated by arrows, are shown in higher magnification in (A–D) and (F–I). Arrowheads indicate the lateral borders used to measure GAD terminal contact lengths. Arrows (A and D) indicate the lateral borders identifiable as synaptic junctions, based on the parallel alignment of the plasma membranes of the axon terminal and of the cell body. The contacts showing only one or the other of the salient features of synaptic junctions could be verified to be synaptic by viewing adjacent ultrathin sections, examples of which are shown in Figure 3. Sometimes, an endoplasmic reticulum saccule abutted the intracellular surface of the plasma membrane of the contact site (C). ER, endoplasmic reticulum; Ast, astrocytic profile. Calibration bar = 2 \( \mu \)m in (E); 500 nm in (I), and applies to all other panels as well, except for E.
of the animals’ wheel running revealed that once food access becomes limited to certain hours of the day, animals begin to exhibit particularly high wheel running during the 6-h bin that immediately precedes feeding, referred to as the food anticipatory activity (FAA). The remaining 18 h also contribute significantly, albeit less, to the overall increase in wheel running that is evoked by FR (Chowdhury et al. 2013). Analysis of this cohort’s wheel running indicated that these animals also exhibited FAA during the 6-h preceding feeding (Fig. 4C). The 4 animals exhibiting the greatest vulnerability (7, 5, 8, and 11, ranked in the order of ABA severity) exhibited strong correlation between FAA running and their 24 h activity during the first ABA induction ($R = 0.9524$; $P < 0.05$) as well as a marginal correlation with their total wheel activity, spanning from the baseline days to the end of the second ABA ($R = 0.8771$; $P = 0.12$).

To determine whether animals would reduce the running behavior to gain more food during the period of limited food access (7 PM to 9 PM) under FR, the wheel running activity during 7 PM to 9 PM in baseline, first ABA, re-acclimation, and second ABA were measured. No difference in the ABA group mean values of wheel running was found in first ABA and second ABA, compared with baseline (Fig. 4D). This indicates that animals voluntarily chose to run on the wheel, rather than eat, even during the hours of food availability, after they had become entrained to the restrictive feeding schedule and had lost, on average, 18% of their body weight.

If the emergence of increased wheel activity during the 6-h preceding feeding is a reflection of animals’ entrainment to the limited hours of food access, then those animals that exhibit heightened FAA might also be the ones to maximize feeding by suppressing wheel running during the hours of 7 PM to 9 PM, when food is available. However, no correlation was found between individual animals’ FAA and wheel activity during food access ($R = 0.22$; $P = 0.63$).

**GAD Terminal Contacts onto Cell Bodies of Layer V Pyramidal Neurons in the PFC of Animals Following ABA**

We assessed the extent of axo-somatic contacts formed by GAD terminals onto layer V pyramidal neurons by quantifying the proportion of the perikaryal plasma membrane contacted directly by axon terminals immunoreactive for GAD, the rate-limiting enzyme for the synthesis of GABA (Fig. 1B). These GAD-immunoreactive axon terminals formed only symmetric synaptic junctions, characterized by the parallel alignment of the presynaptic plasma membranes and vesicles on the postsynaptic side (Figs 1–3). With the use of EM, these contacts were verified to be direct, that is, not interposed by astrocytic or other axonal processes that can be as fine as 30 nm in diameter (Figs 1 and 2). Symmetric synaptic specializations (synaptic junctions) were typically flanked by plasma membranes that lacked the clearly parallel pre-to-postsynaptic alignment, but nevertheless maintained direct contact. Measurements of the GAD terminal contacts included these non-parallel flanking portions, so long as the 2 plasma membranes were not interposed by astrocytic processes (Figs 1–3).

EM revealed that in all 3 regions of the PFC of CON animals, GAD terminals contacted 11.9 ± 0.95% of the cell body plasma membrane lengths. In contrast, the extent of contacts formed by GAD terminals onto cell bodies of the ABA animals was 17.3 ± 0.64%, reflecting a 45% increase, relative to the CON values [$t_{12} = 4.762$, $P < 0.05$]. This difference was significant ($P < 0.05$), as were differences when comparing each PFC area separately (Fig. 5A, all $P < 0.05$). This difference, in percentage, could have arisen due to changes in the presynaptic GAD terminals or the postsynaptic perikaryal plasma membranes. The mean lengths of the ultrastructurally analyzed perikaryal plasma membranes were 26710.4 ± 1080.45 nm for the ABA group and this was not significantly different from the values of the CON groups (27466.8 ± 1544.15 nm), whether pooled across the 3 groups or analyzed separately for each PFC area (Fig. 5B, $P > 0.05$ for all), thereby pointing to the change being presynaptic.

Differences in the proportion of plasma membrane covered by GAD terminals across the groups could have arisen due to differences in GAD terminal contact sizes and/or the frequency of GAD terminals contacting the somata. The mean number of GAD terminals encountered along the perikaryal plasma membrane was 4.07 ± 0.18 for the PFC of the ABA group and this was not significantly different from the values for the CON group ($t_{12} = 0.12$), whether analyzed across the 3 groups or analyzed separately ($P > 0.05$; Fig. 4). The possibility that percentage differences in the lengths of contact formed by GAD terminals

**Figure 3.** Ultrastructure of synaptic profiles re-captured in an adjacent ultrathin section. Contacts 3 (Panel A) and 1 (Panel B), captured from an ultrathin section adjacent to the one shown in Figure 2, revealed that these contacts are associated with synaptic specializations, based on the clearer visualization of synaptic vesicles, due to the lightened DAB reaction product intensity (contact 3), and of the more clearly parallel alignment of the plasma membranes of the axon terminals and of cell body (arrows, contacts 3 and 1). The portions of the plasma membrane with synaptic specializations contrast the longer contact lengths (arrowheads at the lateral-most borders) that include plasma membranes lacking the definitively parallel alignments (arrowheads). Calibration bar = 500 nm applies to both panels.

### Ultrastructure of synaptic profiles re-captured in an adjacent ultrathin section.

Contacts 3 (Panel A) and 1 (Panel B), captured from an ultrathin section adjacent to the one shown in Figure 2, revealed that these contacts are associated with synaptic specializations, based on the clearer visualization of synaptic vesicles, due to the lightened DAB reaction product intensity (contact 3), and of the more clearly parallel alignment of the plasma membranes of the axon terminals and of cell body (arrows, contacts 3 and 1). The portions of the plasma membrane with synaptic specializations contrast the longer contact lengths (arrowheads at the lateral-most borders) that include plasma membranes lacking the definitively parallel alignments (arrowheads). Calibration bar = 500 nm applies to both panels.
onto cell bodies reflected differences in GAD terminal contact sizes was tested by analyzing the mean lengths and cross-sectional areas of the boutons formed by GAD terminals directly contacting the somatic plasma membrane of layer V pyramidal cells. The mean value of individual GAD terminal contact profile lengths was 4381.8 ± 154.9 nm for the ABA group, representing a 40% enlargement compared with the value from the CON tissue (3125.6 ± 216.9 nm). Each PFC area also exhibited a significant difference between ABA and CON groups (Fig. 5C, all P < 0.05). Correspondingly, the mean cross-sectional area of GAD boutons forming contacts onto cell bodies of pyramidal cells was 1816 ± 70886.55 nm² for the ABA group, representing a >60% enlargement compared with the value from the CON tissue (1137 ± 128575.4 nm²). The group difference was also significant for each of the 3 PFC regions that were analyzed separately (Fig. 5D).

This difference in GAD contact terminal lengths and areas at sites of contact with cell bodies of layer V pyramidal neurons indicates that enlargement of GAD terminals is likely to have contributed most significantly to the differences in the proportion of the perikaryal plasma membrane receiving GAD terminal contact.
Correlation between the Running Wheel Activity and GAD Terminals’ Axo-Somatic Contact Lengths on Layer V Pyramidal Neurons

Previous studies indicated that the anterior cingulate cortex (ACg) (Cg1), located at the dorsal-most region of the PFC, is linked to motor behaviors (Heidbreder and Groenewegen 2003; Vertes 2006). We investigated whether the inter-animal differences in behavioral responses to the FR might correlate with differences in the inhibitory input onto layer V pyramidal neurons in Cg1.

The relationship between lengths of GAD terminal contact with the somatic plasma membrane of Cg1 layer V pyramidal cells and running wheel activity in the ABA paradigm was examined using the Pearson correlation analysis. There was no correlation between running activity prior to FR and GAD terminal contact lengths (Fig. 6A). In contrast, GAD terminal contact lengths were negatively correlated with the wheel-running activity during the first ABA (Table 1 and Fig. 6B), meaning that the animals exhibiting the greatest increase in wheel activity were the ones that exhibited the lowest increase of GAD terminal contact lengths at layer V cell bodies. The negative correlation was even stronger during the second ABA exposure (Table 1 and Fig. 6C). The negative correlation between GAD terminal contact length and wheel running was also strong for the total running activity (sum of running from baseline through the first as well as the second ABA; Table 1 and Fig. 6D).

GAD terminal size analysis further confirmed that the cytoplasmic area of GAD terminals at the site of contact with cell bodies was also negatively correlated with the total wheel-running activity (Table 1).

In contrast to Cg1, which is linked to motor behaviors, the ventral regions of the PFC areas PrL and Mo are associated with diverse emotional, cognitive, and mnemonic processes and less to motor behaviors (Heidbreder and Groenewegen 2003; Vertes 2006). The strong correlations observed between the contact by GAD terminals onto cell bodies of layer V pyramidal neurons and running activity were specific for the Cg1, in that the PrL (Fig. 6E) and MO (Fig. 6F) did not exhibit these correlations (Table 1).

Correlation between the FAA, Running during Food Access, and GAD Terminal Contact Lengths with Somatic Plasma Membranes of Layer V Pyramidal Neurons

Although axo-somatic contact lengths formed by GAD terminals in the PrL and MO did not exhibit correlation with wheel running during the first or second ABA, a possibility remained that some other subcomponent of the wheel activity might. Indeed, correlation was also found between GAD terminal contacts onto somata in the PrL and the running during the hours of food access, from 7 PM to 9 PM, of the second ABA (Table 1 and Fig. 7B). The
correlation was negative and significant, meaning that animals exhibiting the least wheel activity during the food access period were the ones that exhibited the greatest increase of GAD terminal contact lengths. GAD terminal size analysis further confirmed that the area of GAD terminals at axo-somatic contact sites in the PrL was also negatively correlated with the running during the hours of food access (Table 1). In contrast, GAD terminal contact lengths onto cell bodies in the Cg1 and MO did not correlate significantly with wheel running during food access in the second ABA (Fig. 7A for Cg1; Fig. 7C for MO; and Table 1). None of the 3 PFC regions exhibited correlation between GAD terminal contact lengths onto cell bodies of layer V pyramidal cells and running during the hours of food access of the first ABA (Table 1).

Interestingly, correlation was also found between GAD terminal contact lengths in the PrL and FAA, spanning the 6 h that preceded feeding during the first ABA (Fig. 7E and Table 1). The correlation was “positive,” meaning that animals exhibiting the greatest wheel activity during the FAA period were the ones that exhibited the greatest increase of GAD terminal contact lengths at cell bodies. MO, the most ventral part of PFC, also exhibited marginally positive correlation between GAD terminal contact lengths at cell bodies and FAA of the first ABA (Fig. 7F and Table 1). In contrast, GAD terminal contact lengths onto pyramidal cells in layer V of Cg1 did not correlate significantly with FAA during the first ABA (Fig. 7D).

**Discussion**

The present study is consistent with the idea that induction of FR-evoked hyperactivity on the wheel (i.e., ABA) caused enlargement of GAD axon terminals targeting layer V pyramidal cell bodies in the PFC. However, contrary to our expectation, this circuitry change is not likely to have supported the FR-evoked hyperactivity, since the 2 parameters correlated negatively and strongly ($R = -0.87$, $P < 0.01$): Individuals with the most enlarged GAD terminal contacts onto somata exhibited the greatest suppression of FR-evoked hyperactivity, whereas those animals with terminal contact sizes no different from controls’ exhibited

![Figure 6. Correlation between the running wheel activity and GAD terminal lengths at sites of contact with the somatic plasma membrane of layer V pyramidal neurons.](https://academic.oup.com/cercor/article-abstract/26/6/2574/1754151)
Table 1 Summary of correlation coefficients and P-values between running activities and contact sizes

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Running during baseline</th>
<th>Running during the first ABA</th>
<th>Running during the second ABA</th>
<th>Total running activity</th>
<th>Contact size: length (mm)</th>
<th>Contact size: area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cg1</td>
<td>Pearson: r = 0.81, P = 0.03</td>
<td>Pearson: r = 0.07, P = 0.75</td>
<td>Pearson: r = 0.51, P = 0.24</td>
<td>Pearson: r = 0.55, P = 0.11</td>
<td>Pearson: r = 0.68, P = 0.08</td>
<td></td>
</tr>
<tr>
<td>PrL</td>
<td>Pearson: r = 0.53, P = 0.02</td>
<td>Pearson: r = 0.57, P = 0.02</td>
<td>Pearson: r = 0.64, P = 0.08</td>
<td>Pearson: r = 0.69, P = 0.05</td>
<td>Pearson: r = 0.69, P = 0.08</td>
<td></td>
</tr>
<tr>
<td>MO</td>
<td>Pearson: r = 0.21, P = 0.78</td>
<td>Pearson: r = 0.24, P = 0.69</td>
<td>Pearson: r = 0.28, P = 0.78</td>
<td>Pearson: r = 0.30, P = 0.54</td>
<td>Pearson: r = 0.30, P = 0.54</td>
<td></td>
</tr>
</tbody>
</table>

Bold values indicate statistically significant, p < 0.05.

ABA, activity-based anorexia; Cg1, cingulate cortex, area 1; PrL, prelimbic cortex; MO, medial orbital cortex; FAA, food anticipatory activity.

Another unexpected finding was that although individual differences in GAD terminal contact sizes at cell bodies correlated most strongly with those in hyperactivity during the second ABA induction, this correlation was already detectable 10 days prior, during the first ABA induction. Although identification of the molecular cascades underlying this change was beyond the scope of this study, an idea compatible with the current finding is that some yet-to-be-identified individual difference supporting activity-dependent changes in GABAergic synapses existed prior to ABA induction. A number of previously published findings point to individual differences in the activity-dependent brain derived neurotrophic factor (BDNF) expression as a possible mechanism linking the FR-evoked stress or heightened exercise mechanism to the upregulation of GABA receptors and expansion of contact sites formed by GABA terminals onto somata (Neeper et al. 1998; Gomez-Pinilla et al. 2002; Lee et al. 2002; Duman 2005; Stanton et al. 2007, 2009; Jiao et al. 2011).

Functional Significance of the Increased Lengths of Contacts Formed by GABAergic Axon Terminals onto Layer V Pyramidal Neurons in the PFC

PFC is a site of integration of multiple sensory modalities in addition to signals associated with visceral states, rewards, social conflicts, stress, attention, and “affective qualities” [reviewed by Vertes (2006)]. The PFC converts these signals into effenter signals that contribute toward behavior that are in accordance with internal goals. The PFC has been described as contributing to executive functioning which, for rodents, includes attentional selection, resistance to interference, reduction in impulsive behavior. For rodents, an impulsive initial response to starvation is to enhance foraging behavior. It has been hypothesized that the increased wheel activity that was elicited by all 7 of our experimental animals during the initial ABA induction may a substitute for the foraging behavior elicited by hungry rodents in captivity (Gutierrez 2013). Conversely, the dampened wheel activity that was observed by some of the mice during the second ABA induction may reflect a learned behavior of suppressing what turned out to be an unproductive behavior during the first ABA induction, mediated through enhanced GABAergic inhibition of excitatory outflow from the PFC to the motor centers. As for the mechanism supporting enhanced...
GABAergic inhibition, elevated levels of BDNF in the PFC have been hypothesized for another learned behavior—the extinction of fear memory (Bredy et al. 2007), and may be at work for the ABA-induced mice as well.

Functional Significance of the Regional Differences in the Correlation between GAD Terminals’ Axo-Somatic Contact Lengths and Wheel Activity

Cg1, PrL, and MO are all parts of the medial PFC, but receive different afferents (Hoover and Vertes 2007). Specifically, the dorsally located Cg1 receives primarily sensorimotor cortical input, whereas the more ventrally located PrL and MO receive primarily limbic inputs that are both cortical and subcortical, including the amygdala, hippocampus, basal forebrain area, the monoaminergic nuclei, and the midline thalamus. All 3 of these medial PFC regions exhibited increased GAD terminal sizes in response to ABA induction. However, we also noted individual differences in GAD terminal contact sizes among the ABA-induced mice and discovered that GAD terminal sizes within specific subregions correlate with distinct components of the FR-evoked hyperactivity. Specifically, within the area PrL, GAD terminal sizes correlated negatively and strongly with wheel activity during the 2 h of food availability of the food-restricted days. In other words, animals with the largest GAD terminals in the PrL were the ones that were able to suppress wheel activity the most during the 2 h of food availability. Although the PrL region is part of the medial PFC, as is the Cg1, highly localized lesion studies within the medial PFC have revealed that there are behavioral tasks linked more to the PrL than to the Cg1. These include the delayed non-matching-to-sample response (Delatour and Gisquet-Verrier 1999), extra-dimensional and cross-modal set-shifting, and shifts between new strategies or rules (Dalley et al. 2004). As was described earlier, cage-housed mice that are resilient and survive ABA induction are those that can learn a new rule, namely that wheel running does not yield a new source of food and is detrimental within the confines of a cage. The enlargement of GAD terminal contact sizes at the PrL cell bodies may
have contributed toward the animal’s learned behavior and/or shifting to this new rule, even if running is intrinsically rewarding (Meijer and Robbers 2014) and helpful for foraging.

GAD terminal sizes in Cg1 also correlated with individuals’ wheel activity. However, in contrast to the PrL, GAD terminal sizes in the Cg1 correlated most strongly with the animals’ total wheel activity during the second ABA, and not specifically to the 2 h of food availability. Although weaker, Cg1 GAD terminal contact sizes also correlated significantly with the 24-h activity during the first ABA, even though these preceded euthanasia by more than 10 days. Within the medial PFC, the most dorsally located Cg1 (corresponding to Acg in rats; Paxinos and Watson 1998; Ongur and Price 2000) projects most prominently to the dorsal striatum (Gabbott et al. 2005). Based on this trajectory, it is not surprising that inhibition of these excitatory outflows from the PFC would also suppress locomotor activity.

Among the 3 animals—3, 4, and 6—that exhibited the greatest resilience to ABA induction by reducing wheel activity, it was Animal 4 that exhibited the largest GAD terminal contact lengths and bouton areas in PrL and the least wheel activity during the hours of food availability. The other 2 animals (3 and 6) exhibited the largest GAD terminal contact lengths and areas in Cg1 and minimal overall wheel activity during the FR days. These observations indicate that the cellular response for attaining resilience to ABA induction varied across animals—enlargement of GAD terminal contacts in PrL or in Cg1. For both the Cg1 and PrL, the correlation between GAD terminal contact lengths and wheel activity suppression was more robust for the second ABA than the first. Perhaps, the GAD terminal enlargement was cumulative through the first ABA, the recovery phase, and the second ABA.

It is reasonable to consider that those animals exhibiting the most heightened FAA are the ones most strongly entrained to the restricted feeding schedule and have undergone the most robust alteration in PFC circuitry. This idea is supported by our observation that a positive correlation exists between FAA and GAD terminal sizes—those individuals with largest GAD axo-somatic contact lengths in the PrL and MO are the ones that exhibited the highest FAA. Interestingly, there was no correlation between FAA and suppression of activity during the non-FAA period, including the 2 h of food access. This indicates that entrainment to the feeding schedule (as expressed by the heightened FAA) was not sufficient to elicit behavior according to the new rule—to suppress the intrinsically rewarding wheel activity during the hours of food availability, when food access is limited. The lack of correlation between FAA and wheel running suppression during the non-FAA hours is explained by the diversity among the mice in terms of attaining resilience. As described above, one attained resilience by reducing wheel activity during the 2 h of food availability, while 2 attained resilience by reducing overall activity during the FR days. The remaining 4 did not attain resilience because they did not reduce activity during the non-FAA hours, even though their FAAs were high. These 4 that failed to elicit behavior according to the new rule were the ones with smaller GAD terminal sizes, no different from controls’.

Prior to this study, we showed that ABA induction during adolescence evokes a change in the GABAergic system within 2 other brain regions—the hippocampal CA1 and the basolateral amygdala. The change evoked in the hippocampal CA1 was to increase GABAergic inhibition through increased contact lengths by GABAergic axon terminals onto cell bodies and dendrites of pyramidal cells (Chowdhury et al. 2013). After just one exposure to ABA, we also observed increased plasmalemmal expression of non-synaptic GABAergic receptors at spines in the hippocampus (Aoki et al. 2012, 2014). Since upregulation of GABAergic terminal contact sizes onto cell bodies and of non-synaptic GABAergic receptors at spines was greater among animals exhibiting suppression of the FR-evoked hyperactivity (Chowdhury et al. 2013; Aoki et al. 2014), these changes to the hippocampal GABAergic system may have contributed, together with the medial PFC, toward reducing the FR-evoked hyperactivity. Whether other cortical areas also undergo changes in the GABAergic system and may contribute toward suppression of hyperactivity or whether the changes evoked in the hippocampus and/or PFC are sufficient to alter behavior remains to be determined.

Basolateral amygdala, a hub of emotion circuits in the brain (LeDoux 2000), was analyzed within brains of rats, immediately after they underwent single ABA induction (Wable et al. 2014). Here, the change evoked by ABA induction was an increase in the expression of non-synaptic GABA receptor expression by inhibitory interneurons, which would be expected to increase the excitatory outflow and possibly contribute toward increased anxiety and the initial FR-evoked hyperactivity (Wable et al. 2015). In the current study, we observed a modest positive correlation between GABAergic axon terminal contact sizes (lengths and areas) impinging upon layer V pyramidal neurons of the MO and FAA wheel activity. MO is a visceromotor limbic cortex with projections to the dorsal motor nucleus of vagus, nucleus of the solitary tract and amygdala, through which it is proposed to modulate autonomic activity and emotion (Fisk and Wyss 2000; Vogt et al. 2004). Assuming the murine basolateral amygdala undergoes similar GABAergic reorganization as observed in rats, the remodeling of the GABAergic system in MO may be a compensatory mechanism to dampen the heightened anxiety brought about by the increased excitatory outflow from the basolateral amygdala.

Conclusions

It is well established that food deprivation evokes robust and reliable increases in voluntary wheel running by rodents (Sherwin 1998). Among the many ideas about why food deprivation evokes wheel running in rodents, one prevalent idea is that this reflects the adaptive behavior of foraging within the wild by hungry animals, but could also be related to the maladaptive, over-exercise response elicited by a large proportion of patients diagnosed with AN (Epling et al. 1983; Davis et al. 1997, 1999; Hebebrand and Bulik 2011; Zunker et al. 2011; Gutierrez 2013). This idea is the basis for using ABA as an animal model for understanding and developing treatments for AN (Gutierrez 2013). AN is a complex psychiatric illness with biological bases. Twin studies indicate that there is a genetic basis for vulnerability to AN (Bulik et al. 2007), and animal models have pointed to a link between genes modulating anxiety and ABA vulnerability (Gelegen et al. 2006, 2007, 2008, 2010). However, unlike other mental and neurological illnesses such as schizophrenia, autism, epilepsy, or depression, the largest scale search for a genetic link to AN has yet to identify candidate genes (Boraska et al. 2014). In our opinion, one important contribution we have made to the field of ABA is the utility of correlating individual differences in vulnerability to ABA with cellular and molecular markers.

Our findings indicate that ABA induction among adolescent female mice has an impact on PFC circuitry and could be contributing toward prevention of weight loss caused by FR-evoked hyperactivity. The individual differences in response to ABA induction and AN vulnerability may arise due to GAD gene polymorphism, which exists (Addington et al. 2005; Hettema et al. 2006; Zhao et al. 2007), or epigenetic regulations of the GAD gene promoter (Kundakovic et al. 2007; Satta et al. 2008; Zhang
et al. 2010) in the medial FPC. More studies are needed to identify the molecular mechanisms underlying the differential responses of GAD terminals in the FPC and whether it is mediated through the trophic-terminal-dependent release of BDNF or other neurotrophic factors, such as NGF1-A (Zhang et al. 2010). What is also needed is a direct test of whether increased GABAergic inhibition of layer V pyramidal neurons in the FPC will enable individuals to exert cognitive control over the impulsive desire to exercise, rather than to eat.

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Enlarged GABA Synapses in the Medial Prefrontal Cortex Correlates with ABA Resilience

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