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# Abnormal Stress Responsivity in a Rodent Developmental Disruption Model of Schizophrenia

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Although numerous studies have implicated stress in the pathophysiology of schizophrenia, less is known about how the effects of stress interact with genetic, developmental, and/or environmental determinants to promote disease progression. In particular, it has been proposed that in humans, stress exposure in adolescence could combine with a predisposition towards increased stress sensitivity, leading to prodromal symptoms and eventually psychosis. However, the neurobiological substrates for this interaction are not fully characterized. Previous work in our lab has demonstrated that rats born to dams administered with the DNA-methylating agent methylazoxymethanol acetate (MAM) at gestational day 17 exhibit as adults behavioral and anatomical abnormalities consistent with those observed in patients with schizophrenia. Here, we examined behavioral and neuroendocrine responses to stress in the MAM model of schizophrenia. MAM-treated male rats were exposed to acute and repeated footshock stress at prepubertal, peripubteral, and adult ages. Ultrasonic vocalizations (USVs), freezing, and corticosterone responses were quantified. We found that juvenile MAM-treated rats emitted significantly more calls, spent more time vocalizing, emitted calls at a higher rate, and showed more freezing in response to acute footshock stress when compared with their saline (SAL) treated counterparts, and that this difference is not present in older animals. In addition, adolescent MAM-treated animals displayed a blunted HPA axis corticosterone response to acute footshock that did not adapt after 10 days of stress exposure. These data demonstrate abnormal stress responsivity in the MAM model of schizophrenia and suggest that these animals are more sensitive to the effects of stress in youth.
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## INTRODUCTION

Stress is believed to contribute to the pathogenesis of a variety of psychiatric illnesses (Belujon and Grace, 2011; Roozendaal *et al*, 2009; Touma, 2011). In particular, evidence suggests that early life stress is an important factor in the etiology of schizophrenia, a developmental disorder that typically manifests in adolescence or early adulthood. Stressful life events can precipitate or exacerbate the psychotic symptoms of schizophrenia (Corcoran *et al*, 2003; Meyer-Lindenberg and Tost, 2012) and psychosocial stressors increase the risk for developing the disease (Lim and Chong, 2009). It has been suggested that individuals who are at risk for schizophrenia are more susceptible to the effects of stress and that the interaction between a genetic or developmental predisposition and stress in early life could promote symptom onset (Benes, 1997; Tsuang, 2000; Walker *et al*, 2008). Indeed, in children at risk for schizophrenia, those that show abnormally high responses to stress tend to be those that convert to schizophrenia (Johnstone *et al*, 2002; Owens *et al*, 2005). However, the neurobiological substrate for this susceptibility remains unclear.

In order to address this question, we examined behavioral and neuroendocrine responses to stress in a well-validated animal model of schizophrenia. Rats born to dams administered with the mitotoxin methylazoxymethanol acetate (MAM; Nagata and Matsumoto, 1969) on gestational day (GD) 17 display multiple neural and behavioral abnormalities that mirror those seen in schizophrenia, including thinning of limbic cortices, deficits in rhythmic activity in frontal cortex, decreased prepulse inhibition of the startle reflex, and deficits in latent inhibition, among others (Flagstad *et al*, 2004; Grace and Moore, 1998; Le Pen *et al*, 2006; Lodge and Grace, 2008; Moore *et al*, 2006). Importantly, previous work from our laboratory suggests that adult MAM animals are more susceptible to alterations in synaptic plasticity associated with stress (Belujon *et al*, 2013; Goto and Grace, 2006).

In this study, we examined stress responsivity in MAM-treated animals at prepubertal, peripubertal, and adult ages using two objective and quantifiable measures: USVs and HPA axis activation. USVs are closely linked to affective states in rats (Kim *et al*, 2010; Portfors, 2007; Schwarting and

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Wöhr, 2012). Rats exposed to footshock, airpuff startle, and intermittent cold-water swim stress, as well as alcohol or heroin withdrawal, emit robust 22 kHz vocalizations (Brudzynski, 2001; Williams *et al*, 2012). In addition, USVs have been shown to predict stress resilience in rats challenged with intermittent cold-water swim stress (Drugan *et al*, 2009). The HPA axis serves to maintain physiological homeostasis in response to stress, a function that is robustly influenced by regulatory input from the limbic cortices (Jankord and Herman, 2008; McEwen and Gianaros, 2010). Numerous studies have highlighted limbic system abnormalities in patients diagnosed with schizophrenia and in animal models of the disease (Chance *et al*, 2002; Grace, 2010; Lisman *et al*, 2010). In addition, patients with schizophrenia show abnormal HPA axis responses to stressors (Walker *et al*, 2008, 2004).

Given these findings, we examined USV and HPA axis responses to acute and repeated stress in the MAM model of schizophrenia. We hypothesized that juvenile and adolsecent MAM-treated animals would exhibit altered physiological responses to both acute and repeated stressors as compared with their SAL counterparts. Single cohorts of MAM and SAL rats were exposed to inescapable footshock stress throughout development at prepubertal, peripubertal, and adult time points. In these animals, we recorded and quantified USVs emitted in response to acute footshock and analyzed total number of calls, call duration, and call frequency, as well as freezing behavior exhibited during recording sessions. In a separate experiment, we measured plasma corticosterone levels following acute as well as repeated footshock in peripubertal MAM and SAL rats. Together, our findings suggest that USV responses to stress are exaggerated and the neuroendocrine stress response is abnormal in the MAM model of schizophrenia.

## METHODS

### Animals and MAM Treatment

All experiments were performed in accordance with the guidelines outlined in the United States Public Health Service *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. MAM animals were treated following a previously established protocol (Moore *et al*, 2006). Briefly, timed pregnant female Sprague–Dawley rats (Harlan Research Models, Frederick, MD) were obtained at GD15 and housed individually in plastic breeding tubs. MAM (diluted in saline, 20 mg/kg, i.p.) was administered on GD17. Matched control pregnant rats received injections of saline (1 ml/kg, i.p.) on GD17. On day 21, offspring from 4 total litters (2 SAL and 2 MAM) were culled to 10 by removal of female pups to control for effects of the estrous cycle. Male pups were weaned on day 21, housed on a reverse light cycle, and given free access to food and water in groups of two or three with littermates.

### Measuring Vocalizations and Freezing in Response to Footshock Stress

Recording sessions were scheduled for postnatal day (PND) 22 (juvenile), 28 (weanling), 34 (mid adolescent), 37 (late adolescent), 42 (post-adolescent young adult), 50 (post-adolescent young adult), and 54 (adult). Vocalizations were recorded from the same individuals at each of the above time points, SAL $N=12$, MAM $N=14$. Animals were selected in random order for 15-min recording sessions in a dimly-lit, soundproof operant box. The $16(\frac{1}{12})'' \times 16(\frac{1}{12})'' \times 15(\frac{1}{2})''$ Plexiglass chamber was fitted with a grid floor comprised of 0.48 cm stainless steel rods spaced 1.6 cm apart and housed within a sound-attenuating cubicle (Med Associates, St Albans, VT). Each trial was initiated with a 60-s habituation period. Then five scrambled footshocks (1.0 mA, 2 s) were delivered at random intervals (60 ± 20 s). Analog audio was acquired with a Pettersson D200 bat detector with display accuracy: ± 0.15 kHz and bandwidth: 8 ± 4 kHz (Pettersson Elektronik, Carlisle, PA; Del Punta *et al*, 2002) tuned to 22 kHz and digitized at 100 kHz (AD Instruments, Colorado Springs, CO). All behavioral equipment was cleaned with ethanol between animals. Frequency-shifted signals were fed to speakers and behavior was monitored via a video camera. Vocalizations were counted and arranged in single-minute bins to measure rates. Intervals of 4 s were removed from the time period of shock delivery to eliminate noise associated with footshock-induced hyperlocomotion. Freezing, defined as an absence of all non-respiratory movement for at least 3 s, was measured from videotape playback both during the footshock and during the entire test period.

### Measuring Plasma Corticosterone in Response to Footshock Stress

MAM and SAL animals were exposed to one session of footshock per day for 10 days in the peripubertal period, from PND 31–40. The same individuals were exposed to footshock at each timepoint. In each session, rats were placed in a Plexiglas chamber equipped with a grid floor (see above). Twenty-five scrambled footshocks (1.0 mA, 2 s) were delivered every 60 ± 20 s using Med-PC IV software (Med Associates, St Albans, VT). In the control condition (SHAM), rats were exposed to the same protocol but the grid floor was not connected to the current generator. All behavioral equipment was cleaned with ethanol between animals.

Blood was collected from the lateral tail vein before the first session of footshock (PND 31), immediately ($<5$ min) after the second session (PND 32) and immediately after the last session (PND 40). Blood was collected in both groups in morning and afternoon time periods (counterbalanced) into EDTA-coated plastic tubes, immediately centrifuged, and stored at $-20\,^{\circ}$C. An enzyme immunoassay was used to determine plasma concentrations of corticosterone (Assay Designs, Ann Arbor, MI). Corticosterone samples were diluted 1 : 40 and analyzed in duplicate.

### Statistics

The effect of footshock on total number of calls, time spent vocalizing, rate of vocalization, and on freezing behavior was assessed using repeated measures two-way ANOVA. The effects of footshock on weight gain and plasma corticosterone were assessed using repeated measures two-way

ANOVA and Tukey's *post-hoc*. All statistical analyses used $p > 0.05$ and were presented as mean ± SEM.

## RESULTS

### Footshock-Evoked Vocalization

Inescapable footshock stress reliably evoked 22 kHz vocalizations in both SAL- and MAM-treated rats at each developmental time point. No animals were found to vocalize before the first footshock, ie, in the 60 s habituation period (see Methods). The signals were analyzed with regard to call duration and number of calls. Animals vocalized in short clusters or in continuous chains (Figure 1b). The frequency of 22 kHz vocalizations occasionally shifted within the monitoring range of the detector. This modulation was most often present during the first call within a cluster and less evident in continuous vocalization chains, a pattern that is consistent with previous reports showing a similarly clustered 22 kHz call frequency profile in rats responding to airpuff startle (Brudzynski and Holland, 2005).

Footshock provoked a broad range in the number of vocalizations across all animals within the 15-min trial period (210.0 ± 13.7, mean ± SEM). Of the 26 rats tested, 18 did not respond in at least one session and only one animal did not respond at all over the course of the experiment. In any given session, an average of 6.4 ± 0.8 animals did not respond, but these animals did respond vigorously and often beyond the test duration in other sessions. No differences in treatment (MAM *vs* SAL) or litter were observed between responders and non-responders. Vocalizing over the entire 15 min range was less common (21 occurrences): 13 animals vocalized over the entire test duration in at least one session, no more than 7 animals vocalized for over 15 min during a session, and there was only one instance in which an animal greater than 34 days old vocalized beyond the testing interval. The latency to first vocalization declined steadily from the first session (190.9 ± 15.2 s) to the last (76.8 ± 12.8 s). The duration of vocalization in MAM and SAL groups peaked in session 2 (weanling, 610.2 ± 52.2 s; Figure 2a) and declined in session 4 (late adolescent, 240.6 ± 36.42 s), corresponding more closely to the length of the stimulus (five footshocks, interval 60 ± 20 s). Overall, clear group differences were observed across sessions, despite some degree of within-session variability.

### MAM Rats Demonstrated Higher Levels of Vocalization and More Freezing Behavior in Response to Repeated Footshock

In response to footshock, juvenile (PND 22) MAM rats elicited more 22 kHz vocalizations throughout the 15-min recording session than SAL controls (SAL $n = 12$, MAM $n = 14$; $F(1,23) = 2.582$, $p = 0.119$, session 1: $t(2.868)$, $p = 0.005$; Figure 1c). Although juvenile MAM animals did not vocalize more than their SAL counterparts during the period of intermittent, active footshock (ie, the first 5 min of each session; repeated measures two-way ANOVA, $t = 1.447$, $p > 0.05$) they did emit a higher number of vocalizations following footshock ie, the last 10 min of each session (repeated measures two-way ANOVA, $F(1, 23) = 4.049$, $p < 0.05$, significant effect in juvenile session, $t = 2.312$, $p < 0.05$). This effect was not present at any other developmental timepoint.

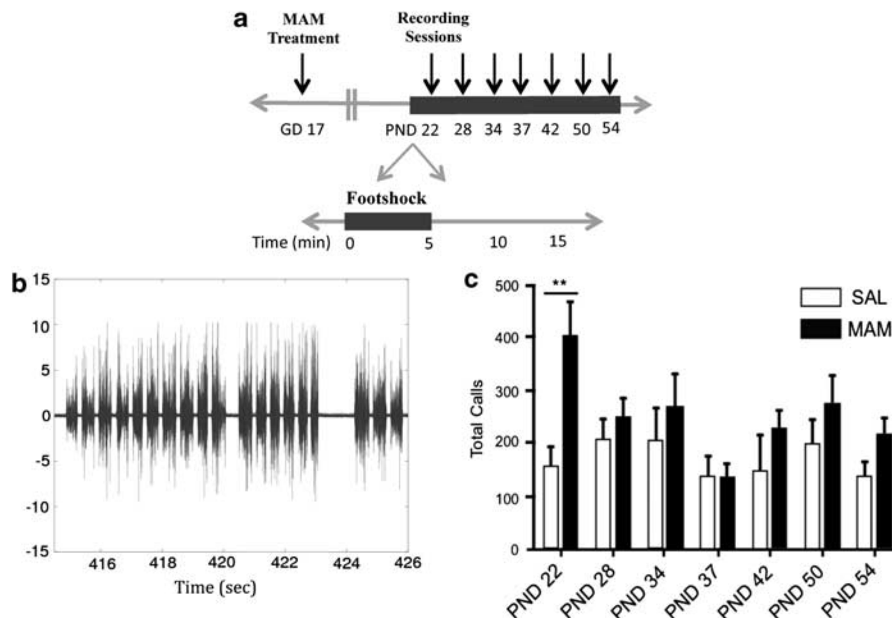


**Figure 1** Juvenile MAM animals emitted more calls following footshock than SAL controls. (a) Design of USV experiment. MAM-treated animals tested at specific developmental time points were exposed to five footshocks delivered at random intervals (60 ± 20 s) and 22 kHz vocalizations were recorded throughout each 15-min session. Note that the same individuals were exposed to footshock at each developmental timepoint. (b) Sample trace illustrating the typical pattern of vocalization emitted by rats exposed to footshock stress. (c) MAM-treated animals in the juvenile period (PND 22) emitted more 22 kHz vocalizations per 15-min session compared with SAL controls (**$p < 0.01$, $F(1,23) = 2.582$, $p = 0.119$, session 1: $t(2.868)$, $p = 0.005$). SAL $n = 12$, MAM $n = 14$.

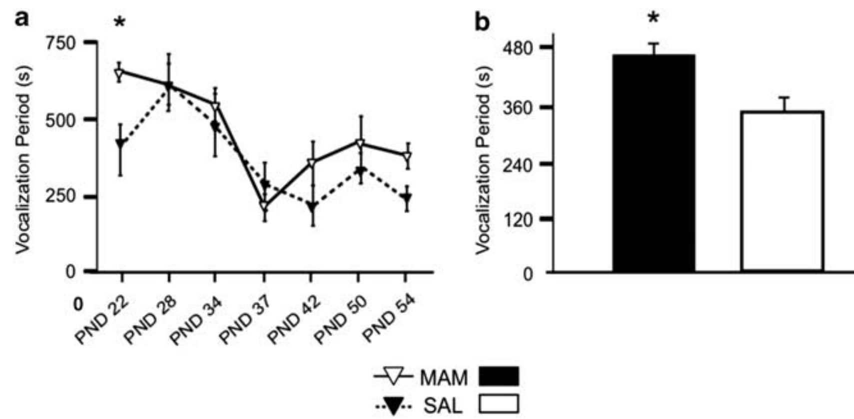


**Figure 2** Juvenile MAM animals spent more time vocalizing following footshock than SAL controls. (a) MAM-treated animals in the juvenile period spent more time vocalizing compared to SAL controls, as measured from the beginning of the first vocalization to the end of the final vocalization in each 15-min session (*$p<0.05$, repeated measures two-way ANOVA, $F(1,23)=7.569$). (b) MAM animals spent more time vocalizing when measured across all ages tested (repeated measures two-way ANOVA, $F(1, 23)=7.569$, $p<0.01$) SAL $n=12$, MAM $n=14$.

In addition to producing more vocalizations, juvenile MAM animals also spent more time vocalizing than SAL controls, measured from the beginning of the first vocalization to the end of the final vocalization within each 15 min session. Furthermore, when measured across all developmental time points, MAM animals spent more time vocalizing than SAL controls overall (repeated measures two-way ANOVA, $F(1, 23)=7.569$, $p<0.01$; Figure 2b). There was no difference in the latency to first vocalization between MAM and SAL animals (repeated measures two-way ANOVA, $F(1, 23)=2.657$, $p>0.05$).

Juvenile MAM animals vocalized at a higher rate than SAL control animals following the period of active footshock, an effect not observed in older rats (repeated measures two-way ANOVA, $F(1,23)=4.049$, $p>0.05$, session no. 1: $t=2.312$, $p=0.023$; Figure 3). In addition, MAM animals vocalized at a higher rate than SAL controls during footshock across all sessions (repeated measures two-way ANOVA, $F(1, 23)=7.392$, $p<0.01$). In particular, MAM rodents vocalized at a higher rate during the period of active footshock, ie, the first 5 min of each session, in the mid-adolescent phase (session 3: $t=2.143$, $p<0.05$) and during the young adult (session 5: $t=2.879$, $p<0.01$) phases.

Freezing behavior was measured both during the footshock, as well as across the entire 15-minute session. Both MAM and SAL rats exhibited similar levels of freezing during the footshock (repeated measures two-way ANOVA, $F(1, 24)=0.08$, $p=0.77$). There was a significant increase in freezing during the footshock period in both groups across sessions (Figure 4a; repeated measures ANOVA, $F(6,24)=9.18$, $p<0.01$). When measured across the entire 15 min session (both footshock and post-footshock), there were no group- or session-dependent differences between MAM and SAL rats (ANOVA, $F(1, 24)=0.13$, $p=0.72$). However, MAM rats at PND22 showed significantly more freezing, owing to increased post-shock freezing compared with controls (Figure 4b; $t(2.2137)$, $p=0.04$).

### Physiological Responses to Repeated Footshock were Blunted in MAM Animals

Statistical analysis of weight gain in MAM and SAL animals over the course of footshock treatment revealed a significant effect of time (SAL-SHAM $n=7$, SAL-FS $n=7$, MAM-SHAM $n=5$, MAM-FS $n=12$; repeated measures two-way ANOVA, $F(9351)=1092$, $p<0.01$; Figure 5b) and treatment ($F(3,39)=11.25$, $p<0.01$) as well as a significant interaction between time and treatment ($F(27\,351)=7.629$, $p<0.01$). SAL controls exposed to repeated footshock in the peripubertal period gained significantly less weight over the course of treatment than SAL animals exposed to SHAM (Tukey's *post-hoc*, $p<0.05$). In contrast, MAM animals exposed to repeated footshock gained a similar amount of weight over the course of treatment as MAM animals exposed to SHAM (Tukey's *post-hoc*, $p>0.05$, Figure 5b).

Measurement of plasma corticosterone levels revealed divergent responses to SHAM exposure in SAL and MAM animals. Before the first treatment session, corticosterone levels in all groups were low ( ~13.35 ng/ml on average), consistent with a basal level of HPA axis activation (Figure 5; Lightman *et al*, 2008). In SAL animals exposed to SHAM treatment, there was an overall significant effect of day (repeated measures one-way ANOVA, $F(2,6)=19.33$, $p<0.01$). Following the second session of SHAM exposure, corticosterone levels in SAL animals were significantly elevated ($77\pm11$ ng/ml; Tukey's *post-hoc*, $p<0.01$). However, plasma corticosterone levels measured after the tenth day of SHAM exposure had returned to near-baseline levels, ($11.5\pm1.1$ ng/ml; Figure 5c), suggesting adaptation of the HPA axis response to SHAM exposure/blood draw. In contrast to their SAL counterparts, following the second session of SHAM exposure corticosterone levels in MAM animals remained unchanged (repeated measures one-way ANOVA $F(2,8)=2.351$, $p>0.05$, 95% CI $=-18.22$ to 62.78). These levels remained unchanged even after the tenth session of SHAM exposure ($43.0\pm19$ ng/ml; Figure 5c), suggesting a lack of initiation and adaptation

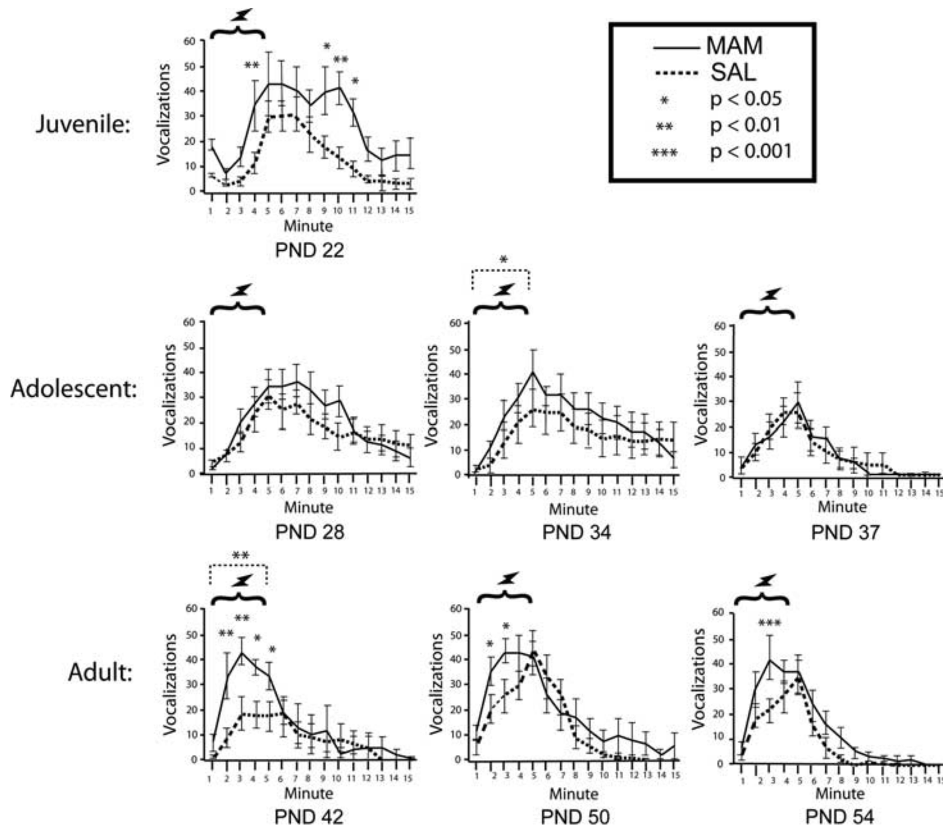


**Figure 3** Juvenile MAM animals vocalized at a higher rate following footshock compared with SAL controls. Juvenile MAM animals emitted 22 kHz vocalizations at a significantly higher rate following the period of active footshock as compared with SAL controls, an effect not observed in other stages of development (repeated measures two-way ANOVA, $F(1,23) = 4.049$, $p < 0.05$, $t = 2.312$, $p < 0.05$). Across all sessions, MAM-treated animals vocalized at a higher rate compared with SAL controls during the period of active footshock ie, the first 5 min of each session (repeated measures two-way ANOVA, $F(1, 23) = 7.392$, $p < 0.05$), with significant interactions in the mid-adolescent (PND 34) and young adult (PND 42) stages (PND 34: $t = 2.143$, $p < 0.05$; PND 42: $t = 2.879$, $p < 0.01$).

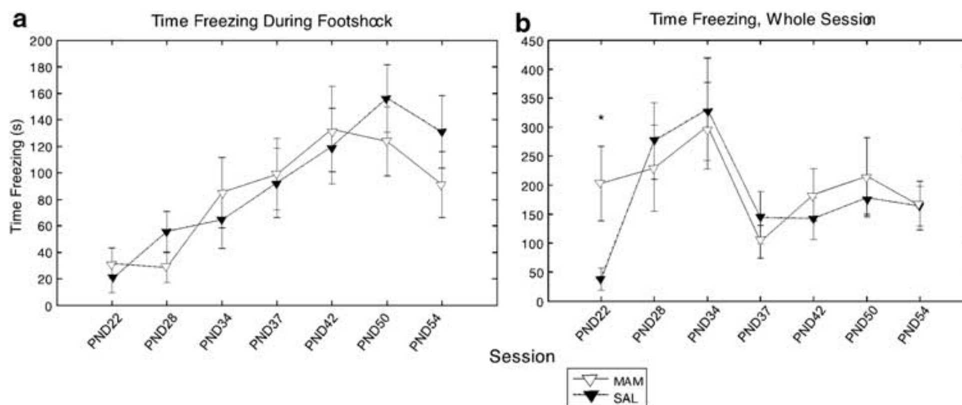


**Figure 4** MAM animals showed increased freezing during post-footshock periods. Freezing was measured during the footshock, as well as across the entire 15 min session. (a) When measured only during the footshock, both MAM-treated rats and SAL-treated rats showed increased levels of freezing across sessions. No differences between MAM and SAL animals were observed. (b) When measured across the entire 15 min period, there were no differences overall between MAM and SAL rats; however, the MAM rats sshowed significantly greater time freezing at PND22, which is consistent with the increased vocalizations observed beyond the period of footshock exposure.

of corticosterone responses to SHAM exposure in MAM animals.

Stress responses in SAL and MAM animals exposed to footshock treatment mirrored the SHAM pattern but were much greater in amplitude. In SAL animals exposed to footshock, there was an overall significant effect of day (repeated measures one-way ANOVA, $F(2,5) = 21.43$, $p < 0.01$). SAL animals exposed to footshock displayed an increase in plasma corticosterone levels following the second session of footshock ($421 \pm 67$ ng/ml); a nearly

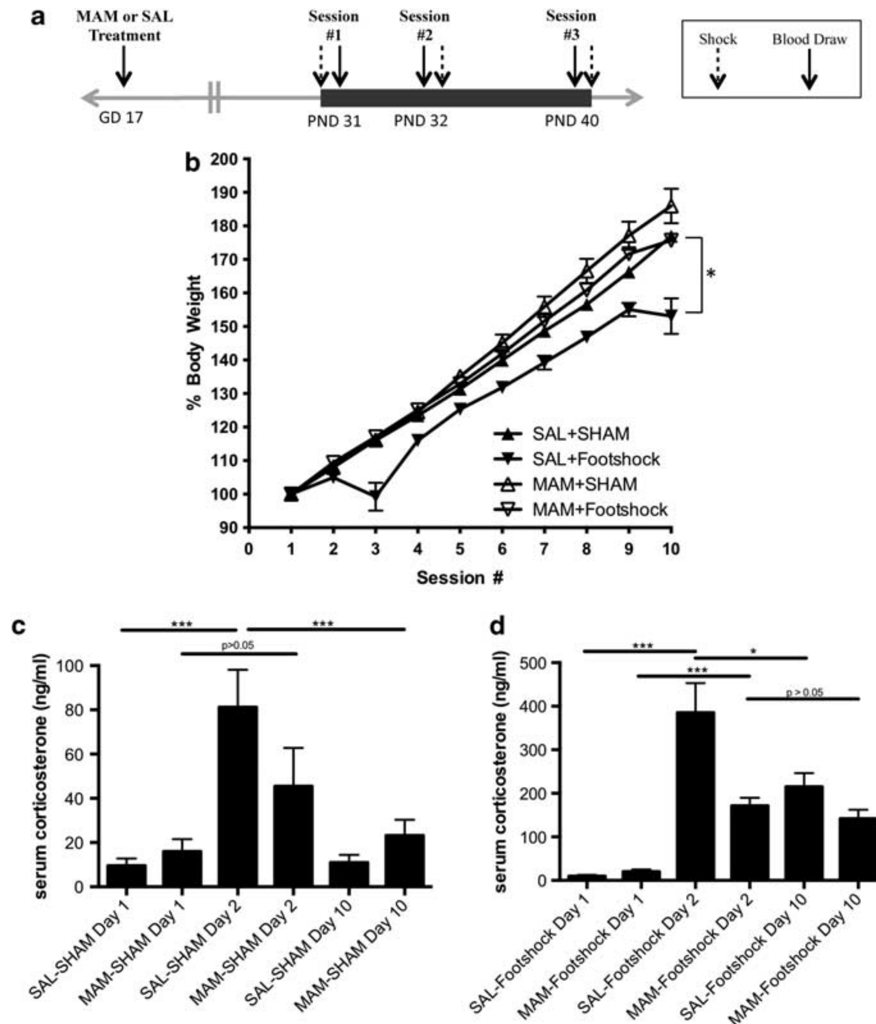


**Figure 5** MAM animals displayed abnormal weight gain and HPA axis responses to acute and repeated footshock exposure. (a) Design of corticosterone experiment. A separate cohort of MAM-treated animals were exposed to 10 days of repeated footshock stress (dashed arrow) in the peripubertal period (PND 31–40) and plasma corticosterone levels were measured in blood drawn (solid arrows) before the first session (PND 31), immediately after the second session (PND 32) and immediately after the last session (PND 40) in the same animals, as indicated by the position of the arrows. (b) SAL-treated animals gained significantly less weight in response to repeated footshock as compared with SHAM controls (repeated measures two-way ANOVA, Tukey's *post-hoc*, $*p<0.05$). In contrast, MAM animals exposed to repeated footshock gained a similar amount of weight compared with their SAL counterparts (repeated measures two-way ANOVA, Tukey's *post-hoc*, $p>0.05$). (c) SAL animals exposed to the SHAM condition displayed a moderate but significant increase in plasma corticosterone levels following the second session that adapted over the course of 10 sessions (repeated measures one way ANOVA $F(2,6)=19.33$, $p<0.01$, Tukey's *post-hoc*). (Note differences in *y* axis between SHAM and footshock treatments.) MAM animals exposed to the SHAM condition showed no differences in plasma corticosterone levels following the second or tenth session (repeated measures one-way ANOVA $F(2,8)=2.351$, $p>0.05$, Tukey's *post-hoc*). (d) Corticosterone levels in SAL animals were elevated ($\sim$40-fold increase) after the second session (repeated measures one-way ANOVA $F(2,5)=21.43$, Tukey's *post-hoc* $***p<0.001$) and had adapted by the tenth session (repeated measures one-way ANOVA $F(2,5)=21.43$, Tukey's *post-hoc* $*p<0.05$). In contrast, MAM animals exposed to footshock displayed a significant, but attenuated increase in plasma corticosterone ($\sim$8-fold increase) following the second session of treatment (repeated measures one-way ANOVA $F(2,11)=24.34$, Tukey's *post-hoc*, $*p<0.05$) that did not adapt by the tenth session (repeated measures one-way ANOVA $F(2,11)=24.34$, Tukey's *post-hoc*, $p>0.05$). For all, SAL-SHAM $n=7$, SAL-FS $n=7$, MAM-SHAM $n=5$, MAM-FS $n=12$.

200-fold increase over baseline levels (Tukey's *post-hoc*, $p<0.01$; Figure 5d). After the tenth session of footshock, this response was significantly reduced ($249\pm21$ ng/ml; Tukey's *post-hoc*, $p<0.05$), consistent with an adaptation of the corticosterone response to stress in SAL animals. In MAM animals exposed to footshock treatment, there was a significant effect of day on corticosterone responses (repeated measures one-way ANOVA $F(2,11)=24.34$, $p<0.01$). In contrast to SAL animals, however, plasma corticosterone levels following footshock exposure were blunted in MAM animals. After the second session of footshock, corticosterone levels were increased 8-fold over baseline levels (Tukey's *post-hoc*, $p<0.05$, $163.0\pm15$ ng/ml; Figure 5d), a much less robust response than observed in SAL animals. In addition, this response did not adapt over time. Corticosterone measured in MAM animals after the tenth session remained elevated near levels observed following the second session ($130.0\pm17$ ng/ml) even after 10 days of footshock exposure (Tukey's *post-hoc*, $p>0.05$). This is in contrast to the pronounced decrease in plasma

corticosterone observed in SAL animals following the tenth session of footshock.

## DISCUSSION

Our measurements of USVs emitted in response to acute footshock stress show that MAM rats in the juvenile period are more responsive to stressors as compared with matched SAL rats, at least as represented by our footshock stress paradigm. In response to acute footshock, juvenile MAM-treated rats emitted more 22 kHz vocalizations than their SAL counterparts, vocalized for a longer duration, and emitted vocalizations more frequently compared with SAL controls. In the same experimental protocol, both MAM and SAL rats showed equivalent amounts of freezing during the footshock, but juvenile MAM rats exhibited more non-shock freezing. The fact that both groups showed similar freezing during the footshock suggests that MAM and SAL rats are equally sensitive to the footshock. Moreover, adolescent MAM rats showed a disruption in HPA axis responses to stress, exhibiting attenuated corticosterone levels after acute footshock that, unlike in SAL counterparts, did not change after 10 days of stress exposure. Adolescent MAM rats also did not show diminished weight gain due to footshock exposure, unlike their SAL counterparts. Although we have not observed evidence to date of differences in litters of MAM rats, it should be noted that 2 l of SAL and 2 l of MAM animals were used for our experiments, as described in the Methods. Taken together, the USV findings suggest that juvenile and adolescent MAM animals are more sensitive to the effects of stress, while the weight gain and corticosterone data suggest that they are unable to integrate appropriate responses to acute stress and unable to adapt to repeated stress over time.

Previous studies have demonstrated that 22 kHz USVs and HPA axis responses to stress change with developmental disruption. Kosten *et al* (2005) demonstrated that rats exposed to neonatal isolation show increased USV responses to footshock stress. In addition, rats exposed to neonatal ventral hippocampal lesion display an attenuated increase in plasma corticosterone levels as compared with sham-lesion controls after 20 min of footshock, and this response fails to adapt after 60 min of footshock (Chrapusta *et al*, 2003). Similarly, the offspring of dams exposed to chronic unpredictable stress during pregnancy display an exaggerated corticosterone response to an open field test (Clinton *et al*, 2008). Taken together, these data suggest that USV and HPA axis responses to stress are sensitive to various developmental disruptions and that HPA axis dysregulation could contribute to other abnormal phenotypes seen in animal models of psychiatric disease.

In addition to our data in juvenile MAM animals, we also observed an increase in USV frequency in adult MAM animals in response to footshock. Although these data are difficult to interpret given the relative lack of previous work concerning USV emission across the rat lifespan, we believe that this effect is independent of the effect we observed in juvenile animals. Although call frequency was significantly elevated in this group, call number and duration were not. In addition, the increases in USV frequency and freezing occurred during the period of active footshock, in contrast to juvenile animals that showed heightened vocalizations and freezing following the footshock. Finally, the effect appears to be unrelated to any sort of learning response, as no similar effect was observed in the adolescent or young adult time points. Instead, this may reflect a continuation of increased stress reactivity in the adult, albeit at a lower intensity.

MAM animals exposed to footshock stress for 10 consecutive days did not show any differences in weight gain as compared with their SHAM counterparts. In contrast, SAL animals exposed to the same footshock paradigm did in fact show a significant reduction in weight gain over the 10-day paradigm, as is typically observed in chronic stress protocols (Dess and Minor, 1989; Dias-Ferreira *et al*, 2009; Jankord and Herman, 2008). Indeed, the effects of stress on the HPA axis and the subsequent endocrine response are known to influence appetite as well as metabolic processes (McEwen, 2007). We interpret this unexpected lack of response in adolescent MAM animals to the stress paradigm as an aberrant metabolic response to stress that could be related to the HPA axis abnormalities we have identified.

Although the same animals were exposed to stressors at each developmental time point in our USV experiment, we do not believe that this impacted the differences we observed between the MAM and SAL groups for several reasons. Exposures were very brief and separated by several days. In addition, adult MAM animals were found to vocalize at a higher frequency in response to footshock. This finding suggests against the occurrence of a learning response and/or habituation, and even more so given that it was observed during the period of active footshock, in contrast to juvenile animals who vocalized at a higher rate following active footshock. Finally, if repeated stress exposure on its own had an effect on USV number, duration, or frequency, we would expect that such an effect would be observed in both the SAL and MAM groups. However, this was not the case: while SAL animals responded in a similar pattern across the lifespan, MAM animals displayed a characteristic increase in call number, duration, and frequency at PND 22.

Perhaps most convincingly, we observed no differences in freezing behavior during the footshock across all treatment groups and sessions, although both MAM and SAL rats showed increased time freezing during the footshock across developmental periods. Although the difference in USVs in the youngest cohort is robust, it is difficult to evaluate whether the effects on vocalization seen in the adult may have been due to differences in learning with repeated exposure between MAM and SAL rats, or were due solely to the MAM treatment. Nonetheless, the differences in MAM rats at the juvenile stage at first footshock exposure and the lack of an apparent difference in learning response between SAL and MAM animals suggests that any differences we observed in USVs in MAM animals were likely due to the MAM treatment itself.

Interestingly, the abnormal response to stress identified in our work is present pre- and peripubertally; a time that precedes the point of transition to psychosis in susceptible individuals (Addington and Heinssen, 2012; Miller *et al*, 2001) and also precedes the time point that MAM rats show increased amphetamine-induced locomotor responses

(Flagstad *et al*, 2004; Moore *et al*, 2006). The finding of a blunted corticosterone response to acute footshock exposure is of particular interest. Studies have shown that a substantial corticosterone response is necessary for homeostatic adaptation to stressors (McEwen and Gianaros, 2010; Rao *et al*, 2012), and a pathologically blunted cortisol response to stress in humans is believed to contribute to disorders such as post-traumatic stress disorder (Baker *et al*, 2005; LaBar and Cabeza, 2006; Luo *et al*, 2012). Therefore, a blunted initial corticosterone response and a failure to show changes with repeated exposure could contribute to the pathological responses to stress seen in the MAM model.

In summary, in pre- and peripubertal periods there is an increase in USVs in response to stress as well as disruption of the corticosterone response in MAM rats. However, the source of this increased responsivity is unclear. Although this study does not directly address cellular and molecular mechanisms of this phenomenon, one possibility is that it could result from a failure of higher cortical processes to limit the impact of stressors. The prefrontal cortex is a region that has been linked to the pathophysiology of schizophrenia (Knable and Weinberger, 1997; Lewis *et al*, 2005), and it has been suggested that deficits in this region could be present in the prodromal state of schizophrenia (Phillips and Seidman, 2008; Thompson *et al*, 2004). Moreover, the mPFC has been shown to limit the response to stress in rats and in humans (Finlay *et al*, 1995; Hariri *et al*, 2003). Therefore, a failure of the mPFC to regulate stress in the MAM-treated rat or in the prodromal stage of schizophrenia could contribute to pathological changes in the hippocampus observed in this disorder. It has been shown that activation of the amygdala, which occurs during stress, will lead to parvalbumin interneuron loss in the hippocampus (Berretta *et al*, 2001) and loss of hippocampal parvalbumin interneurons is proposed to lead to the DA hyper-responsivity in the MAM model (Lodge and Grace, 2007). Thus, a hypersensitivity to stress potentially secondary to decreased mPFC function could initiate a cascade of events during juvenile/adolescent stages to lead to hippocampal dysfunction and the emergence of the schizophrenia-like phenotype observed in the MAM model (Grace, 2004; Thompson *et al*, 2004).

These findings, when considered in light of work demonstrating hippocampal dysfunction in MAM animals, suggest a mechanism for schizophrenia pathogenesis whereby the deleterious effects of stress on the hippocampus are driven by a failure of the mPFC to regulate stress. The resulting profound hippocampal dysfunction would in turn prevent an individual from mounting appropriate homeostatic responses to stressors, rendering them more vulnerable to the effects of stress, and increasing their risk for developing schizophrenia. This is also consistent with human epidemiological data showing that, in children at risk for schizophrenia, those that show the greatest response to stressors tend to convert (Johnstone *et al*, 2002; Owens *et al*, 2005). If stress during puberty and adolescence is indeed a contributing factor to the transition to psychosis, controlling stress at this vulnerable period may circumvent these pathological changes and prevent the emergence of psychosis later in life (Du and Grace, 2013; Thompson *et al*, 2004).

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