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Adolescent olanzapine sensitization is correlated with hippocampal stem cell proliferation in a maternal immune activation rat model of schizophrenia

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Abstract

Previous work established that repeated olanzapine (OLZ) administration in normal adolescent rats induces a sensitization effect (i.e. increased behavioral responsiveness to drug re-exposure) in the conditioned avoidance response (CAR) model. However, it is unclear whether the same phenomenon can be detected in animal models of schizophrenia. The present study explored the generalizability of OLZ sensitization from healthy animals to a preclinical neuroinflammatory model of schizophrenia in the CAR. Maternal immune activation (MIA) was induced via polyinosinic:polycytidilic acid (PolyI:C) administration into pregnant dams. Behavioral assessments of offspring first identified decreased maternal separation-induced pup ultrasonic vocalizations and increased amphetamine-induced hyperlocomotion in animals prenatally exposed to PolyI:C. In addition, repeated adolescent OLZ administration confirmed the generalizability of the sensitization phenomenon. Using the CAR test, adolescent MIA animals displayed similar increase in behavioral responsiveness after repeated OLZ exposure during both the repeated drug test days as well as a subsequent challenge test. Neurobiologically, few studies examining the relationship between hippocampal cell proliferation and survival and either antipsychotic exposure or MIA have incorporated concurrent behavioral changes. Thus, the current study also sought to reveal the correlation between OLZ behavioral sensitization in the CAR and hippocampal cell proliferation and survival. 5′-bromodeoxyuridine immunohistochemistry identified a positive correlation between the magnitude of OLZ sensitization (i.e. change in avoidance suppression induced by OLZ across days) and hippocampal cell proliferation, and a negative correlation between OLZ sensitization magnitude and hippocampal short term cell survival. The implications of the relationship between behavioral and neurobiological results are discussed.

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Author Disclosures

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Keywords

Olanzapine; Conditioned avoidance response; Amphetamine; Locomotor activity; Prepulse inhibition; Maternal immune activation; Sensitization; Neurogenesis; Polyinosinic:polycytidilic acid; 5′-bromodeoxyuridine

INTRODUCTION

The use of antipsychotic drugs (APD) in children and adolescents has increased dramatically worldwide in recent years (Aparasu and Bhatara, 2007; Kalverdijk *et al*, 2008; Rani *et al*, 2008; Wong *et al*, 2004). APD use for patients under the age of 18 now accounts for about 15% of total usage in the U.S., not only for the management of psychosis, but also for bipolar disorder, depression, disruptive behavior, and anxiety (Domino and Swartz, 2008). Clinical studies have established that children and adolescents tend to be more susceptible than adults to the adverse consequences of APD use, including prolactin elevation and motor side effects (Haddad and Sharma, 2007; Wudarsky *et al*, 1999). Preclinical studies in animal models suggest that this may be due to the differential biological effects APD have on adolescents versus adults (Choi *et al*, 2010; Spear *et al*, 1980). For instance, chronic treatment of various APD for 21 days significantly reduced the number of dopamine (DA) D_1 receptors in both the medial prefrontal (mPFC) and dorsolateral frontal cortices of adolescent rats, but not adult rats. In contrast, DA D_2 receptor levels were elevated in the mPFCs of adult rats after chronic APD infusion, but not in adolescent rats (Moran-Gates *et al*, 2006).

Animal studies have also illustrated that application of APD during adolescence, a critical period for neurodevelopment, can stimulate neuronal and behavioral functional changes that are reflected in adulthood (Vinish *et al*, 2013). Adolescent rats chronically exposed to the atypical APD olanzapine (OLZ) therapy released significantly less DA upon stimulation during adulthood than vehicle treated rats. Interestingly, our group has also shown that adolescent APD exposure (postnatal days [P] 35–60, Andersen et al., 2000) can increase APD response in adulthood, a phenomenon known as “antipsychotic sensitization” (Qiao *et al*, 2013a, 2013b, 2014). Although not well understood, APD behavioral sensitization due to chronic treatment is well documented. For example, the exponential curve of psychotic symptoms improvement over the first 4 weeks of treatment; the antipsychotic effect growing with the passage of time (i.e. time-dependent sensitization), and the worsening of extrapyramidal side effects after years of medication are all well-known demonstrations of APD sensitization (Agid *et al*, 2003; Fallon and Dursun, 2011; Kapur *et al*, 2006). Clinically, APD sensitization may play an important implication in prescription considerations, especially after drug abstinence. Furthermore, concerning the adolescent patient population, APD sensitization may interact with developmental trajectories to alter adulthood behavior and brain functions.

In a previous study, we showed that OLZ, an APD widely prescribed in the adolescent population, induces an increased behavioral sensitivity over repeated treatment during adolescence (Pakyurek *et al*, 2013). This increase in sensitivity remains evident when rats are re-exposed to OLZ in adulthood (Qiao *et al*, 2013a). The study employed the rat

conditioned avoidance response (CAR) test to measure APD response. This test is widely used and well-validated for assessing APD activity in rodents, and numerous studies have verified that clinically relevant doses of APD suppress avoidance behavior in animals (Wadenberg and Hicks, 1999; Wadenberg, 2010; Wadenberg *et al*, 1997). The model is commonly used to predict the clinical potency of APD, and thus an ideal measure of behavioral sensitization over repeated exposure (Arnt, 1982; Wadenberg *et al*, 2001).

One mechanism that may be associated with long term APD sensitization is drug-induced changes in cell proliferation and survival. While the effects of APD on cell growth are still inconsistent across studies, long-term administration of APD has been reported by some to increase cell proliferation in the subventricular zone as well as the subgranular zone of the hippocampus in adult animals (Halim *et al*, 2004; Keilhoff *et al*, 2010; Maeda *et al*, 2007; Wakade *et al*, 2002; Wang *et al*, 2004). However, no prior study has considered this effect of APD as a potential mechanism of APD sensitization in adolescence. Also, much of our APD sensitization work has used otherwise healthy rats, and it remains to be elucidated whether APD sensitization is generalizable and can also be demonstrated in preclinical disease models. In the present study, we addressed this issue by testing OLZ in a rat maternal immune activation (MIA) model of schizophrenia, a well-studied model that gained much popularity in the recent decade (Meyer and Feldon, 2010). Adult rodent offspring born to infected mothers displayed specific histological and behavioral abnormalities relevant to those seen in human patients with schizophrenia, including increased prefrontal cortical pyramidal cell density (Fatemi *et al*, 1999), deficits in learning (Shi *et al*, 2003), sensorimotor gating (Wolff and Bilkey, 2008), latent inhibition (Zuckerman and Weiner, 2003), and working memory (Ozawa *et al*, 2006).

Examination of hippocampal cellular changes associated with OLZ sensitization using a preclinical model is relevant to the treatment of schizophrenia. First, impaired hippocampal cell proliferation is thought to contribute to cognitive deficits in schizophrenia, and induction of hippocampal cell growth has been suggested as a possible therapeutic mechanism of APD treatment (Kempermann *et al*, 2008; Reif *et al*, 2006, 2007; Toro and Deakin, 2007). However, it is unclear whether the induction of cell proliferation by antipsychotics is related to behavioral phenotypes that are representative of drug efficacy. Secondly, MIA as a preclinical model of schizophrenia has been shown to reduce neurogenesis in some studies, and one previous study suggested that APD treatment may reverse this dysfunction (Cui *et al*, 2009; Piontkewitz *et al*, 2012b; Vuillermot *et al*, 2010; Wolf *et al*, 2011). However, no study has connected this cellular change in MIA animals with behavioral effects, which warrants the current idea of examining the relationship between cell proliferation and survival associated with APD treatment and the CAR behavior observed in a preclinical model.

In this study, we investigated the potential connection between the induction and expression of OLZ sensitization in adolescent rats and OLZ-induced cell proliferation and survival in the dentate gyrus (DG) of rat hippocampi, using the CAR task for behavioral assessment and 5′-bromodeoxyuridine (BrdU) immunohistochemistry for cellular examination, respectively. In addition, various behavioral assays were used to assess MIA-induced behavioral impairments. These included maternal separation-induced 40 kHz pup ultrasonic

vocalizations (USV) on P11, an assay for early communicative behavior and stress response (Ming *et al*, 2011); prepulse inhibition (PPI) in adolescence (P43), an assay for sensorimotor gating ability (Geyer and Braff, 1987; Swerdlow *et al*, 2008); and amphetamine-induced hyperlocomotion in adulthood (P84), an assay for both DAergic defect as well as APD efficacy (Arnt, 1995; Laruelle *et al*, 1996, 1999). In assessing ultrasonic vocalizations, we expect MIA animals to exhibit decreased total number of vocalizations after maternal separation, as previous studies have indicated communication deficits in MIA offspring using a similar paradigm (Malkova *et al*, 2012). While PPI results have been somewhat inconclusive regarding MIA offspring assessments due to variations in experimental procedure, we hypothesize a deficit in sensory-motor gating based on previous report using parameters similar to the current study (Wolff and Bilkey, 2010). Finally, Zuckerman *et al*, 2003 previously reported an increase in amphetamine-induced hyperlocomotion in adult MIA offspring, and we hypothesize a similar finding in the current study.

Thus, the overall goals of the study were twofold. First, we sought to identify whether OLZ, an APD widely prescribed in the adolescent population, produces behavioral sensitization in adolescent MIA animals. This confirms whether the adolescent OLZ sensitization phenomenon in healthy animals can generalize into a preclinical animal model of psychiatric disorders. Secondly, we also sought to investigate whether changes in cell proliferation and survival in the DG of the hippocampus are associated with the CAR sensitization induced by repeated OLZ exposure. We hypothesized that OLZ sensitization is a generalizable phenomenon that can be observed in MIA animals, and that the CAR sensitization induced by repeated OLZ exposure is associated with changes in cell proliferation and survival within the DG of the hippocampus.

RESULTS

Experiment 1: Assessment of MIA behavioral alterations during early postnatal period and adulthood

We first established the validity of our MIA model by assessing phenotypic alterations at three time points: one day post PolyI:C treatment, early postnatal period, and adulthood. During gestation, we measured the percentage of pregnant dam weight change one day following saline or PolyI:C injection (Fig. 1a). One way ANOVA revealed a main effect of prenatal treatment, $F(1,66)=10.910$, $p=0.002$. This suggest that treatment of PolyI:C during gestation transiently decreased the amount of weight gain in pregnant females, demonstrating the effectiveness of PolyI:C.

Next, we further examined schizophrenia related phenotypes in MIA animals during the early postnatal period. Pup USV serve as an important communication tool from pups to mothers, and MIA is reported to cause an alteration in this behavior (Malkova *et al*, 2012). Here we assessed the number of maternal separation-induced USV made by pups on P11 (Fig. 1b). Repeated measures two-way ANOVA revealed a main effect of call type, $F(3,94)=53.75$, $p<0.001$, and a call type × prenatal treatment interaction, $F(3,94)=3.221$, $p=0.026$. One way ANOVA on the individual call types revealed a main effect of prenatal treatment for long calls, $F(1,98)=9.491$, $p=0.003$ and total number of calls, $F(1,98)=4.011$, $p=0.048$. These findings strengthen the hypothesis that prenatal exposure to MIA may

impair pup social communications, and provide extended information on specific call type alterations.

Finally, as sensitivity to amphetamine is shown to be elevated in adult MIA offspring (Piontkewitz et al., 2011), we examined differences between prenatal treatment conditions in amphetamine-induced hyperlocomotion during adulthood. Figure 1c shows the mean locomotor activities as measured by the number of photobeam breaks throughout the 60-min test period after the amphetamine injections. Repeated measures two-way ANOVA revealed a main effect of time blocks, $F(11,30)=14.001$, $p<0.001$, and a marginal effect of prenatal treatment, $F(1,40)=3.925$, $p=0.054$. These findings suggest that prenatal exposure to MIA increased sensitivity to amphetamine in adult offspring, mimicking increased vulnerability to psychotomimetic drugs.

Experiment 2: Evaluation of OLZ sensitization in the conditioned avoidance response test and hippocampal cell proliferation and survival in MIA and saline rats

Using the validated MIA model, we next examined OLZ sensitization using the CAR test. The procedure consisted of four phases: *avoidance training*, *post training PPI assessment*, *induction of OLZ sensitization*, and *expression of OLZ sensitization*.

The first phase of CAR testing is the avoidance training. Repeated measures two-way ANOVA of percentage avoidance responses throughout the 10 CAR training days revealed only a main effect of day, $F(9,82)=25.224$, $p<0.001$, but no main effect of prenatal treatment (Fig. 2a). While performance from the first two days of CAR training suggests possible differences, the effect is not significant.

Following 10 days of avoidance training, all animals were assessed for PPI to examine differences in sensorimotor gating functions. Figure 2b and 2c shows the percentage of PPI and the magnitude of ASR one day following CAR training (P 43), respectively. There was no main effect of prenatal treatment on PPI, nor a prenatal treatment × prepulse level interaction. There was also no significant difference between the ASR of the two conditions.

Next, animals underwent five days of OLZ administration for induction of drug sensitization. Figure 3a and b shows the percentage of avoidance responses throughout the five test days. During this sensitization induction phase, OLZ treatment consistently disrupted avoidance response. Repeated measures two-way ANOVA analysis on the overall avoidance data for each drug treatment condition revealed a main effect of day, $F(4,83)=25.301$, $p<0.001$, drug groups, $F(2,86)=426.523$, $p<0.001$, and a significant day × drug group interaction, $F(8,168)=3.111$, $p=0.003$ (Fig. 3b). *Post hoc* LSD tests revealed that the 3 drug groups all differed significantly from each other, all $p<0.001$. There was no significant difference between prenatal treatments (Fig. 3a).

Prior to challenging the animals with low dose OLZ to assess sensitization expression, all animals underwent two days of drug free retraining sessions, the first with only the conditioned stimulus of noise present, and the second session with both the conditioned stimulus of noise and the unconditioned stimulus of shock. This ensured that all animals return to a high baseline of CAR behavior before the OLZ challenge. Figure 3c and d shows

the percentage of avoidance responses on the two retraining days and the OLZ sensitization challenge day. There was no group difference on the retraining days between prenatal treatment and drug groups, although repeated measures two-way ANOVA of the retraining days did reveal a main effect of day, $F(1,86)=30.747$, $p<0.001$.

Finally, after two days of drug free sessions, animals were challenged with OLZ 0.5 mg/kg to assess the expression of OLZ sensitization. Multifactorial ANOVA revealed a significant effect of drug group, $F(2,86)=26.749$, $p<0.001$ (Fig. 3). *Post hoc* LSD tests showed that the OLZ 1.0 and 2.0 mg/kg groups were significantly lower in percent avoidance than the VEH group, $p<0.001$ for both, confirming the OLZ sensitization effect. The two OLZ groups did not differ significantly from each other. There was also no significant difference between prenatal treatments (Fig. 3d). These results suggest that the OLZ sensitization is a general behavioral pharmacological phenomenon irrespective of prenatal treatment conditions.

To assess the relationship between OLZ sensitization and hippocampal cell proliferation and survival, all animals were sacrificed after the OLZ challenge, and 5′-bromodeoxyuridine (BrdU) immunoreactivity was measured. Figure 4a depicts a sample micrograph of BrdU labeling in the subgranular zone of the hippocampal dentate gyrus.

Cell proliferation was assessed in animals injected with BrdU during the drug free retraining days, while survival was assessed in animals injected with BrdU immediately prior to the repeated drug test days. Separate repeated two-way ANOVAs found no significant difference in the number of BrdU+ cells between prenatal or drug treatment groups in both cell proliferation (Fig. 4b) or short term cell survival (Fig. 4c). Interestingly, however, there was a differential association between OLZ sensitization and cell proliferation between the MIA and SAL offspring. While the magnitude of OLZ sensitization from day one to two of drug administration had no correlation with the number of BrdU+ cells assessed for proliferation (Fig. 4d, Person's $r=-0.005$, $p=0.983$) in SAL animals, the highest percent avoidance suppression from day one to two of drug administration corresponded to the highest number of BrdU+ cells (Fig. 4e, Pearson's $r=0.527$, $p=0.030$) in MIA animals injected with BrdU 48 hours prior to sacrifice. On the contrary, correlational analysis further revealed a different pattern of association between the magnitude of OLZ sensitization and short term cell survival between MIA and SAL animals. While the magnitude of OLZ sensitization from day one of drug administration to the challenge assessment had no correlation with the number of BrdU+ cells assessed for survival (Fig. 4f, Person's $r=0.162$, $p=0.533$) in MIA animals, the highest percent of avoidance suppression from day one of drug administration to the challenge assessment corresponded to the lowest number of BrdU + cells in SAL animals injected with BrdU 9 days prior to sacrifice (Fig. 4g; Pearson's $r=-0.910$, $p<0.001$). These results suggest that the magnitude of OLZ sensitization, as assessed by the extent of the CAR disruption over days, is associated with the increase in acute cell proliferation rate for MIA animals and the decrease in short term cell survival rate for SAL animals.

DISCUSSION

The present study investigated the association between hippocampal cell growth and survival and adolescent OLZ sensitization in the CAR test using the MIA model of SZ. We found decreased pup USV and increased amphetamine sensitivity in MIA animals, validating that MIA animals do in fact display altered behaviors in preclinical assays relevant to SZ (Malkova *et al*, 2012; Piontkewitz *et al*, 2011). Using a two-phase paradigm we developed, we showed that repeated OLZ exposure sensitized MIA animals not only throughout the repeated drug test days, but also in the subsequent challenge test, with rats previously treated with OLZ having a stronger response to OLZ than drug naïve animals. So far, few groups have studied the effects of APD in MIA (Arad and Weiner, 2012; Piontkewitz *et al*, 2009, 2011, 2012b). Here we established that adolescent OLZ behavioral sensitization is a stable phenomenon irrespective of disease state. Furthermore, the present study extended our work on the neurobiological basis of OLZ sensitization by investigating hippocampal cellular changes (Zhao and Li, 2010; Zhao *et al*, 2012). The results indicated that cell proliferation correlated positively with the magnitude of OLZ induced CAR sensitization in MIA animals, while cell survival correlated negatively with sensitization magnitude in SAL animals.

MIA is a widely accepted disease model of SZ (Meyer and Feldon, 2010; Patterson, 2009). Epidemiological surveys in the human population indicate that bacterial and viral infections during the second trimester increase the risk of neurodevelopmental psychiatric disorders in the offspring by two fold (Boksa, 2008; Brown, 2006; Brown *et al*, 2000). These infections may activate the mother's immune system, increasing cytokine expressions, disrupting offspring neurodevelopment, and causing SZ related phenotypes (Meyer and Feldon, 2009; Smith *et al*, 2007). Preclinical studies show that *in utero* exposure to immunostimulants such as lipopolysaccharide (LPS) and PolyI:C disrupts SZ relevant behaviors, including latent inhibition ([LI]; Meyer *et al*, 2010; Zuckerman and Weiner, 2003; Zuckerman *et al*, 2003), amphetamine-induced hyperlocomotion (Vorhees *et al*, 2012; Zager *et al*, 2012), and sensory-motor gating (Basta-Kaim *et al*, 2012; Wolff and Bilkey, 2008). Here we showed that rat pups prenatally exposed to PolyI:C exhibited abnormal USV patterns on P11 after 20 minutes of maternal separation. So far there have been few studies that assessed this social communication deficit (Malkova *et al*, 2012; Yee *et al*, 2012), and our results complement current knowledge with detailed analyses of the call types, namely the long, short, and frequency modulating calls in the 40kHz range of pup vocalizations (Brudzynski *et al*, 1999; Ming *et al*, 2011). While PolyI:C offspring made fewer calls overall, the number of long calls were especially decreased in MIA offspring compared to control animals. In addition, our findings of increased amphetamine-induced hyperlocomotion in adult MIA animals are consistent with others (Meyer *et al*, 2010; Vorhees *et al*, 2012; Zager *et al*, 2012; Zuckerman *et al*, 2003). On instrumental learning and pre-attentive processing, we found no differences between adolescent control and MIA animals in either CAR acquisition or PPI, though the first and second days of avoidance training suggested a lower performance in the MIA group. Various reviews have alluded to differences in MIA procedures and age of assessment as sources of discrepancies (Fortier *et al*, 2007; Meyer and Feldon, 2012). For example, Basta-Kaim *et al*, 2012 saw PPI deficits in offspring exposed to LPS only on P90,

but not P30, while Wolff and Bilkey, 2010 observed PPI deficits on P35 in offspring exposed to PolyI:C. In learning, Zuckerman *et al*, 2003 found LI disruption in 3 month-old offspring, but not P35. Meyer *et al*, 2006 further confirmed that behavioral inconsistencies may be attributed to MIA timing, as offspring from dams treated with PolyI:C on G6, 9, and 13 exhibited LI deficits, but not those on G17. These discrepancies suggest that while MIA is a valid preclinical model, different manipulations can produce variable results.

In this study, we demonstrated behavioral sensitization to repeated OLZ exposure in MIA offspring using the CAR assay, as we have previously shown in normal animals (Mead and Li, 2010; Qiao *et al*, 2013a; Sparkman and Li, 2012; Swalve and Li, 2012; Zhang and Li, 2012). As most patients on APD receive chronic treatments (Kalverdijk *et al*, 2008), and in recent years there has been a decrease in the age of treatment onset (Aparasu and Bhatara, 2007; Olfson *et al*, 2006; Rani *et al*, 2008; Wong *et al*, 2004), preclinical studies addressing adolescent chronic APD use are critical for translational values. We have shown previously that repeated APD exposure alters the behavioral sensitivity of animals to subsequent treatments (Gao and Li, 2013; Mead and Li, 2010; Qiao *et al*, 2013a; Qin *et al*, 2013; Swalve and Li, 2012), and that this phenomenon is persistent from adolescence to adulthood (Qiao *et al*, 2013a, 2013b). Here we further determined that OLZ sensitization is independent of the disease state of the animal, suggesting that long term neural changes can occur rapidly with repeated APD exposure in this population also.

Using a pharmacological approach, we previously identified that dopamine D_2 and serotonin (5HT) 2a receptors are involved in APD sensitization (Li *et al*, 2010, 2012; Qiao *et al*, 2014). The current study targeted hippocampal cell growth as a potential contributor. While APD are largely developed according to DA and 5HT receptor antagonism, changes in cell growth and survival may be of significance in enhancing therapeutic efficacy. Current literature regarding this issue remains inconsistent, perhaps due to discrepancies in paradigms, dosages, and assessment time points (Keilhoff *et al*, 2012). For example, Halim *et al*, 2004 found that 28 days of clozapine (CLZ) increased dentate gyrus (DG) cell proliferation in healthy animals, but not survival. In contrast, Maeda *et al*, 2007 showed that while 14 days of CLZ protected against PCP-impaired cell proliferation, CLZ alone produced no change. Here we identified correlations between cellular measures and OLZ sensitization. Namely, that sensitization is positively correlated with cell proliferation for MIA animals and negatively correlated with survival for SAL animals.

In the present work, the number of BrdU-labeled cells that survived for nine days in SAL animals correlated negatively with the magnitude of OLZ sensitization. Here, a larger decrease in avoidances over days corresponded with a smaller number of newborn cells that survived, suggesting that individual OLZ sensitivity is associated with drug-related changes in cell survival. In addition, the number of BrdU-labeled cells two days post injections correlated positively with the magnitude of avoidance suppression (i.e. OLZ sensitization in CAR) in MIA animals. Thus, a larger decrease in avoidances over days corresponded with a larger number of newborn cells, suggesting that individual OLZ sensitivity in MIA is associated with drug-related changes in cell growth. The increase in MIA cell proliferation echoes previous findings(Halim *et al*, 2004; Kodama *et al*, 2004). For example, Kodama *et al*, 2004 showed that 21 days of OLZ treatment in healthy subjects was able to increase cell

proliferation in the DG. Here, our concurrent examination of hippocampal cellular changes and behavioral responsiveness suggests that differential sensitivity to OLZ is associated with individual variability in cell growth and pruning. As patient evaluation studies such as Stroup, 2007 indicate that patients with schizophrenia exhibit diverse reactions to APD treatment, our observation highlights the role of cell proliferation and survival as a marker of individual sensitivity to APD.

So far only Piontkewitz *et al*, 2012b have explored the effects of APD on MIA hippocampal changes. While the group reported increased cell survival in MIA, the study assessed survival of cells born post-APD treatment and at three weeks post BrdU injection, thus differing from our cell survival throughout the drug treatment period. Our result of a positive correlation between the magnitude of OLZ behavioral sensitization and MIA hippocampal cell proliferation suggests possible differences in adolescent brain response to repeated OLZ exposure between disease and healthy animals despite similar behavioral outputs. Indeed, while Piontkewitz *et al*, 2012b did not examine cell proliferation, the authors found that risperidone differentially affected 21-day cell survival between adolescent MIA and control animals. The underlying mechanism of how APD treatment alters MIA cell proliferation and survival is unknown, and our findings suggest the increase in hippocampal cell proliferation as a possibility.

Various studies have considered the effects of APD on cell survival under different durations – 14, 21, or 28 days. The duration of nine days was fitting for this study as it covered only the CAR assessment and drug treatment period. This prevented potential environmental confounds post OLZ treatment and CAR testing. While no parametric study exists regarding the progression of hippocampal cell survival over time under the influence of APD, it is likely that APD related changes in cell survival vary over time.

One limitation of this study is the uncertainty of the phenotypes of BrdU+ cells. While the focus here was not to determine hippocampal cell proliferation and survival specific to the neuronal lineage, it is important to remember that hippocampal stem cells not only give rise to neurons, but various supporting cell types including oligodendrocytes and endothelial cells (Kodama *et al*, 2004; Wang *et al*, 2004). Some above-mentioned studies investigating APD and hippocampal alterations have found that most BrdU+ cells are also NeuN+. However, studies have also suggested that disease mechanisms of SZ may involve deficits in non-neuronal cells (Benes and Berretta, 2001; Zhang and Reynolds, 2002), lending the notion that proliferation of all cell types may contribute to APD therapeutic efficacy.

In summary, results from the current study provided clinically relevant information. First, we verified that OLZ behavioral sensitization occurs in a disease model of SZ, confirming that this phenomenon is irrespective of disease state and offers a reference in creating therapeutic regimen. Secondly, the association between the magnitude of behavioral sensitization and hippocampal cell proliferation and survival provides novel additional insight regarding mechanisms associated with APD sensitization. Further investigation into whether hippocampal cell proliferation and pruning are necessary and sufficient to induce APD sensitization and generate increased therapeutic efficacy may lead to superior pharmacological management.

METHODS

Animals

Sixty-eight pregnant Sprague-Dawley dams from Charles River (Portage, MI; gestation day [G] 6 on delivery date) and their offspring were used. Pregnant dams were single housed in 48.3 × 26.7 × 20.3 cm transparent polycarbonate cages under 12h light/dark conditions (light on between 0630 and 1830 h). Rat pups remained with dams until weaning (P21), upon which they were separated and housed two per cage in 182 × 50 × 188.1 cm transparent polysulfone individually ventilated cages. Room temperature was maintained at 22 ± 1°C with a relative humidity of 45–60%. Food and water was available *ad libitum*. All experiments were run during the light cycle. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln.

Drugs and choice of doses

Polyinosinic:polycytidylic acid (PolyI:C, 4.0 mg/kg, intravenous [iv]; Sigma Aldrich, St. Louis, MO) was dissolved in 0.9% saline. This dose of PolyI:C was chosen based on previous studies using the same dose and route of administration to generate schizophrenia-like phenotypes (Piontkewitz *et al*, 2009, 2012a, 2012b; Wolff and Bilkey, 2008, 2010; Yee *et al*, 2012; Zuckerman and Weiner, 2005; Zuckerman *et al*, 2003). Olanzapine (OLZ) 0.5, 1.0, and 2.0 mg/kg (subcutaneous [sc]; gifts from the NIMH drug supply program) were dissolved in distilled sterile water with 1.0–1.5% glacial acetic acid. The 1.0 and 2.0 mg/kg OLZ acutely inhibit CAR without inducing catalepsy based on our previous work (Feng *et al*, 2013; Li *et al*, 2004, 2007, 2009; Mead and Li, 2010; Qiao *et al*, 2013a, 2013b; Sun *et al*, 2009; Zhang and Li, 2012; Zhao and Li, 2010). The lower challenge dose of 0.5 mg/kg OLZ has been successfully used in our previous studies (Mead and Li, 2010; Qiao *et al*, 2013a; Sparkman and Li, 2012; Swalve and Li, 2012; Zhang and Li, 2012) and prevents the floor effect (i.e. a high dose may cause maximal avoidance disruption and obscures the sensitization effect). The injection solution of amphetamine (1.5mg/kg, sc; Sigma Aldrich, St. Louis, MO) was obtained by mixing the drug with 0.9% saline. The dosage was chosen based on previous work showing robust hyperlocomotion without severe stereotypy (Sun *et al*, 2009). PolyI:C, OLZ and amphetamine were all administered at 1.0 ml/kg. 5′-bromodeoxyuridine (BrdU, 75 mg/kg, ip; Sigma Aldrich, St. Louis, MO) was dissolved in 0.9% saline (10 mg/ml) and administered at 7.5 ml/kg. This dosage and administration protocol of BrdU was chosen based on previous studies indicating clear labeling of dividing cells (Halim *et al*, 2004; Maeda *et al*, 2007; Wang *et al*, 2004).

Prenatal treatment

Prenatal PolyI:C treatment was performed on G15, when pregnant dams were anesthetized with 3% isoflurane (Fisher Scientific, Denver, CO) in 98% O_2 and given a single iv injection at the tail vein. Weight changes of pregnant dams were measured and recorded one day post PolyI:C administration. For control comparison, half of the G15 pregnant dams were injected with saline at the tail vein, creating offspring that served as control animals without prenatal immune activation.

Two-way avoidance conditioning apparatus

Eight identical two-way shuttle boxes (Med Associates, St. Albans, VT) were used. Each box (64 cm W × 24 cm D × 30 cm H from grid floor) was housed in a ventilated, sound-insulated isolation cubicle (96.52 cm W × 35.56 cm D × 63.5 cm H). Each box was divided into two equal-sized compartments by a partition with an arch style doorway (15 cm H × 9 cm D at base) and a barrier (4 cm H from grid floor) so that rats had to jump in order to cross from one compartment to the other. The grid floor consisted of 40 stainless-steel rods measuring 0.48 cm in diameter spaced 1.6 cm apart center to center, through which a scrambled footshock (unconditioned stimulus [US], 0.8mA, maximum duration: 5 s) was delivered by a constant current shock generator (Model ENV-410B) and scrambler (Model ENV-412). The rat location and crossings between compartments were monitored by a set of 16 photobeams (ENV-256-8P) affixed at the bottom of the box (3.5 cm above the grid floor). Illumination was provided by two houselights mounted at the top of each compartment. The conditioned stimulus (CS; i.e. 76 dB white noise) was produced by a speaker (ENV 224 AMX) mounted on the ceiling of the cubicle, centered above the shuttle box. Background noise (~74 dB) was provided by a ventilation fan affixed at the top corner of each isolation cubicle. All training and testing procedures were controlled by Med Associates programs running on a computer.

Pup ultrasonic vocalization (USV) recording apparatus

In each CAR box, an USV microphone (P 48/Emkay Microphone, Avisoft Bioacoustics, Berlin, Germany) was mounted on the ceiling of the two-compartment chamber. The microphone was connected via an E-MU 0404 USB Audio device to a computer. Acoustic data were displayed in real time by the Avisoft RECORDER, a multi-channel triggering hard-disk recording software (version 3.4; Avisoft Bioacoustics), and were recorded at a sampling rate of 192 kHz in 16 bit format. For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (Version 4.51) and a fast Fourier transformation (FFT) was conducted. Spectrograms were generated with an FFT-length of 256 points and a time window overlap of 50% (100% Frame, FlatTop window). The spectrogram was produced at a frequency resolution of 750 Hz and a time resolution of 0.6667 ms. Call detection was provided by an automatic single threshold-based algorithm (threshold: −20 dB) and a hold-time mechanism (hold time: 0.02 s).

Prepulse inhibition (PPI) of acoustic startle response (ASR) apparatus

The PPI test was performed using six Startle Monitor Systems (Kinder Scientific, Julian, CA). Each system, controlled by a PC, was housed in a compact sound attenuation cabinet (36 cm wide × 28 cm deep × 50 cm high). A speaker (diameter: 11 cm) mounted on the cabinet's ceiling was used to generate acoustic stimuli (70 dB-120 dB). The startle response was measured by a piezoelectric sensing platform on the floor, which was calibrated daily. During testing, rats were placed in a rectangular box made of transparent Plexiglas (19 cm wide × 9.8 cm deep × 14.6 high) with an adjustable ceiling positioned atop the box, providing only limited restraint while prohibiting ambulation.

Locomotor activity monitoring apparatus

Sixteen 48.3 cm × 26.7 cm × 20.3 cm transparent polycarbonate activity boxes were housed in a quiet room. Each was equipped with a row of 6 photocell beams (7.8 cm between two adjacent photobeams) placed 3.2 cm above the floor of the cage. A computer with recording software (Aero Apparatus Sixbeam Locomotor System v1.4) was used to detect the disruption of the photocell beams and recorded the number of beam breaks.

Experiment 1: Assessment of MIA behavioral alterations during early postnatal period and adulthood

To establish the validity of our MIA model, we examined offspring behavior in two behavioral assays that have previously been reported to show abnormal phenotypes by MIA: pup USV (Malkova *et al*, 2012) and adult amphetamine-induced hyperlocomotion (Piontkewitz *et al*, 2011).

Pup USV induced by maternal separation—Pup USVs serve as an important social communication tool from pups to mothers. MIA is reported to cause an alteration in this behavior (Malkova *et al*, 2012). This experiment was designed to validate this behavioral abnormality associated with MIA. The procedure was similar to that previously used in our lab (Ming *et al*, 2011). Experimentally naïve rats (1 male and 1 female pup; SAL, n=50; MIA, n=50) from each litter was used on P11 for the experiment. Pups were separated from their dams and placed in plastics bowls (one pup per bowl, 14.5 and 9.0 cm wide at the top and bottom, 7.5 cm high) on heating pads (~35°C). Twenty minutes later, the bowls and pups were placed in the CAR chambers directly underneath the microphone. USV were recorded for 3 minutes. For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (Version 4.51) and a fast Fourier transformation (FFT) was conducted. Spectrograms were generated with an FFT-length of 256 points and a time window overlap of 50% (100% Frame, FlatTop window). The spectrogram was produced at a frequency resolution of 750 Hz and a time resolution of 0.6667 ms. Call detection was provided by an automatic dual threshold-based algorithm and a hold-time mechanism (hold time: 0.02 s). The primary threshold was defined at −40 dB, while the start/end threshold was defined at −20 dB. Pup USV were classified into three types by a custom-built computer program. “Long” USV were defined as waveforms that were greater than 50 ms long, with a frequency deviation of less than 3 kHz. “Short” USV weny waveforms that were less than 50 ms long. “Frequency-modulated” USV were greater than 50 ms long and had a frequency deviation of greater than 3 kHz. A comparison between this automatic quantification method and human scoring on four randomly selected tests revealed a high degree of agreement, with a Pearson’s product moment correlation ranging from 0.979 to 0.998.

Adult amphetamine-induced hyperlocomotion—Sensitivity to amphetamine is elevated in adult MIA offspring as a measure of hyperDAergic function (Piontkewitz et al., 2011). This experiment examined possible differences between prenatal treatment conditions in amphetamine-induced hyperlocomotion during adulthood. Separate groups of male and female adult offspring that were not used in OLZ testing were tested on P84, which also include some rats tested for pup USVs. The procedure was similar to that previously used in our lab (Sun *et al*, 2009). Rats were first habituated to the locomotor

activity apparatus for 30 min. They were then taken out of the activity monitoring boxes, injected with 0.9% saline and replaced immediately back into the boxes. At the end of another 30 min period, rats were taken out and injected with amphetamine and placed back in the boxes for another 60 min. Locomotor activity (number of photobeam breaks) was measured and recorded in 5 min intervals throughout the entire 120 min testing session.

Experiment 2: Evaluation of OLZ sensitization and hippocampal cell proliferation and survival in the conditioned avoidance response test using MIA and control animals

This experiment examined whether OLZ sensitization was differentially expressed in the MIA and saline male rats and whether cell proliferation in the dentate gyrus (DG) was altered by OLZ exposure to mediate OLZ sensitization in the CAR test. No more than 2 male pups from each litter were used in this experiment in order to minimize litter effects (Zorrilla, 1997). The overall experimental procedure was similar to that previously reported (Mead and Li, 2010; Qiao *et al*, 2013a; Swalve and Li, 2012) and consisted of four phases: *avoidance training, post training PPI assessment, induction of OLZ sensitization, OLZ sensitization assessment*, and cell proliferation and survival assay by BrdU immuohistochemistry

Avoidance training—Adolescent Sprague-Dawley males (P31) were first habituated to the CAR boxes for 2 days (30min/day) and then trained for conditioned avoidance responding for 10 consecutive days/sessions. Each session consisted of 30 trials. Every trial started by presenting a white noise (CS) for 10s, followed by a continuous scrambled foot shock (0.8 mA, US, maximum duration = 5 s) on the grid floor. An avoidance response was recorded if the subject crosses into the adjacent compartment within the duration of the CS presentation. An escape response was recorded if the subject crosses into the adjacent compartment during the US presentation. If the rat made no response throughout both CS and US, the trial was terminated and recorded as an escape failure. The total number of avoidance responses was recorded for each session. Inter-trial intervals varied randomly between 30 and 60s.

PPI of ASR assessment—One day after the CAR training (P43), rats were tested for a single session of PPI in order to examine possible differences between prenatal treatment conditions. Rats were placed in the PPI boxes and tested according to procedures adapted from Culm and Hammer, 2004. The PPI session lasted approximately 18 min and began with a 5 min period of 70 dB background noise that continued throughout the duration of the session. The 5 min background was followed by four different trial types: PULSE ALONE trials and three types of PREPULSE+PULSE trials, which consisted of a 20 ms 73, 76, or 82 dB prepulse (3, 6, and 12 dB above background) followed 100 ms later by a 120 dB pulse. The session was divided into 4 blocks. Blocks 1 and 4 were identical, each consisting of 4 PULSE ALONE trials. Blocks 2 and 3 were also identical and each consisted of 8 PULSE ALONE trials and 5 of each PREPULSE+PULSE trial type. A total of 54 trials were presented. Trials within each block were presented in a pseudorandom order and were separated by a variable intertrial interval averaging 15 s (ranging from 9 to 21 s). Startle magnitude of the acoustic startle response (ASR) was defined as the maximum force (measured in Newtons) applied by the rat to the startle apparatus recorded over a period of

100 ms beginning at the onset of the pulse stimulus. Between each stimulus trial, 100 ms of activity was recorded when no stimulus was present. These trials were called NOSTIM trials and were not included in the calculation of intertrial intervals. Responses recorded during NOSTIM trials are considered a measure of gross motor activity within the PPI boxes. Startle responses from testing blocks 2 and 3 were used to calculate percent prepulse inhibition (%PPI) for each acoustic prepulse trial type:

$$\%\mathrm{PPI}=100-\left[\left(\frac{\text{average startle response to PREPULSE+PULSE trials}}{\text{average startle response to PULSE ALONE trials}}\right)\times 100\right]$$

Repeated OLZ testing in CAR (i.e. induction phase of sensitization) (P 44–48)—Following PPI assessment (P44), rats were assigned to one of three drug treatment groups: vehicle (VEH, 1% glacial acetic acid in sterile water), OLZ 1.0 mg/kg, and OLZ 2.0 mg/kg and tested daily for avoidance response for 5 consecutive days. Rats were assigned such that offspring from the same litter were placed in different groups to minimize litter. The complete factorial design thus consisted of two prenatal treatments (MIA and saline offspring) × three drug doses (VEH, OLZ 1.0 mg/kg, and OLZ 2.0 mg/kg), with the saline offspring treated with vehicle during adolescence serving as the main control comparison group. The CS-only (no shock, 30 trials/daily session) condition was used to eliminate any relearning effect caused by the presence of the US. During each drug test, rats were first injected with OLZ or VEH and placed in the CAR boxes and tested 1 h later.

OLZ challenge test (i.e. expression phase of sensitization) (P51)—At the end of the drug test session (P49) all rats were retrained drug-free for two days, the first under CS-only condition (no shock) and the second under CS-US, to ensure all groups had a comparable level of avoidance responding before the sensitization assessment. OLZ sensitization was assessed 1 day later (P51), when all rats were injected with a challenge dose of OLZ 0.5 mg/kg and tested for avoidance performance 1 h later.

BrdU immunohistochemistry for hippocampal cell proliferation and survival—The BrdU immunohistochemistry technique was modified based on Cameron, 2006. Half of the animals in each prenatal and drug treatment group received 3 BrdU injections spaced 2 h apart following the PPI assessment (P43), while the other half received the injections on the first CAR retraining day (P49). The P43 injection allowed for short term cell survival assessment as the animals were sacrificed nine days later, while the P49 injection allowed for cell proliferation assessment as the animals were sacrificed after 48 hours. BrdU is a synthetic thymidine analogue preferentially incorporated into actively replicating cells. It is commonly used as a measure of cell proliferation as well as cell survival depending on the duration between BrdU injection and the time of examination of BrdU immunoreactivity in tissues (Gratzner, 1982).

The time points chosen in the current study is based on two considerations. First, for cell proliferation, we chose to inject BrdU 48 hours prior to sacrifice, one day after the last day of repeated OLZ administration, and animals were immediately sacrificed after the final OLZ challenge test. This allows for examination of how repeated OLZ treatment affects cell

proliferation without the contamination of the acute effect of OLZ exposure in the last challenge, as the time between challenge and sacrifice is too short for complete mitosis. Secondly, for cell survival, we chose to inject BrdU 9 days prior to sacrifice, one day prior to the start of repeated OLZ administration. This allows for examination of how repeated OLZ treatment affects cell survival without the contamination of other environmental factors post repeated treatment to alter cell survival. The durations were also chosen based on a parametric study by Dayer *et al*, 2003. The study concluded that BrdU signals two days post injection were highly co-expressed with Ki-67, a marker present in cells in all active phases of the cell cycle, and also frequently used as a marker of cell proliferation (Kee *et al*, 2002; Muskhelishvili *et al*, 2003). This suggests that BrdU+ cells identified two days post BrdU injection are representative of actively dividing cells (i.e. cell proliferation). The study also concluded that BrdU signals eight days post injection were not co-expressed with Ki-67, indicating that these cells were no longer in the mitotic phase and not representative of newly added daughter cells. This suggests that BrdU+ cells identified at eight days (nine in the current study) post BrdU injection are representative of cells that survived after leaving the cell cycle.

Following OLZ sensitization challenge test (P51), all rats were deeply anesthetized with sodium pentobarbital (100.0 mg/kg, ip) and transcardially perfused with 0.02M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA, pH 7.4) in 0.1M PBS. Brains were then harvested, post-fixed in 4% PFA overnight and cryoprotected in 30% sucrose for 72 hours. Serial coronal sections (35μm) of the hippocampus (Bregma −2.0 to −4.5; Paxinos and Watson, 2007) were cut on a cryostat, and every twelfth section in series (6 sections per animal) were processed for BrdU labeling. Free floating sections were incubated in 2M HCl at 37°C for 30 min, followed by two 15 min incubations of 40mM borate buffer. Sections were then incubated in blocking solution (0.3% Triton X-100 and 10% normal horse serum in 0.02M PBS) for 1 h, followed by 3% H_2O_2 with 100% methanol for 30 min, and then in primary antibody at 4°C overnight (mouse anti-BrdU, 1:15,000; Millipore, Billerica, MA). Secondary antibody (biotinylated horse anti-mouse IgG, 1:500; Vector Laboratories, Burlingame, CA) incubated sections for 1 h, followed by avidin-biotinylated enzyme complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for another hour. 3,3′-diaminobenzidine (DAB peroxidase substrate, Vector Laboratories, Burlingame, CA) was used to reveal BrdU labeling. Normal horse serum substitution for primary antibody was used as negative control.

Cell counting was conducted by an experimenter blinded to the conditions. Cells were viewed with an Olympus CX41RF microscope (Japan) under 200× magnification, and those with clear delineation of staining in the granule cell layer of the hippocampal DG were counted. A modified stereological procedure adapted from previous reports (Cameron and McKay, 2001; Eisch *et al*, 2000; Gould *et al*, 1999; Malberg *et al*, 2000; West *et al*, 1991) was used for analysis, which consisted of counting all BrdU-labeled cells bilaterally for each section, then multiplying the sum of the 6 sections by 12 in order to estimate the total number of BrdU+ cells per DG.

Statistical analysis

All data were expressed as mean ± standard error of mean. One way analysis of variance (ANOVA) was used to compare pregnant dam weight changes one-day after PolyI:C or saline treatment. Repeated measures two-way ANOVA (between-subjects factor: prenatal treatment; within-subjects factor: call type) was conducted on pup USV. Repeated measures two-way ANOVA (between-subjects factor: prenatal treatment or drug groups; within-subjects factor: days) was also used for CAR training and drug test sessions. In addition, percent PPI data for the 3 prepulse intensities (e.g. 73, 76 and 82 dB) were analyzed with repeated measures two-way ANOVA (between-subjects factor: prenatal treatment; within-subjects factor: prepulse types). Data from CAR retraining, sensitization assessment, and cell counting were analyzed by multi-factorial ANOVA (prenatal treatment × drug groups). Pearson product-moment correlational analysis was conducted between percentage of avoidance suppression, as assessed by calculating the percent decrease in the number of avoidances made during either day two of drug administration or challenge day compared to day one of drug administration, and number of BrdU+ cells. All significant differences were followed by *post hoc* LSD tests. For all analyses, $p < 0.05$ was considered statistically significant.

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Highlights

1. Adolescent olanzapine use causes sensitization in immune activated offspring.
2. Sensitization magnitude correlates negatively with cell survival in controls.
3. Magnitude correlates positively with cell growth in immune activated offspring.

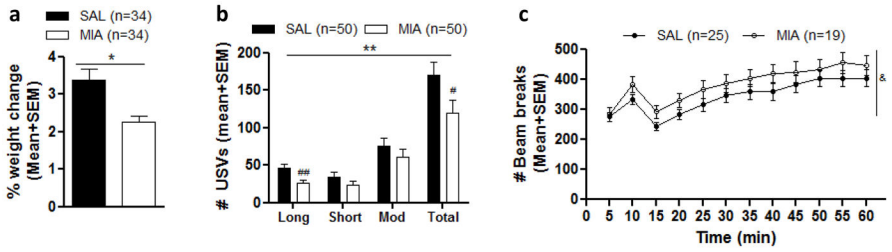

Figure 1.

Assessment of MIA induced alterations in infancy and adulthood. Percentage of pregnant dam weight changes one day following saline (SAL) or PolyI:C (MIA) treatment on G15 (a). Maternal separation induced pup ultrasonic vocalizations on P11 (b). Numbers of ultrasonic vocalizations for each call type (short, long, frequency modulating [mod], total) made by offspring from the prenatal SAL and MIA treatment groups. Amphetamine-induced hyperlocomotion on P84 (c). Mean locomotor activities measured as numbers of photobeam breaks during the 60-min test period after the amphetamine injection made by offspring from the prenatal SAL and MIA treatment groups. All data are expressed as mean ± SEM. *p<0.005 and &p=0.054 between prenatal treatment conditions; **p<0.001 between call types; ##p<0.005 and #p<0.05 relative to the saline group.

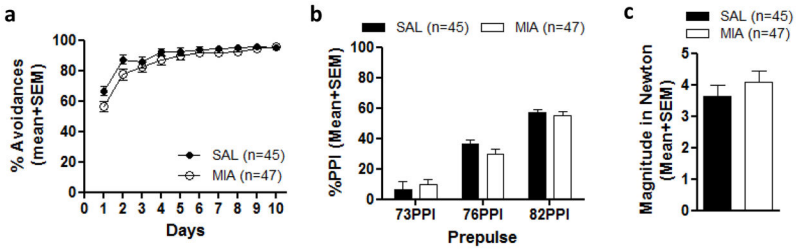

Figure 2.

Conditioned avoidance response acquisition. Percentages of avoidance responses made by offspring from the prenatal SAL and MIA treatment groups during the ten days of conditioned avoidance training (a). Prepulse inhibition (b) and acoustic startle response (c) assessment post conditioned avoidance response training. All data are expressed as mean ± SEM. *p<0.05 between prenatal treatment conditions.

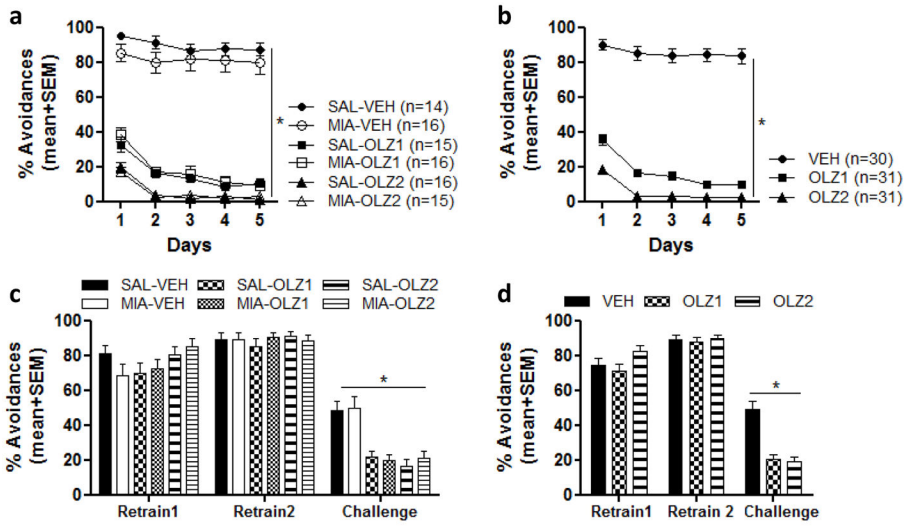

Figure 3.

Olanzapine sensitization and assessment. Repeated olanzapine administration in conditioned avoidance response (a,b). Percentages of avoidance responses made by rats from each drug group (VEH, OLZ 1.0, and 2.0 mg/kg) in the two separate prenatal treatment conditions (a, saline [SAL] and PolyI:C [MIA]) and with the prenatal conditions combined (b) throughout the five drug test days. Assessment of olanzapine sensitization in conditioned avoidance response (c,d). Percentages of avoidance responses made by rats from each drug group (VEH, OLZ 1.0 and 2.0 mg/kg) in the two separate prenatal treatment conditions (c, SAL and MIA) and with the prenatal conditions combined (d) on the retraining days and the OLZ sensitization assessment day. All data are expressed as mean ± SEM. *p<0.001 between drug treatment conditions.

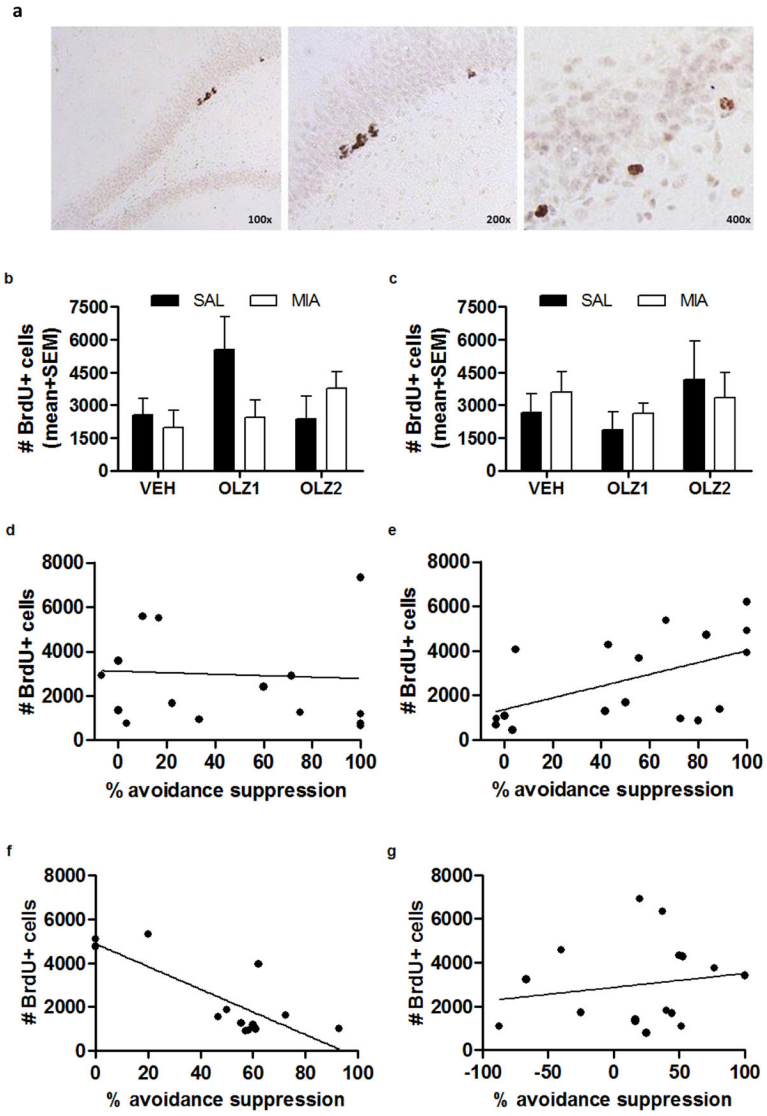

Figure 4.

BrdU analysis of olanzapine associated cell proliferation and survival. Sample micrograph of BrdU+ cells (dark brown) in the granule cell layer of the hippocampal dentate gyrus (a). Assessment of cell proliferation (b). Estimated numbers of BrdU+ cells throughout the entire dentate gyrus for animals in each prenatal and drug treatment condition treated with BrdU two days prior to sacrifice. Assessment of cell survival (c). Estimated numbers of BrdU+ cells throughout the entire dentate gyrus for animals in each prenatal and drug treatment condition treated with BrdU nine days prior to sacrifice. All data in b and c are expressed as mean ± SEM. Correlational analyses of olanzapine sensitization and cell proliferation in the hippocampal dentate gyrus (d,e). Number of BrdU+ cells two days post BrdU injection was positively associated with the percent of avoidance suppression induced by olanzapine in MIA offspring, r=0.527, *p*=0.030 (e). Correlational analyses of olanzapine sensitization and cell survival in the hippocampal dentate gyrus (f, g). Number of BrdU+ cells nine days post

BrdU injection was negatively associated with the percent of avoidance suppression induced by olanzapine in SAL offspring, $r = -0.910$, $p < 0.001$ (f).