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OLANZAPINE ANTIPSYCHOTIC TREATMENT OF ADOLESCENT RATS CAUSES LONG TERM CHANGES IN GLUTAMATE AND GABA LEVELS IN THE NUCLEUS ACCUMBENS

Su Xu¹, Rao P. Gullapalli¹, and Douglas O. Frost^{2,3}

¹Dept. of Diagnostic Radiology and Nuclear Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, USA

²Dept. of Pharmacology, University of Maryland School of Medicine, Baltimore, MD 21201, USA

³Dept. of Psychiatry, University of Maryland School of Medicine, Baltimore, MD 21201, USA

Abstract

Atypical antipsychotic drugs (AAPDs) are widely used in children and adolescents to treat a variety of psychiatric disorders. However, little is known about the long-term effects of AAPD treatment before the brain is fully developed. Indeed, we and others have previously reported that treatment of adolescent rats with olanzapine (OLA; a widely prescribed AAPD) on post-natal days 28–49, under dosing conditions that approximate those employed therapeutically in humans, causes long-term behavioral and neurobiological perturbations.

We have begun to study the mechanisms of these effects. Dopamine (DA) and serotonin (5HT) regulate many neurodevelopmental processes. Currently approved AAPDs exert their therapeutic effects principally through their DAergic activities, although in schizophrenia (SZ) and some other diseases for which AAPDs are prescribed, DAergic dysfunction is accompanied by abnormalities of glutamatergic (GLUergic) and γ -aminobutyric acidergic (GABAergic) transmission. Here, we use proton magnetic resonance spectroscopy (¹H MRS) to demonstrate long-term reductions in the levels of both GLU and GABA in the nucleus accumbens (NAc) of adult rats treated with OLA during adolescence. The NAc is a key node in the brain's "reward" system, whose function is also disrupted in schizophrenia. Further research into potential, OLA-induced changes in the levels of GLU and GABA in the NAc and other brain areas, and the dynamics and mechanisms of those changes, are an essential step for devising new adjunct therapies for existing AAPDs and for

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Corresponding Author: Prof. Douglas Frost, Dept. of Pharmacology, University of Maryland School of Medicine, 655 West Baltimore St., Baltimore, MD 21201, Tel: 1-410-706-0413, FAX: 1-410-706-0032, dfrost@umaryland.edu.

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designing new drugs that increase therapeutic effects and reduce long-term abnormalities when administered to pediatric patients.

Keywords

antipsychotic; development; neuroplasticity; magnetic resonance spectroscopy; reward system

1. INTRODUCTION

There is increasing awareness among the clinical and research communities of the potential for long-term sequelae arising from psychotropic medication of pediatric patients. Antidepressants have been most widely studied in this regard (Oberlander et al., 2009; Simpson et al., 2011). Atypical antipsychotic drugs (AAPDs), including OLA, are commonly prescribed to treat multiple psychiatric disorders in pediatric patients (Cheng-Shannon et al., 2004; McCracken et al., 2008; McKnight and Park, 2010; Sikich, 2008; Strawn and Delbello, 2008; Weller and Weller, 2009). Patients with psychotic illness often require long-term maintenance therapy, but most pediatric AAPD treatment is for other, off-label indications (Alexander et al., 2011; Scheltema Beduin and de Haan, 2010), for which consensus guidelines (Pappadopulos et al., 2003; Pappadopulos et al., 2011; Thomas et al., 2011) recommend trial off-medication after stability is achieved. Although short-term tolerance of AAPDs is reasonably good in adult and pediatric patients (Zuddas et al., 2011), the long-term sequelae that follow cessation of AAPD treatment in pediatric patients are unknown (Cheng-Shannon et al., 2004).

Although AAPDs vary in the spectra of their pharmacologic actions, most AAPDs potentially inhibit dopamine type 2 (D2) family receptor function; most AAPDs have a broad spectrum of other activities, most commonly, antagonism of serotonergic (5HTergic) transmission (Baldessarini, 2001; Bymaster et al., 1996; Yadav et al., 2011). Neurotransmitters, including glutamate (GLU), γ -aminobutyric acid (GABA), DA and 5HT, also regulate a variety of neurodevelopmental processes (Azmitia, 2001; Frost and Cadet, 2000; Hensch, 2005; Huang, 2009; Levitt et al., 1997; Lu et al., 2009; Moreau and Kullmann, 2013; Stoneham et al., 2010). Thus, even time-limited AAPD therapy can, potentially, alter the subsequent developmental trajectory of the brain, resulting in long-term abnormalities of brain function and behavior. The DAergic system (Wahlstrom et al., 2010) and other cerebral substrates upon which AAPDs act continue to undergo pronounced ontogenetic changes into the third decade in humans (Giedd et al., 2009; Petanjek et al., 2011). Rodents used most commonly in pre-clinical studies of drug and disease mechanisms undergo parallel changes on a greatly compressed time scale. Based on measures of brain development, the rat brain reaches maturity by ~90 days of age. The protracted development of the brain raises the possibility that the behavioral and neurobiological sequelae of early life AAPD therapy vary with age of treatment and/or the age at which effects are assessed. This underscores the necessity of independently evaluating the enduring sequelae of AAPD treatment at different ontogenetic stages.

Animal models have begun to shed light on these issues. In the mature brain, DAergic signaling is one of the mechanisms of cognition, learning and memory (Kehagia et al., 2010; Robbins and Arnsten, 2009). AAPDs administered to adolescent rodents at doses that produce D2 receptor occupancies in the human therapeutic range result in a variety of long-term behavioral and neurobiological effects. We (Milstein et al., 2013; Vinish et al., 2013) and others (Llorente-Berzal et al., 2011) have recently shown that adult rats treated with OLA at these doses during adolescence exhibit abnormalities in a subset of learning tasks that are sensitive to DAergic function. These rats also exhibit changes in DAergic transmission, and in the organization and function of neuronal networks, in several brain regions that are mediators of the affected behaviors (Milstein et al., 2013; Vinish et al., 2013) (also, Brooks, O'Donnell and Frost, manuscript in preparation).

The DAergic, GLUergic and GABAergic systems are disrupted in SZ and other disorders with a psychotic component (Egerton and Stone, 2012; Eyles et al., 2012; Howes and Kapur, 2009; Lisman et al., 2008; Szulc et al., 2013). They and the 5HTergic system mutually interact and are direct or indirect targets of the AAPDs used to treat those disorders (Del Arco and Mora, 2005, 2008; Di Pietro and Seamans, 2007; Gao et al., 2003; Howes and Kapur, 2009; Kapur and Remington, 1996; Lisman et al., 2008; Loskutova, 2001; Marek, 2007; Missale et al., 2006; Sun and Wolf, 2009; Wang et al., 2002; West et al., 2003). These interactions can include the formation of heterodimers consisting of receptors for different transmitters (de Bartolomeis et al., 2013; Missale et al., 2010). Indeed, abnormalities of GLUergic transmission can induce a cascade of events that lead to disruption of DAergic and GABAergic signaling and, in humans and animal models, phenotypes of psychosis and cognitive dysfunction (Egerton and Stone, 2012; Howes and Kapur, 2009; Lisman et al., 2008; O'Donnell, 2012; Szulc et al., 2013). Therefore, we hypothesized that in adult rats treated with OLA as adolescents, GLU and GABA levels should be abnormal in brain regions in which we demonstrated long-lasting alteration of DAergic transmission.

We emphasize that the present experiments are aimed at understanding the mechanisms by which adolescent OLA treatment exerts its long-lasting, behavioral effects, rather than at elucidating the mechanisms by which the drug exerts its therapeutic effects in psychiatric patients. In this study, we focused on the NAc because: 1) we have shown that conditioned place preference (a task of which the NAc is a key mediator) is altered in mature rats following adolescent OLA treatment (Vinish et al., 2013); 2) adolescent OLA treatment induces enduring changes of DA release and DA receptor binding in the NAc (Vinish et al., 2013); 3) in the NAc, there are important interactions among the GLUergic, GABAergic and DAergic systems (Floresco et al., 2003; Goto and O'Donnell, 2003; Huppé-Gourges and O'Donnell, 2012; Huppé-Gourges and O'Donnell, 2012); 4) some of these interactions are ontogenetically modified between pre-pubertal stages and maturity (Benoit-Marand and O'Donnell, 2008; Huppé-Gourges and O'Donnell, 2012; Huppé-Gourges and O'Donnell, 2012), thus rendering the developmental trajectory of these systems susceptible to modification by environmental stimuli (including drugs) during adolescence (review in Andersen and Navalta, 2011); 5) indeed, at least one interaction between the DAergic and GABAergic systems in the NAc matures abnormally as a result of adolescent OLA treatment (Brooks, O'Donnell and Frost, manuscript in preparation) and may be one mechanism by which OLA exerts some of its enduring behavioral effects. These facts led us

to hypothesize that adolescent OLA treatment would alter levels of GABA and/or GLU in the NAc.

2. METHODS

2.1 Subjects

We studied male, colony-bred Long-Evans rats (breeding stock obtained from Charles River, Wilmington, MA). Litters were culled to 10–12 pups on postnatal day 7 (first 24h after birth = P0). On P21, subjects were weaned and pair- or triple-housed with same-sex littermates. Rats were maintained under standard laboratory conditions on a light cycle of 14h light/ 10h dark. Water and food were available *ad libitum*.

2.2 Drug treatment

In the present experiments, on P28–49 the drinking water was replaced with an aqueous solution of OLA in 1 mM acetic acid or vehicle (VEH), alone (8 rats/ treatment group). The OLA solution was mixed fresh daily at a concentration calculated to deliver a target dose of 7.5 mg/kg/d, based on the weights and water consumption of the rats over the previous 24h. This regimen achieved ~96% of the target dose. On P49, all rats were switched to normal drinking water. We used this procedure because; 1) it produces plasma OLA concentrations that result in D2 receptor occupancies in the human therapeutic range (65–80%; Kapur et al., 2003; Vinish et al., 2013); 2) it reproduces as closely as possible the clinical administration of AAPDs to humans and avoids difficulties associated with other treatment procedures (Vinish et al., 2013); 3) it induces long-lasting cognitive changes and neurobiological alterations in behaviorally relevant brain regions (Llorente-Berzal et al., 2011; Milstein et al., 2013; Vinish et al., 2013). Although we did not perform formal tests for catalepsy during treatment, the ED50 doses (Kolaczowski et al., 2014) and D2 receptor occupancies (Johnson et al., 2014) for OLA and other atypical antipsychotics to induce catalepsy and other predictors of extrapyramidal effects in rats are consistently higher than those required to produce behavioral changes that are predictive of antipsychotic therapeutic efficacy. Indeed, in our regular handling of numerous rats during and after adolescent OLA treatment, they all appeared to be normally active and responsive to external stimuli.

2.3 *In vivo*, high resolution ^1H MRS

In vivo ^1H MRS experiments were performed on P90–120, when the rats were fully mature, young adults. All experiments were performed on a Bruker Biospec 7.0 Tesla 30 cm horizontal bore scanner, interfaced to a Bruker Paravision 5.1 console. A four-element ^1H surface coil array was used as the receiver and a 72 mm linear-volume coil as the transmitter. Rats were initially anesthetized using isoflurane (3–4% in O_2 ; 1L/ min), then placed prone in a Bruker animal bed. The RF receiver coil was then centered over the brain and the animal bed was moved to the center of the magnet. An MR-compatible system was used to monitor respiration rate (which was maintained at 55–70 breaths/min by adjusting the isoflurane level between 1.5 and 2.0 %) and body temperature was maintained at 36–37°C, using a circulating warm water heater. At the completion of the imaging procedure, the rats were taken off anesthesia and warmed until they were fully reanimated. All

protocols were approved by the Institutional Animal Care and Use Committees of the University of Maryland Baltimore.

A scout image consisting of three slices (one each in the axial, mid-sagittal, and coronal planes) was used to center the rat's brain in the imaging field of view. A rapid shimming protocol (FASTMAP) was used to reduce external magnetic field inhomogeneity within the region of interest. (Gruetter, 1993). Both proton density- and T₂-weighted images were obtained using 2D rapid acquisition with a relaxation enhancement (RARE) sequence for anatomic reference. The sequence parameters were: repetition time/effective echo time (TR/TE_{eff1}/TE_{eff2}) = 5500/18.9/56.8 ms, number of echoes = 4, matrix size = 256 × 256, slice thickness = 1 mm, number of averages = 2, field of view (FOV) = 3.0 × 3.0 cm², in-plane (coronal) resolution = 117 μM. For *in vivo* high resolution ¹H MRS, adjustments of all first- and second-order shims over the voxel of interest were accomplished with the FASTMAP procedure. A custom modified PRESS (Point-RESolved Spectroscopy) pulse sequence (TR/TE = 2500/10 ms) (Xu et al., 2013) was used for MRS data acquisition from the NAc. The region of interest in the NAc was centered ~1.7 mm anterior to bregma (Paxinos and Watson, 1998) and data obtained within a 2 mm (dorsal-ventral) × 6 mm (medial-lateral) window (Fig. 1, inset) from 3 adjacent, 1 mm thick coronal slices were averaged for each case. The unsuppressed water signal from the prescribed voxel was collected as a metabolite concentration reference for each scan.

Quantification of the MRS was based on frequency domain analysis using a "Linear Combination of Model spectra" (LCModel; Provencher, 2001). In order to obtain an index of the reliability of our calculation of the contribution of GABA and GLU to the measured spectra in the LCModel, we determined the percent standard deviation (the Cramér-Rao lower bounds [CRLB]) of each component (Bolliger et al., 2013; Cavassila et al., 2001). In the rat brain, we consider a CRLB value of <20 % as an acceptable level of quantitation reliability (Provencher, 1993, 2001). In the current study, the CRLB values for GABA (12.5 ± 1.05%) and GLU (5.25 ± 0.41%) both fulfilled this criterion. For each transmitter, we calculated effect size using the Cohen's d statistic ($= [2 \cdot t] / \sqrt{df}$; Cohen, 1988), the amount of variance in the data attributable to the effects of OLA treatment (the variance estimator, $r^2 = t^2 / (t^2 + df \cdot 1)$) and the significance of treatment effects using two-tailed, unpaired t-tests, corrected for multiple comparisons by the Bonferroni-Holm procedure (Holm, 1979).

3. RESULTS

Fig. 1A illustrates high resolution, localized *in vivo* ¹H spectra obtained from the NAc (inset) of representative, adult rats treated as adolescents with OLA and VEH, with the peaks corresponding to GABA and GLU indicated. In the NAc (Fig. 1B, Table 1), OLA treatment reduced the concentration of GABA to 82.5% of control values (Bonferroni-Holm corrected p=0.024) and the concentration of GLU to 86.9% of control values (Bonferroni-Holm corrected p=0.040). The effect sizes (Cohen's d; 1.534 for GABA; 1.207 for GLU) are considerably greater than the criterion for large effects (> 0.8; Cohen, 1988). Furthermore, treatment effects account for 60.8% and 51.7%, respectively, of the total variance in the data for GABA and GLU, as estimated by r^2 . This analysis demonstrates that in the NAc,

adolescent OLA treatment causes large, significant, long-term reductions in GABA and GLU concentrations.

4. DISCUSSION

The main finding of this study is that adolescent OLA treatment causes enduring reductions in the concentrations of GLU and GABA within the NAc. These data, and our recent finding that adolescent OLA treatment causes long-lasting reductions in DA release in the NAc (Vinish et al., 2013) constitute the first demonstration that *adolescent* AAPD treatment can cause long-term changes in neurotransmitter levels. These findings raise important questions concerning the benefits and risks of AAPD therapy in adolescent patients and define mechanistic questions whose answers can guide the development of improved treatment regimens.

4.1 What are the functional correlates of the OLA-induced reductions in GLU and GABA levels in the NAc?

During the peripubertal period, there are dramatic changes in the functional organization of neuronal networks in the NAc: In mature rats, DA increases the magnitude of excitatory post-synaptic potentials (EPSPs) evoked in NAc medium spiny neurons (MSNs) by the activity of GLUergic cortical afferents (Brady and O'Donnell, 2004). By contrast, prior to puberty, DA decreases the magnitude of these EPSPs (Benoit-Marand and O'Donnell, 2008). The developmental switch from reduction to potentiation is evident by P50 - shortly after puberty (~P42 in male rats) and is due to the emergence of a D2-facilitated GABAergic component in cortico-NAc responses during adolescence; GABAergic interneurons in the NAc that express parvalbumin (PV) are the likely source of this component (Benoit-Marand and O'Donnell, 2008). We have recently found that in adult rats treated with OLA on P28–49 (as in the present study), DA reduces EPSPs evoked in MSNs by the activity of cortical afferents, as in normal, pre-pubertal rats (Brooks, O'Donnell and Frost, manuscript in preparation). This outcome strongly suggests that the GABAergic neurons that mediate the potentiation of the EPSPs have not developed normally. This interpretation is supported by the reduction of NAc GABA levels induced by adolescent OLA treatment in the present study. Reductions in GABA levels in other populations of inhibitory neurons in the NAc cannot be excluded. Thus, we have begun to investigate the effects of adolescent OLA treatment on distinct populations of NAc GABAergic neurons.

Interpretation of the reduction in GLU levels in the NAc is more difficult. The NAc contains few GLUergic neurons. Thus, in the NAc, GLU is distributed principally in the metabolic pool of cell bodies, or in the axon terminals of the numerous populations of excitatory afferent axons that project there. We are not aware of any studies of the impact on GLU levels in the NAc of AAPD treatment of adult patients or animal models, with which our data might be compared.

We (Vinish et al., 2013) and others (Llorente-Berzal et al., 2011) have shown that, in contrast to the effects of OLA in humans and in adult rats (Baldessarini, 2001; Llorente-Berzal et al., 2011), OLA treatment of *adolescent* rats does not significantly increase weight or alter plasma glucose levels (Llorente-Berzal et al., 2011) during or after treatment;

triglyceride levels are unchanged during adolescent OLA treatment and fall afterwards (Llorente-Berzal et al., 2011). Thus, although these metabolic measures are altered during treatment with OLA (and other AAPDs) in humans and in adult rats, such changes do not occur in adolescent rats and thus, cannot be upstream mediators of the long-term effects of adolescent OLA administration reported here and in our previous studies.

4.2 Are the long-term effects of adolescent OLA treatment specific to that developmental stage?

An extensive literature (from which only a few illustrative citations are presented here) demonstrates that the impacts on the brain and behavior of pharmacologic (Ansorge et al., 2008; Kolb et al., 2012; Milstein et al., 2013) and experiential (Hubel and Wiesel, 1970; Hubel et al., 1977; Innocenti et al., 1985) challenges, and of lesions (Goldman, 1971; Goto and O'Donnell, 2002; Kolb et al., 2012; Miller et al., 1973), depend upon the developmental stage at which treatment is begun, the magnitude (dose) and duration of the treatment, the developmental stage at which outcomes are assessed and the outcome measures used. The present experiments were not designed to determine the critical period for OLA treatment to induce the observed effects. Indeed, this is an important direction for future studies. Neural circuitry is changing rapidly through the end of adolescence, and, in humans, continues to develop into the third decade (Introduction). This makes the brain's ontogenetic trajectory especially vulnerable to environmental influences, including psychotropic drug therapy (review in Andersen and Nalva, 2011). AAPDs are moderately well tolerated in adult- and pediatric patients in the short term (Kapetanovic and Simpson, 2006; Shekelle et al., 2007; Zuddas et al., 2011), but the long-term sequelae that follow cessation of APD treatment in pediatric patients are unknown (Cheng-Shannon et al., 2004) and are likely to differ from those of adult treatment, due to the numerous differences between the mature and developing brains. This expectation is supported by the fact that when OLA and other atypical AAPDs are chronically or sub-chronically administered to adult humans (Sedvall, 1990), the effects of treatment dissipate when the drugs are withdrawn. By contrast, the present study and our previous ones (Frost et al., 2010; Milstein et al., 2013; Vinish et al., 2013) show that adolescent OLA treatment induces a variety of enduring, behavioral and neurobiological effects. Although our data do not reveal which of those effects are specific to adolescent treatment, their identification, and the elucidation of their mechanisms, is translationally important for pediatric patient populations, independent of whether or not OLA treatment of adults induces the same effects. Even if some long-term phenotypes of OLA treatment are similar for adolescents and adults, those effects may arise by distinct mechanisms at different developmental stages.

4.3 Do the observed changes in glutamate and GABA levels reflect a developmental process?

The rat brain is fully mature by post-natal day 90 (humans reach similar stages of development some time in the early- to mid-third decade of life (Giedd et al., 2009; Petanjek et al., 2011). Our MRS data show that some time between post-natal day 28 (pre-puberty, when OLA treatment begins) and P90 (young adulthood), when we begin to obtain our experimental measures, GABA and GLU levels in the NAc have been altered. This is positive evidence of an altered developmental trajectory. Better temporal resolution of the

time course of the OLA-induced changes reported here and in our previous studies (Milstein et al., 2013; Vinish et al., 2013) is another important avenue of further investigation. Knowledge of the temporal dynamics of OLA-induced effects in the NAc (and other brain regions) 1) identifies the developmental stages and brain loci at which to investigate the sequence of mechanistic changes by which OLA alters neural circuitry and behavior and 2) defines “windows of opportunity” in which supplemental interventions might be applied to mitigate the deleterious effects of adolescent OLA therapy. MR spectroscopy is non-invasive, which permits repeated observations in each subject and thus makes it an ideal tool for such studies.

How stable are the neurobiological and behavioral phenotypes of adolescent OLA exposure? A generalization that emerges from studies of the sequelae of early life pharmacologic and experiential factors, and lesions (examples cited above), is that the responses to these challenges can have dynamic developmental trajectories that can, in some instances, progress (and/ or remain modifiable by other factors) up to the time the brain is fully mature. (This is true of changes in dendritic spine density caused by adolescent OLA treatment; Milstein et al., 2013). Once maturity is reached, outcomes remain relatively stable unless further interventions are performed to drive the brain into a more plastic state. In this experiment and our previous ones, outcome measures were obtained from rats aged 3–8.5 months, i.e., rats that were fully mature, but considerably younger than the age (12–15 months) at which the rat brain begins to show geriatric changes (eg. Suzuki et al., 2001). Thus, although we have not studied individual outcome measures at multiple ages after subjects reach maturity, it seems likely that the OLA-induced changes we have studied are fully expressed by that time and that they endure at least to the time when the brain begins its age-related decline. This is especially true in the current study, for which all the data were obtained from rats 90–120 days old.

4.4 Future directions and translational significance

A key issue for future research is to apply neurophysiological, biochemical and imaging techniques to investigate the developmental trajectory, between the onset of adolescent OLA administration and maturity, of molecular, cellular and neurobiological measures altered by OLA treatment. The data of such studies will identify the mechanistic cascades that produce long-term changes in the neural networks that mediate the behaviors adversely affected by adolescent OLA therapy. At the translational level, such data would permit the identification of cellular and molecular targets for adjunct therapies to mitigate the adverse effects of AAPD treatment, and the temporal windows during which those interventions are likely to be most effective. A related issue will be to determine how the spectrum of long-term effects resulting from treatment changes as a function of the developmental stage at which AAPDs are administered. For example, we would predict that the spectrum of effects of pre-pubertal treatment differs significantly from that of post-pubertal treatment, because the substrate on which the drugs act – the brain – changes dramatically at puberty. It will also be important to expand our MR spectroscopy studies to other brain regions that are targets of AAPDs and that are part of the neural networks that mediate behaviors impacted by adolescent AAPD treatment. Finally it is critical to investigate the extent to which the behavioral and neurobiological changes induced by early life AAPD therapy are sexually dimorphic. Some

degree of sexual dimorphism is to be expected because sex differences in the brain begin to arise early in development (Lenz et al., 2012) and because females reach puberty earlier than males. Sexual dimorphism in the long-term effects of AAPD therapy might suggest different guidelines for the use of these agents in males and females, and distinct adjunct therapies to mitigate adverse effects.

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Figure 1.

A: Representative *in vivo* ¹H MR spectra at 7 T from the NAc of adult rats treated as adolescents with VEH or OLA. The signals assigned in the spectra refer to γ -aminobutyric acid (GABA) and glutamate (GLU). Inset: The proton-density-weighted image of a rat brain, *in vivo*, illustrates the region-of-interest ($2 \times 6 \times 3$ mm). B: GABA and GLU concentrations, measured by *in vivo* ¹H MR, in the NAc of adult rats treated as adolescents with VEH or OLA (n=8/group). Concentrations are shown as mean $\pm$ standard error. Asterisks indicate that the OLA-induced changes in GABA and GLU concentrations are significant at the p=0.024 and p=0.040 levels, respectively.

Summary of the statistical analysis of OLA-induced changes in GABA and GLU concentrations in the NAc of adult rats treated as adolescents with VEH or OLA.

Table 1

TRANSMITTER	CONCENTRATION OLA-TREATED RATS (mM) MEAN ± SE	CONCENTRATION VEH-TREATED RATS (mM) MEAN ± SE	n	df	t	EFFECT SIZE (Cohen's d)	VARIANCE ESTIMATOR (r ²)	p (Bonferroni-Holm Corrected)
GABA	2.200 ± 0.125	2.668 ± 0.104	8	14	2.869	1.534	0.608	0.024
GLU	6.736 ± 0.399	7.749 ± 0.205	8	14	2.259	1.207	0.517	0.040