Published in final edited form as:

Neuroscience. 2011 April 14; 179: 1–8. doi:10.1016/j.neuroscience.2011.01.032.

Age-Dependent Effects of Initial Exposure to Nicotine on Serotonin Neurons

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Abstract

Adolescence is a critical vulnerable period during which exposure to nicotine greatly enhances the possibility to develop drug addiction. Growing evidence suggests that serotonergic (5-HT) neurotransmission may contribute to the initiation and maintenance of addictive behavior. As the dorsal raphe (DR) and median raphe (MnR) nuclei are the primary 5-HT source to the forebrain, the current study tested the hypothesis that there are age-dependent effects of acute nicotine administration on activation of 5-HT neurons within these regions. Both adolescent (Postnatal day 30) and adult (Postnatal day 70) male Sprague-Dawley rats received subcutaneous injection of either saline or nicotine (0.2, 0.4, or 0.8 mg/kg). Subsequently, the number of 5-HT cells that were double-labeled for Fos and tryptophan hydroxylase was counted in 7 subregions within the DR and the entire MnR. The results show that acute nicotine injection induces Fos expression in 5-HT neurons in a region-specific manner. In addition, adolescents show broader regional activations at either a lower (0.2 mg/kg) and a higher (0.8 mg/kg) dose of nicotine, displaying a unique U-shape response curve across doses. In contrast, 5-HT cells with activated Fos expression were restricted to fewer regions in adults, and the patterns of expression were more consistent across doses. The results reveal dose-dependent effects of nicotine during adolescence with apparent sensitization at different ends of the dosage spectrum examined compared to adults. These data indicate that initial exposure to nicotine may have unique effects in adolescence on the ascending 5-HT system, with the potential for consequences on the affective-motivational qualities of the drug and the subsequent propensity for repeated use.

Keywords

raphe; adolescence; serotonin; addiction

Nicotine addiction begins in adolescence more often than in any other ages (Adriani *et al.*, 2003; 2004; Schramm-Sapota *et al.*, 2009). Smoking at this developmental stage is associated with a greater tendency to develop drug dependence (Taioli and Wynder, 1991; Breslau and Peterson, 1996; Chen and Millar, 1998; Kandel and Chen, 2000). In animal models of nicotine addiction, several behavioral differences between adolescents and adults have been reported. Adolescent rodents are more prone to acquire place preference than

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adult rats with the same dose of nicotine (Vastola *et al.*, 2002; Belluzi *et al.*, 2004; Torres *et al.*, 2008; Shram and Lê, 2010) and are more motivated to self-administer nicotine after a period of abstinence (Adriani *et al.*, 2002; Levin *et al.*, 2006). In addition, adolescents are more resistant to the aversive effects of nicotine than adults (Wilmouth and Spear, 2004; Shram *et al.*, 2006). However, the neurobiological mechanism underlying the developmental vulnerability to nicotine addiction is unclear.

A growing body of evidence suggests that modulation of the central serotonergic (5-HT) system contributes to the effects of nicotine. Studies have shown that acute injections of nicotine increase the levels of extracellular 5-HT or promotes [³H]5-HT uptake in several regions including the cingulate and frontal cortices, hippocampus and nucleus accumbens shell (Toth *et al.*, 1992; Ribeiro *et al.*, 1993; Summers *et al.*, 1996; Awtry and Werling, 2003). In addition, serotonin receptor antagonists block the effects of nicotine on place preference (Carboni *et al.*, 1988) and anxiety state (Cheeta *et al.*, 2000; 2001). Chronic exposure to nicotine also produces adaptive changes in the 5-HT system, altering 5-HT transporter levels and 5-HT receptor signaling in several areas of the adolescent brain (Xu *et al.*, 2001; 2002; Collins *et al.*, 2004).

The aim of the present study was to determine if acute nicotine has age-dependent effects on 5-HT neurons in the dorsal raphe (DR) and the median raphe (MnR), which are the primary source of forebrain 5-HT. Acute nicotine administration was used to model initial exposure, since the subjective experience of the first exposure to nicotine may influence the propensity for repeated use and subsequently the development of addictive behavior. As forebrain afferents of the DR and MnR are known to display a high level of topographic organization (Vertes, 1991; Van Bockstaele *et al.*, 1993; Vertes *et al.*, 1999; for a review; see Michelsen *et al.*, 2007), we examined the topography of nicotine's effect by assaying the protein appearance of the immediate-early gene product Fos within 5-HT neurons in specific subregions within the DR and MnR. Our working hypothesis is that the responses of 5-HT neurons to nicotine is enhanced in adolescents compared to adults and may be topographically organized. We postulate that the age difference in region-specific effects of nicotine on 5-HT release could be relevant for understanding the nature of a biological vulnerability to initiation of nicotine addiction during adolescence.

1. EXPERIMENTAL PROCEDURES

1.1 Subjects

A total of 87 male Sprague-Dawley rats were used in this study. Forty rats were adolescents at postnatal days (PND) 26 (80–120 g) and 47 rats were adults at PND66 (320–360 g) upon arrival. Rats were housed two per cage, maintained on a 12-h light/dark schedule, and given access to food and water *ad libitum*. Care and use of animals was approved by the Institutional Care and Use Committee at Children's Hospital and was consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

1.2 Drugs

Nicotine was administered at 0.2, 0.4, or 0.8 mg/kg, expressed as the base, freshly prepared by dissolving nicotine hydrogen tartrate (Sigma-Aldrich, St. Louis, USA) in 0.9 % normal saline (BD) and adjusting the pH to 7.0. Solutions were prepared such that injected volume was 1 ml/kg body weight. The doses we used were selected to span a behaviorally effective range by comparison with the literature (Matta *et al.*, 2007). A single acute dose of nicotine was used to model the effects of initial drug exposure in order to identify biologically distinct responses at different ages.

1.3 Procedures

All subjects were handled for 4 days before experiments. Subjects were injected subcutaneously with either nicotine or normal saline. Three different doses of nicotine (0.2, 0.4, and 0.8 mg/kg) were injected into 17, 14, 16 subjects, respectively, and the rest received saline injection. Subjects were returned to their home cage and remained there for 15 minutes for the brain nicotine level to be at its peak (Turner, 1975). All experiments started between 9:00 and 10:00 am, 2–3 h after the initiation of the light cycle.

1.4 Immunohistochemistry

Two hours after administration with either nicotine or saline, animals were deeply anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.). Transcardial perfusion was made with 4 % paraformaldehyde in normal saline. Brains were removed and stored in 4 % paraformaldehyde for 24 hr, and equilibrated in 30 % sucrose in 0.1 M phosphate buffer for at least 2 days. Then, each brain was frozen, serially sectioned in the coronal plane (40 μ m) using a freezing microtome, and processed while floating.

Primary antisera were diluted in 0.1 M phosphate-buffered saline with 0.3 % Triton X-100, 0.04 % bovine serum albumin and 0.1 % sodium azide, and incubated with the tissue overnight at room temperature. Fos immunoreactivity was detected by incubating sections with a rabbit polyclonal anti-Fos antibody (1:10,000; PC38, EMD Biosciences, San Diego, CA). 5-HT neurons were detected using an antiserum raised against tryptophan hydroxylase (TPOH), the synthetic enzyme for serotonin, raised in sheep (1: 1000; AB1541, Chemicon International, Temecula, CA, USA). Sections were then incubated with a mixture of CY3-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA) and AlexaFluor 488-conjugated anti-sheep IgG (1:200; Invitrogen Molecular Probes, Carlsbad, CA) secondary antibodies for 90 min at room temperature. Sections were then rinsed with 0.1 M PBS, mounted onto glass slides, and coverslipped with 90 % glycerol.

1.5 Data analysis

Cells that were double-labeled with Fos and TPOH were counted in seven subregions within the DR and the entire MnR (Fig 1). In DR, three rostrocaudal divisions were made: caudal area was from -8.54 to -9.26 mm, middle area was from -7.73 to -8.45 mm, and rostral area was from -6.92 to -7.64 mm relative to Bregma (Paxinos and Watson, 2005). These three levels were then divided at the midpoint between the base of the aqueduct and the ventral extent of TPOH-containing cells into dorsal and ventral subregions. The lateral wings of the DR and the MnR are the remaining two groups that were both sampled in sections comprising mid-levels of the DR. Abbreviations for each region is as follows: DR-RD, rostral dorsal DR; DR-RV, rostral ventral DR; DR-LW, lateral wings of DR; DR-MD, mid dorsal DR; DR-MV, mid ventral DR; MnR, median raphe; DR-CD, caudal dorsal DR; DR-CV, caudal ventral DR.

For each rat, every fourth section was analyzed, representing approximately 3 sections per regions of interest. For each area, a minimum of six rats (average number of rats/group = 7) contributed to the mean number of cells containing Fos and TPOH in each group. For sampling, the relevant area was illuminated for each fluorophore and digitally photographed using conventional fluorescence microscope and a 10 $\times$ objective. Representative images were photographed using a 20 $\times$ objective for preparation of the figure (see Fig. 2). Double-labeled cells were manually enumerated by visualization of the individual and merged images of each fluorophore. To be considered positive for Fos, Fos immunolabeling had to completely fill the nucleus with an intensity of labeling that was easily distinguished from background levels (Fig. 2). The mean number of double-labeled cells per section was

determined for each rat within a group. Group means and standard error of the means were calculated for each subregion.

Each area was statistically analyzed with multifactorial analysis of variances (ANOVA) with repeated measures (SPSS 14.0; Chicago, IL) with dose [0 mg/kg (saline-treated), 0.2 mg/kg, 0.4 mg/kg, and 0.8 mg/kg], and age (adults vs. adolescents) as between-subject factors and brain region as a within-subject factor. Significant interaction effects were followed by either simple interaction effects or simple main effects at each level of the involved factors. *Post hoc* comparisons using Bonferroni-corrected post hoc comparisons were followed with a threshold for significance of $p < 0.05$ where appropriate.

2. RESULTS

Effects of acute nicotine administration on Fos expression in TPOH-containing cells (Fig. 2) were examined in adults versus adolescents at a dose of 0.2 (Fig. 3), 0.4 (Fig. 4), and 0.8 mg/kg (Fig. 5) in the eight regions of interest (Fig. 1): DR-RD, DR-RV, DR-LW, DR-MD, DR-MV, MnR, DR-CD, and DR-CV. The morphology of the raphe nuclei and abundance of 5-HT cells in the region of interests appeared comparable between the two age groups as shown in Figure 1. Figure 2 depicts representative images of DR-RD in which nicotine enhanced Fos expression (red) in TPOH-containing 5-HT cells (green) in both age groups at a dose of 0.2 mg/kg. The labeling of both TPOH and Fos were easily detectable and quantifiable. While there were few 5-HT cells that were double-labeled with Fos (arrow) in saline-injected rats (Fig. 2B and 2D), double-labeled 5-HT cells were commonly found in both adults (Fig. 2A) and adolescents (Fig. 2C) after nicotine injection.

ANOVA revealed significant interaction effects between dose and brain region ($F(21, 553) = 6.93$; $p < 0.005$) as well as significant triple interactions among age, dose and brain region ($F(21, 553) = 1.73$; $p < 0.05$). Simple effects analysis at each subregion showed significant interaction effects of dose and age particularly in DR-RD; $F(3,79) = 2.79$; $p < 0.05$) and MnR ($F(3,79) = 2.90$; $p < 0.05$). Further analysis by simple effects ANOVA revealed a significant main effect of dose in all remaining regions of interests ($ps < 0.05$). The overall results indicate that acute nicotine effects are dose- and age-dependent, and exhibit regional organization

At the lowest dose, 0.2 mg/kg, adolescents had greater numbers of regions where there was a significant increase in the number of 5-HT cells with Fos compared to adults (Fig. 3). Specifically, *post hoc* Bonferroni tests showed that adolescents displayed significant differences in Fos activation between saline-treated and nicotine (0.2 mg/kg)-treated groups in DR-RD, DR-RV, DR-MD, and MnR ($ps < 0.05$), while adults had differences only in DR-RD and DR-CV ($ps < 0.05$; Fig. 3). At the intermediate dose, 0.4 mg/kg, adults showed an increase in Fos expression in two regions, DR-RD and the MnR. In contrast, there were no regions with activated Fos expression in adolescents (Fig. 4). Given 0.8 mg/kg of nicotine administration, adolescents had nicotine-induced 5-HT activation in DR-RD, DR-RV, DR-MD, DR-MV, and DR-CD whereas adults had the same effect only in DR-RD, DR-RV, and DR-CD (Fig. 5).

A notable age difference is related to the dose-response curve for nicotine, particularly in DR-RD, DR-RV, and DR-MD. Specifically, there were robust 5-HT activations at two different ends of the dose spectrum examined (0.2 mg/kg and 0.8 mg/kg) in these regions (Figs. 3 and 5), but lack of a detectable effect of nicotine at the intermediate dose (0.4 mg/kg) of nicotine in adolescents (Fig. 4). In contrast, adults had consistent levels of 5-HT activation across doses in DR-RD, no apparent activation across doses in DR-MD, and 5-HT activation only at a dose of 0.8 mg/kg in DR-RV (Figs. 3–5). These results indicate that the

adolescents have a unique 'U-shape' dose curve of nicotine-induced 5-HT neuronal activations in selective subregions while the adults do not display this pattern (Figs. 3–5).

3. DISCUSSION

The present study examined developmental effects of acute-nicotine-induced activation of Fos within 5-HT neurons located in subregions of DR and MnR. Three main findings were observed in this study: first, acute nicotine injection induced Fos expression in 5-HT neurons in a topographical manner such that only certain subregions had differences in the number of double-labeled 5-HT neurons between the treatment groups (Figs. 3–5); second, age differences were detected in the number of regions that showed Fos expression in 5-HT neurons induced by the same dose of nicotine such that adolescents showed a greater number of 5-HT-activated regions at a dose of either 0.2 mg/kg or 0.8 mg/kg compared to adults; and third, there was also an age difference in the level of nicotine-induced 5-HT activation across doses in a way that adolescents showed a 'U-shape' dose curve of 5-HT neuronal activation whereas adults did not, particularly, in the rostral part (DR-RD, and DR-RV) and mid dorsal pole of the DR. These observations support the conclusion that the effects of initial exposure to nicotine on 5-HT neurons in the DR and MnR are age-dependent.

One of the unique components of the present study is that the appearance of Fos expression was specifically analyzed within 5-HT neurons and further characterized by sub-regional location within the ascending raphe. This approach is important to detect topographically organized effects or subregion-specific effects of nicotine that may be overlooked otherwise. Indeed, the current study shows that nicotine-induced activation of Fos within 5-HT neurons is topographic with the rostral-dorsal pole of DR being the most consistently activated region (Figs. 3–5). However, this study using Fos as an indicator of neural activity has a methodological limitation. Fos is encoded from the immediate-early gene, *c-fos*, which is rapidly induced by extracellular stimuli (Curran and Morgan, 1995). The presence of Fos reflects activation of specific biochemical pathways, but these pathways may not always directly lead to functional activity of a neuron. Thus, the Fos expression analysis may be subject to both false-negative and false-positive errors. Nonetheless, the use of Fos expression has given broad insight into functioning of the serotonin system (Roche *et al.*, 2003; Abrams *et al.*, 2005; Hollis *et al.*, 2006) and the neural response to drugs of abuse (Dayas *et al.*, 2007; Cippitelli *et al.*, 2010; Plaza-Zabala *et al.*, 2010).

At each dose and age, the pattern of activation of Fos within 5-HT neurons was slightly different. Since ascending projections from the DR and MR are topographically organized (for a review; see Michelsen *et al.*, 2007), these findings may help explain the observation that acute nicotine administration changes extracellular 5-HT and 5-HT metabolite (5-HIAA) concentrations in an age- and region-specific manner (Shearman *et al.*, 2008). The DR-RD, having the most pronounced nicotine effects on 5-HT neurons in both age groups, is one of the areas from which 5-HT neurons mainly innervate the striatum, globus pallidus and substantia nigra (Andén *et al.*, 1965; van der Kooy and Hattori, 1980; Imai *et al.*, 1986). The effect of nicotine was also evident in the DR-MD, particularly in adolescents. The 5-HT neurons from this area target the central nucleus of the amygdala and the paraventricular nucleus of the thalamus, the regions involved in anxiety (Petrov *et al.*, 1992; 1994; Commons *et al.*, 2003). The function of central nucleus of the amygdala has also been implicated in the acute reinforcing effects as well as counteradaptation of the reward system during the development of drug dependence (Koob and Le Moal, 1997). The MnR 5-HT neurons showed Fos expression by acute nicotine in both age groups, but at different dosages (see Figs. 3 and 4). The MnR is thought to provide the major innervation to the dorsal hippocampus (Azmitia and Segal, 1978; Vertes *et al.*, 1999) and previous studies

showed that administration of nicotine modifies hippocampal serotonin release (Kenny *et al.*, 2000) and 5-HT_{1A} mRNA expression (Kenny *et al.*, 2001) which is suggested to be associated with anxiogenic effects of nicotine (Seth *et al.*, 2002). Overall, the current findings support the idea that initial exposure to nicotine may have topographically differential effects on adults vs. adolescent 5-HT system, which could impact the affective and motivational qualities of the drug.

A major feature regarding age differences in nicotine-induced 5-HT activation is that adolescents had greater numbers of regions within the raphe in which 5-HT neurons were activated by acute nicotine at a dose of either 0.2 mg/kg or 0.8 mg/kg than adults. The broader activation at either dose in adolescence could be interpreted as a sensitized response of the 5-HT system to nicotine exposure. There is accumulating evidence that chronic exposure to nicotine has unique effects on 5-HT system in adolescence. During chronic nicotine treatment, adolescents show a gender-selective increase in concentration of 5-HT and 5-HIAA in the midbrain (Xu *et al.*, 2002), and upregulation of cerebrocortical 5-HT_{1A} and 5-HT₂ receptors (Slotkin *et al.*, 2006), although the effects are dependent on the periods of treatment and discontinuation of nicotine. In addition, chronic nicotine exposure decreases serotonin transporter densities in regions including nucleus accumbens in adolescents, while the same exposure causes no changes in adults (Collins *et al.*, 2004). Taken together with our findings that more DR subregions were activated in younger rats, these observations raise the possibility that the unique actions of nicotine on 5-HT neurons themselves could participate in initiating these long-term changes in 5-HT neurotransmission.

Another notable age difference observed in this study is that the difference in the effects of nicotine between adolescents and adults in DR-RD, DR-RV, and DR-MD was not a simple shift of the dose curve toward the left; rather, adolescents had a U-shaped dose curve while this effect was not evident in adults, at least within the dose range that we examined (0.2–0.8 mg/kg). The difference may reflect the complex effects of nicotine in these regions by which nicotine both activates and inhibits 5-HT neurons. Electrophysiological studies have shown that nicotine not only facilitates firing rates of 60–70 % of 5-HT neurons in the DR, but also decreases the rates in the remaining 30–40 % of 5-HT neurons (Li *et al.*, 1998; Mihailescu *et al.*, 1998; 2002). Although nicotine induces both 5-HT and/or γ -aminobutyric acid (GABA) release in the brain, the inhibitory effects of nicotine on the firing rates of 5-HT neurons are more likely to be mediated via 5-HT-induced activation of 5-HT_{1A} receptors, rather than via an indirect GABAergic inhibition (Mihailescu *et al.*, 2002). The 5-HT_{1A} receptors are prevalent in dendrites and soma of 5-HT neurons and function as inhibitory autoreceptors (for a review, see Barnes and Sharp, 1999). The level of 5-HT_{1A} receptors peaks at an early, prenatal period, then decreases across age from adolescence (Bar-Peled *et al.*, 1991; Dillon *et al.*, 1991; Dyck and Cynader, 1993). Thus, it may be reasonable to speculate that the greater numbers of 5-HT_{1A} receptors in adolescents contribute to increased inhibition, which could yield the U-shaped curve response to nicotine.

4. CONCLUSIONS

The results of this study show that acute nicotine effects on 5-HT activation are topographically organized within the DR and MnR, indicating that certain ascending 5-HT pathways may preferentially contribute to the effects of nicotine in each case. This study also provides evidence for increased sensitivity of 5-HT system in adolescents, raising the possibility of 5-HT serving as a contributor to the unique developmental effects of nicotine during adolescence. In addition, the unique U-shape dose curve observed only in adolescents might reflect complex dose-dependent effects of nicotine in inducing excitation and

inhibition in different subgroups of 5-HT cells. Examining how the dynamic control of nicotine on subpopulations of the 5-HT neurons changes across age might give us further insights into the neurobiological mechanism by which the 5-HT system contributes to developmental vulnerability of nicotine addiction.

Acknowledgments

We acknowledge Dr. Mariano Soiza-Reilly for useful comments. This work was supported by grants from *NIH grant DA021801*.

Abbreviations

DR	Dorsal raphe
DR-CD	caudal dorsal pole of dorsal raphe
DR-CV	caudal ventral pole of dorsal raphe
DR-LW	lateral wings of dorsal raphe
DR-MD	mid dorsal pole of dorsal raphe
DR-MV	mid ventral pole of dorsal raphe
DR-RD	rostral dorsal pole of dorsal raphe
DR-RV	rostral ventral pole of dorsal raphe
GABA	γ -aminobutyric acid
MnR	Median raphe
PND	Postnatal day
TPOH	Tryptophan hydroxylase

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Figure 1.

Images of coronal sections of dorsal raphe (DR) at three different levels in the A–P axis (A and A', anterior; B and B', mid; D and D', caudal) and median raphe (C and C') in adolescent (A–D) and adult (A'–D') rats. The numbers depict eight sampled areas: 1, rostral-dorsal DR; 2, rostral-ventral DR; 3, lateral wings of DR; 4, mid-dorsal DR; 5, mid-ventral DR; 6, median raphe; 7, caudal-dorsal DR; 8, caudal-ventral DR. The densities of TPOH-labeled cells are comparable between the two age groups at all levels examined. Scale bar = 200 μ m.

Figure 2.

Acute nicotine effects in 5-HT cells within the rostral dorsal pole of the dorsal raphe. (A) In adults (PND70) administered with acute nicotine (0.2 mg/kg), TPOH-containing cells (green) that were double-labeled with Fos (red) were commonly found (some are indicated by arrows). (B) In adults that received saline, there were fewer double-labeled cells (arrows). (C) In adolescents (PND30) that received acute nicotine injection (0.2 mg/kg), TPOH-containing cells labeled with Fos were easily detectable (arrows). (D) In adolescents injected with saline, there were few TPOH-containing cells that were double-labeled with Fos (arrow). Scale bar = 25 μ m. Abbreviations: PND, postnatal day; TPOH, tryptophan hydroxylase

Figure 3.

Effects of acute nicotine in adults versus adolescents at a dose of 0.2 mg/kg on Fos expression in TPOH-containing cells in the eight regions of interest. (A) Average numbers of cells double-labeled with TPOH and Fos in nicotine-injected subjects (filled bar) and saline-injected controls (open bar) in adults. (B) Average number of double-labeled cells in the eight regions in adolescents. Asterisks (*) denote significant differences between the nicotine-treated group and saline-injected group in each region whereas crosses (+) depict the region that had a significant interaction effect between dose and age as well as a significant group difference. Error bars are $\pm$ SEM. Abbreviations: DR-RD, rostral dorsal DR; DR-RV, rostral ventral DR; DR-LW, lateral wings of DR; DR-MD, mid dorsal DR; DR-MV, mid ventral DR; MnR, median raphe; DR-CD, caudal dorsal DR; DR-CV, caudal ventral DR.

Figure 4.

Acute nicotine effects at a dose of 0.4 mg/kg on 5-HT activation in the eight subregions within the DR and the MnR in adults versus adolescents. (A) Average numbers of TPOH-containing neurons double-labeled with Fos in nicotine-injected rats (filled bar) and saline-injected rats (open bar) in adults. (B) Average number of double-labeled cells in the eight regions in adolescents. Note that there were no effects of acute nicotine in any subregion within the DR and the MnR in the adolescents. Crosses (+), error bars, and abbreviations are equivalent to Figure 3.

Figure 5.

Developmental effects of acute nicotine at a dose of 0.8 mg/kg on Fos expression in TPOH-containing cells. (A) Mean numbers of cells double-labeled with TPOH and Fos in the eight sampled regions in adults. (B) Mean number of double-labeled cells in the eight sampled regions in adolescents. Asterisks (*), crosses (+), error bars, and abbreviations are equivalent to both Figures 3 and 4.