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Asenapine sensitization from adolescence to adulthood and its potential molecular basis

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Abstract

Asenapine is a new antipsychotic drug that induces a long-lasting behavioral sensitization in adult rats. The present study investigated the developmental impacts of adolescent asenapine treatment on drug sensitivity and on 3 proteins implicated in the action of antipsychotic drugs (i.e. Brain-derived neurotrophic factor (BDNF), dopamine D₂ receptor, and FosB) in adulthood. Male adolescent Sprague-Dawley rats (postnatal days, P 43-48) were first treated with asenapine (0.05, 0.10 or 0.20 mg/kg, sc) and tested in the conditioned avoidance or PCP (2.0 mg/kg, sc)-induced hyperlocomotion tasks for 5 days. After they became adults (~P 76), asenapine sensitization was assessed in a single avoidance or PCP-induced hyperlocomotion challenge test with all rats being injected with asenapine (0.10 mg/kg, sc). Rats were then sacrificed 1 day later and BDNF, D₂ and FosB in the prefrontal cortex, striatum and hippocampus were examined using Western blotting. In adolescence, repeated asenapine treatment produced a persistent and dose-dependent inhibition of avoidance response, spontaneous motor activity and PCP-induced hyperlocomotion. In the asenapine challenge test, adult rats treated with asenapine (0.10 and 0.20 mg/kg) in adolescence made significantly fewer avoidance responses and showed a stronger inhibition of spontaneous motor activity than those previously treated with saline. However, no group difference in the levels of BDNF, D₂ and FosB expression was found. These findings suggest that although

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adolescent asenapine treatment for a short period of time induces a robust behavioral sensitization that persists into adulthood, such a long-term effect is not likely to be mediated by BDNF, D₂ and FosB.

Keywords

Asenapine; Conditioned avoidance response; Phencyclidine; Locomotor activity; Adolescence; Sensitization; BDNF; Dopamine D₂ receptor; FosB; Ultrasonic vocalization

1 Introduction

Antipsychotic treatment in children and adolescents has increased dramatically in recent decades. Epidemiological surveys conducted in many countries (e.g. UK, US, Germany, Netherlands) indicate a 2- to 6-fold increase in the number of prescribed antipsychotic drugs for young patients (< 20 years) between the 1990s and the mid-2000s [1-3]. As adolescence is a unique period when the brain undergoes dramatic reorganization and frontal maturation, it is conceivable that antipsychotic exposure during this period will alter brain development and behavioral function in the long run. Recent preclinical studies suggest that this is indeed the case. For example, periadolescent exposure to antipsychotic drugs such as olanzapine, risperidone and clozapine is found to alter various neuroreceptors, including dopamine D₁, D₂ and D₄ receptors [4, 5], serotonin 5-HT_{1A} and 5-HT_{2A} receptors [6], and ionotropic NMDA and AMPA glutamatergic receptors [7]. Adolescent antipsychotic treatment is also shown to enhance rodents' sensitivity to amphetamine [5], impair their working memory in a delayed non-match to sample test, delay the extinction process of shock-induced fear memory in adulthood [8], and prevent the development of various psychosis-like behaviors [9-12].

Our research on the long-term effects of antipsychotic treatment on behavioral and brain functions throughout development has focused on the drug-induced alterations in drug sensitivity from adolescence to adulthood [13-16]. As is often the case, repeated administration of a psychotropic drug results in either an increase or decrease of a particular behavioral effect of the drug, termed *sensitization* and *tolerance*, respectively [17]. Our previous antipsychotic work on adult rats (> 70 days old) identify two similar behavioral phenomena, which are termed antipsychotic sensitization and tolerance [18-23]. Using two distinct behavioral tests of antipsychotic activity: conditioned avoidance response (CAR) and PCP-induced hyperlocomotion, we showed that repeated administration of haloperidol, olanzapine, asenapine or risperidone daily for 5-7 days in adult rats progressively increases the drug's efficacy to inhibit avoidance responding and PCP-induced hyperlocomotion over time (a within-subjects index of sensitization). A few days later, when all rats are given a challenge dose of these drugs, they often make significantly *fewer* avoidance responses and exhibit *lower* PCP-induced hyperlocomotion than those that are treated with these drugs for the first time (a between-subjects index of sensitization). In contrast, repeated administration of clozapine causes a decrease in its behavioral efficacy in these tests, indicating a tolerance effect. In addition, our previous studies also indicate that antipsychotic sensitization that can last up to 50 days [19], and are likely mediated by dopamine D₂ and 5-HT_{2A} receptor-related neural plasticity [23].

Recently we expanded our antipsychotic sensitization and tolerance work into the adolescent period. We have demonstrated that adolescent antipsychotic treatment could induce behavioral sensitization and tolerance that maintain into adulthood. Adolescent treatment of olanzapine or risperidone causes a sensitization effect, whereas clozapine treatment in adolescence causes a tolerance in adulthood [13-15, 24]. Rats treated with only 5 days of olanzapine or risperidone in adolescence showed a stronger inhibition of CAR and PCP-induced motor activity than those treated with vehicle when all rats were challenged with the same antipsychotic drug in adulthood, whereas those treated with clozapine for 5 days in adolescence showed a weaker inhibition than the vehicle rats. In addition, adolescent risperidone treatment even altered adulthood responsiveness to other atypical drugs (e.g. olanzapine and clozapine) [15]. Collectively, these findings provide strong evidence that antipsychotic treatment in adolescence can induce a long-term change in drug responsiveness that persists into adulthood.

The primary goal of the present study was to determine the generality of this observation by examining whether asenapine, a newer atypical antipsychotic drug with a distinctive receptor binding profile from other atypical antipsychotic drugs [25, 26], would also cause a sensitization effect that persists from adolescence into adulthood. In addition, we attempted to identify the possible molecular mechanisms underlying such a long-term behavioral effect. Given that adolescent antipsychotic treatment is known to increase dopamine D₂ receptors in certain forebrain regions [4, 5], and repeated antipsychotic treatment is shown to induce a robust long-term change in BDNF and FosB (a transcription factor), two important biomarkers involved in brain plasticity and the action of chronic antipsychotic treatment [27-31], we focused our attention on the D₂, BDNF and FosB levels in the prefrontal cortex (PFC), striatum, and hippocampus in adult rats that had received either asenapine or vehicle treatment in adolescence. These brain areas have been implicated in antipsychotic action and the neuropathology of schizophrenia [32]). Our hypothesis was that adolescent asenapine treatment would induce a sensitization effect that persists into adulthood. In addition, we expected that adolescent asenapine treatment would also cause long-lasting changes in the expression of D₂, BDNF and FosB levels parallel to the behavioral sensitization.

2. Materials and methods

2.1. Animals

Adolescent male Sprague–Dawley rats from Charles River (Portage, MI; postnatal days, P 25-27 or P 35-37 upon arrival, averaged age were assumed to be ~P 26 or ~P 36) were used. They were housed two per cage, in 48.3 cm × 26.7 cm × 20.3 cm transparent polycarbonate cages under 12-h light/dark conditions (light on between 6:30 am and 6:30 pm). Room temperature was maintained at 22 ± 1°C with a relative humidity of 45-60%. Food and water was available *ad libitum*. Rats were allowed at least 5 days of habituation to the animal facility before being used in experiments (~P 31 or ~P 41). All behavioral tests took place between 9 am and 5 pm in the light cycle. The experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln.

2.2. Drugs and choice of doses

Asenapine Maleate (ASE, a gift from the NIMH drug supply program) was dissolved in 0.9% saline. Doses of asenapine (0.05, 0.10 and 0.20 mg/kg) were determined on the basis of our literature review showing that this dose range of asenapine causes a dose-dependent suppression of CAR but does not cause severe motor impairment [33, 34]. These doses were also chosen on the basis of our recent studies showing that asenapine at these doses induces a dose-dependent and long-lasting sensitization in adult rats in the CAR test [18, 19]. Phencyclidine hydrochloride (PCP, gift from the NIDA Chemical Synthesis and Drug Supply Program) was dissolved in 0.9% saline and tested at 2.00 mg/kg. All drugs were administered subcutaneously (sc) at 1.00 ml/kg.

2.3. Two-way avoidance conditioning apparatus and ultrasonic vocalization (USV) apparatus

Eight identical two-way shuttle boxes custom designed and manufactured by Med Associates (St. Albans, VT) were used. Each box was housed in a ventilated, sound-insulated isolation cubicle (96.52 cm W × 35.56 cm D × 63.5 cm H). Each box was 64 cm long, 30 cm high (from grid floor), and 24 cm wide, and was divided into two equal-sized compartments by a partition with an arch style doorway (15 cm high × 9 cm wide at base). A barrier (4 cm high) was placed between the two compartments, so the rats had to jump from one compartment to the other. The grid floor consisted of 40 stainless-steel rods with a diameter of 0.48 cm, 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, 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 (approximately 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.

In each CAR box, a USV microphone (P48 Avisoft Bioacoustics/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 and analyzed by Avisoft SASLab Pro (version 4.51; Avisoft Bioacoustics).

2.4. Locomotor activity monitoring apparatus

Sixteen activity boxes were housed in a quiet room. The boxes were 48.3 cm × 26.7 cm × 20.3 cm transparent polycarbonate cages, which were similar to the home cages but were each 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, Toronto, Canada) was used to detect the disruption of the photocell beams and recorded the number of beam breaks. All experiments were run during the light cycle.

2.5. Experiment 1: Asenapine sensitization and associated D₂ and BDNF protein expression in asenapine-treated rats in the conditioned avoidance response test

This experiment examined whether the sensitization effect induced by asenapine in adolescence could be maintained into adulthood and explored its potential molecular basis in the CAR test. Table 1 details the experimental procedure, which involved avoidance training, repeated asenapine testing, asenapine challenge test and D₂ and BDNF Western blotting.

Avoidance training in adolescence from P 33 to P 42—Forty rats (~P 31) were first habituated to the CAR boxes for 2 days (30 min/day) and then trained to make avoidance responding for 10 consecutive days/sessions. Each daily session consisted of 30 trials. Every trial started by presenting a white noise (CS) for 10 s, followed by a continuous scrambled foot shock (0.8 mA at maximum duration of 5 s) on the grid floor. If a subject moved from one compartment into the other during the CS, it avoided the shock and this shuttling response was recorded as *avoidance*. If the rat made a crossing upon receiving the footshock, this response was considered as *escape*. If the rat did not respond during the entire 5 s presentation of the shock, the trial was terminated and the next trial started after an intertrial interval lapsed (randomly varied between 30 s to 60 s).

Five days of repeated asenapine testing in adolescence from P 43 to P 47—At the end of the training session (~P 42), rats were first matched based on the number of avoidance response on the last training day (i.e. pre-drug) to create blocks of rats (n = 4 rats/block). Within each block, they were then randomly assigned to 1 of 4 groups: ASE 0.05 mg/kg (ASE 0.05, n = 10), ASE 0.10 mg/kg (ASE 0.10, n = 10), ASE 0.20 mg/kg (ASE 0.20, n = 10) and saline (VEH, n = 10), and tested daily under the CS-only (30 trials/session, no shock) condition for 5 consecutive days. During each drug test, rats were first injected with asenapine or saline, 30 min later, they were placed in the CAR boxes and tested. Ultrasonic vocalizations at the 22 kHz range (20-32 kHz) as a validated measure of anxiolytic effect [35] were also recorded for the first 10 min of testing using Avisoft Recorder software (Version 3.4). Settings included sampling rate at 192 kHz, format 16 bit. 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).

Avoidance retraining and asenapine challenge test on ~P 76—Rats remained in their home cages until ~P 68 when all rats were returned to the CAR boxes for 1 habituation session, followed by 7 days of CAR training/retraining under the CS-US condition (30 trials, shock) to ensure all groups had a comparable level of avoidance responding before the

sensitization assessment which was conducted 1 day after the 7th training session (~P 76) with all rats being injected with asenapine 0.10 mg/kg and tested under the CS-only condition (30 trials, no shock) 30 min later. Numbers of avoidance response and 22 kHz USV were recorded.

Western blot analysis—One day after the challenge test (~P 77), 8 rats randomly selected from each group were sacrificed by live decapitation and their brains were quickly removed. The prefrontal cortex (PFC), striatum and hippocampus were dissected out over ice according to the brain atlas [36], and were frozen on dry ice and stored at -80°C until further analysis.

Tissues from these areas were first homogenized in ice-cold RIPA buffer, containing 25 mM Tris/HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS (Thermo Scientific, Rockford, IL) with Protease Inhibitor Cocktail (Thermo Scientific, Rockford, IL). After centrifugation at 16,000g for 15 min, the supernatant was collected and protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Western blots were performed in duplicate with equal amount of proteins (40-50 µg/lane) using the sodium dodecyl sulfate (SDS)-12% polyacrilamide gel (Bio-Rad, Hercules, CA). Proteins were separated by electrophoresis at 80-120 V for 90 min (BDNF, D₂ receptor and β-actin), then electrophoretically transferred onto Polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) for 60 min at 300 mA in Tris/glycine buffer in a tank transfer system (Bio-Rad, Hercules, CA). The PVDF membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 2 h at room temperature, and then incubated with primary antibodies overnight at 4°C. Immunostaining was carried out using the following antibodies: BDNF (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), D₂ (1:1000, EMD Millipore Corporation, Billerica, MA) and β-actin (1:800, Santa Cruz Biotechnology, Santa Cruz, CA or 1:2000, Sigma-Aldrich, St. Louis, MO). After being washed 3 times in TBS with 0.1% Tween 20 (TBST) at a 10-min interval, the membranes were incubated with Odyssey anti-rabbit, anti-goat or anti-mouse secondary antibodies (Li-COR Biosciences, Lincoln, NE), respectively, with 1:3000-1:5000 dilutions in TBST at room temperature. One hour later, the membranes were washed 3 times at a 10-min interval and the bands were visualized and quantified using Odyssey Fc Imager (Li-COR Biosciences, Lincoln, NE) according to the manufacturer's instructions.

2.6. Experiment 2: Asenapine sensitization and associated FosB protein expression in asenapine-treated rats in the PCP-induced hyperlocomotion test

No prior study has demonstrated asenapine sensitization in this test of antipsychotic activity in adolescent rats. In this experiment, we first validated whether asenapine sensitization is persistent into adulthood in the PCP-induced hyperlocomotion test, and then examined the potential involvement of asenapine-induced change in FosB protein in the sensitization. We decided to examine this protein instead of D₂ and BDNF because results from Experiment 1 did not reveal any significant group differences in the expression of D₂ and BDNF in spite of the conspicuous asenapine sensitization effect. FosB is a transcription factor that plays an important role in long-term neuroplasticity. Chronic administration of antipsychotic drugs causes FosB induction in the striatum and frontal cortex [29, 31, 37].

The experiment was comprised of three phases: Repeated asenapine testing during adolescence, asenapine challenge test during adulthood and FosB Western blotting. Table 2 details the experimental procedure.

Five days of repeated asenapine testing from P41 to P 45—Forty-eight adolescent rats (~P 41) were randomly assigned to 1 of 5 groups: VEH+VEH (saline+saline, n = 16), VEH+PCP (saline+PCP 2.00 mg/kg, n = 8); ASE 0.05+PCP (ASE 0.05 mg/kg+PCP 2.00 mg/kg, n = 8), ASE 0.10+PCP (ASE 0.10 mg/kg+PCP 2.00 mg/kg, n = 8), and ASE 0.20+PCP (ASE 0.20 mg/kg+PCP 2.00 mg/kg, n = 8). They were first handled and habituated to the locomotor activity apparatus for 2 days (30 min/day). On each of the next 5 days, they were first injected with saline, asenapine 0.05, 0.10, or 0.20 mg/kg and then immediately placed in the boxes for 30 min. At the end of the 30-min period, they were taken out and injected with saline or PCP (2.00 mg/kg, sc) and placed back in the boxes for another 60 min. Locomotor activity (number of photobeam breaks) was measured in 5 min intervals throughout the entire 90-min testing session.

Asenapine challenge test during adulthood on ~P 76—Twenty-eight days after the last (5th) asenapine test, after the rats became adults (~P 75), all rats were returned to the locomotor activity boxes for 1 re-habituation session (30 min), followed by the asenapine challenge test 1 day later (~P 76). On the challenge day, the VEH+VEH group was split into two subgroups (n = 8/subgroup): the VEH+VEH-1 group and VEH+VEH-2 group. All rats were first injected with asenapine 0.10 mg/kg and then immediately placed in the locomotor activity boxes for 30 minutes. At the end of the 30-min period, they were taken out and injected with either saline (VEH+VEH-1, n = 8) or PCP (2.00 mg/kg) (VEH+VEH-2 and rats in other groups) and placed back in the boxes for another 60 min. Locomotor activity was recorded for the entire 90-min testing session. Due to a mechanical error, data from 1 rat in the asenapine 0.05+PCP group were lost on the 5th day of asenapine testing, and were excluded from data analysis.

Western blot analysis—As described above, 1 day after the challenge test (~P 76), rats were sacrificed by live decapitation and their brain samples were used for Western blotting of FosB (1:200, Cell Signaling Technology, Danvers, MA) following the protocol described in Experiment 1.

2.7. Statistical Analysis

All data were expressed as mean + SEM. Data from the 5 drug test sessions (e.g. avoidance response and PCP-induced hyperlocomotion) were analyzed using a factorial repeated measures analysis of variance (ANOVA) with the between-subjects factor being drug groups and the within-subjects factor being test days, followed by post hoc LSD tests. Differences between groups on the specific drug test days and on the challenge tests were analyzed using one-way ANOVAs, followed by post hoc LSD tests (for more than 3 groups). For all analyses, $p < 0.05$ was considered statistically significant and all data were analyzed using SPSS version 21.

The BDNF, D₂ and FosB proteins were quantified by normalizing to β-actin, and was either re-probed or co-probed on the same membrane and then calculated as percentage of the corresponding control group (deemed to be 100%). Group and regional differences on the BDNF and D₂ expressions were analyzed using repeated measures ANOVA [4 (group) × 3 (region)]. While the FosB expression was analyzed using repeated measures ANOVA [6 (group) × 3 (region)].

3. Results

3.1. Experiment 1: Asenapine sensitization and associated D₂ and BDNF protein expression in asenapine-treated rats in the conditioned avoidance response test

Repeated asenapine treatment suppressed avoidance response in adolescent rats—Figure 1A shows the mean number of avoidance responses on the last training (pre-drug) day and 5 drug test days. There was no group difference on the last training day (pre-drug). Throughout the 5 drug test days, asenapine increased its suppression of avoidance response progressively and dose-dependently. Two-way repeated measures ANOVA revealed a main effect of group, $F(3, 36) = 20.433, p < 0.001$; day, $F(4, 144) = 26.335, p < 0.001$ and a significant group × day interaction, $F(12, 144) = 2.051, p = 0.024$. Post hoc LSD tests showed that all 3 asenapine groups made significantly fewer avoidance responses than the saline group, all $p < 0.001$, and both ASE 0.10 and ASE 0.20 groups also made significantly fewer avoidance responses than the ASE 0.05 group, $p = 0.032$ and $p = 0.001$, respectively. The group × day interaction was due to the faster decline of avoidance responding in the 3 asenapine groups across the test days and a relatively stable high level of avoidance in the saline group.

Figure 1B shows the mean number of 22 kHz USV counts recorded for the first 10 min on the last training (pre-drug) day and 5 drug test days. There were more 22 kHz USVs on the pre-drug day (with shock) than on the drug test days (no shock). Repeated measures ANOVA revealed a main effect of day, $F(4, 144) = 2.735, p = 0.031$, but no significant main effect of group, $F(3, 36) = 0.789, p = 0.508$ and no significant group × day interaction, $F(12, 144) = 0.973, p = 0.478$, possibly due to the floor effect.

Adolescent asenapine exposure potentiated behavioral reactivity to asenapine re-exposure in adulthood—Figure 2A shows the number of avoidance responses on the 7th retraining day (pre-drug day) and the asenapine challenge test day (~P 76). No significant group difference was found on the pre-drug day. On the challenge day when all rats were injected with ASE 0.10 mg/kg, there was a main effect of group, $F(3, 36) = 8.294, p < 0.001$. All 3 asenapine groups made fewer avoidance responses than the saline group. Post hoc LSD tests showed that the ASE 0.10 and ASE 0.20 groups had significantly lower avoidance than the VEH group, $p = 0.003$ and $p < 0.001$, respectively. The ASE 0.10 group and the ASE 0.20 group also had significantly lower avoidance than the ASE 0.05 group, $p = 0.018$ and $p = 0.001$, respectively. In contrast, the group differences on the 22 kHz USVs on the pre-drug day and on the challenge test day were not significant (Figure 2B), $p > 0.584$.

Levels of BDNF and D₂ receptor expression were not different among the groups—As shown in Figure 3, the group difference of the BDNF protein levels among the 4 groups did not appear to be significant. Two-way repeated measures ANOVA with the group as the between-subjects factor and brain site (e. g. PFC, hippocampus and striatum) as the within-subjects factors did not find a main effect of group, $F(3, 20) = 0.635$, $p = 0.601$ or significant interaction between the two, $F(6, 40) = 0.644$, $p = 0.694$. However, there was a significant main effect of brain site, $F(2, 40) = 4.847$, $p = 0.013$, with a relatively higher level of BDNF expression found in the striatum. Similarly, on the D₂ receptor expression (Figure 4), the main effect of group, $F(3, 28) = 0.149$, $p = 0.929$ and group \times brain site interaction, $F(6, 56) = 0.766$, $p = 0.600$, were not significant, while the main effect of brain site was, $F(2, 56) = 3.486$, $p = 0.037$. Again, the striatum appears to have a higher level of D₂ receptors than other brain regions.

3.2. Experiment 2: Asenapine sensitization and associated FosB protein expression in asenapine-treated rats in the PCP-induced hyperlocomotion test

Repeated asenapine treatment suppressed spontaneous and PCP-induced motor activity in adolescent rats—Figure 5A shows the mean motor activity of the 5 groups of rats during the 30-min period before PCP or saline injection throughout the 5 days of drug testing (a measure of spontaneous motor activity). Two-way repeated measures ANOVA revealed a main effect of group, $F(4, 43) = 7.460$, $p < 0.001$; and a main effect of test day, $F(4, 172) = 36.285$, $p < 0.001$; but no significant group \times day interaction, $F(16, 172) = 1.478$, $p = 0.113$. Post hoc LSD analyses revealed that the 3 asenapine groups had significantly lower motor activity than the VEH+VEH group, all $p < 0.001$. The ASE 0.10+PCP group and the ASE 0.20+PCP group also had significantly lower motor activity than the VEH+PCP group, $p = 0.045$ and $p = 0.010$, respectively, confirming the strong inhibitory effect of asenapine on spontaneous motor activity. The main effect of day was attributed to a sharp decline in motor activity from day 1 to day 2 across all groups.

Figure 5B shows the mean motor activity of 5 groups during the 60-min test period after saline or PCP injection throughout the 5 days of drug testing. Two-way repeated measures ANOVA revealed a main effect of group, $F(4, 42) = 30.489$, $p < 0.001$; and a significant group \times day interaction, $F(16, 168) = 2.447$, $p = 0.002$; but no main effect of day, $F(4, 168) = 2.057$, $p = 0.089$. Post hoc LSD tests revealed that the VEH+PCP group had significantly higher motor activity than the VEH+VEH group, $p < 0.001$, confirming the strong psychomotor activating effect of this dose of PCP. The medium ASE (0.10 mg/kg) dose group and the high ASE (0.20 mg/kg) dose group had significantly lower motor activity than the VEH+PCP group, $p = 0.042$ and $p < 0.001$, respectively. In addition, all 3 asenapine groups differed significantly from each other, all $p < 0.004$. The significant group \times day interaction was mainly due to the progressive increase in motor activity in the ASE 0.05 group and a concurrent decline in the ASE 0.10 and 0.20 groups throughout the test days.

Re-habituation session in adulthood on ~P 75—On ~P 75 before the asenapine challenge test, all rats were placed in the motor activity boxes for 30 min with no drug treatment, and one-way ANOVA did not find any significant group difference, $F(5, 41) = 1.355$, $p = 0.261$ (Figure 6A).

Adolescent asenapine treatment enhanced its inhibition of spontaneous motor activity in the asenapine challenge test on ~P 76—Figure 6B shows the mean motor activity during the 30-min test period before the PCP or saline injection on the asenapine challenge test day (~P 76). One-way ANOVA confirmed a main effect of group, $F(5,41) = 6.192, p < 0.001$. Post hoc LSD tests showed that the ASE 0.10 and ASE 0.20 groups were significantly different from the VEH+VEH-1 group and VEH+VEH-2 group, all $p < 0.004$; and different from the VEH+PCP group, $p = 0.033$ and $p = 0.004$, respectively, indicating a potentiated inhibition of spontaneous motor activity due to adolescent asenapine treatment. The ASE 0.20 group are also different from the ASE 0.05+PCP group, $p = 0.011$.

Figure 6C shows the mean motor activity during the 60-min test period after the saline or PCP injection on the asenapine challenge test day. Independent samples t test found that the VEH+VEH-2 group (treated with PCP) had significantly higher motor activity than the VEH+VEH-1 group (treated with saline), $t(14) = -5.516, p = 0.001$, confirming the psychomotor stimulating effect of PCP. One way ANOVA on the PCP-treated groups (5 groups excluding the VEH+VEH-1 group) found no significant main effect of group, $F(4,34) = 1.860, p = 0.140$.

FosB expression did not differ between groups—As shown in Figure 7, the group and regional differences of the FosB expression did not appear to be significant. Two-way repeated measures ANOVA with the group as the between-subjects factor and brain site (e.g. PFC, striatum and hippocampus) as the within-subjects factors did not find a main effect of group, $F(5, 42) = 0.187, p = 0.332$, or the significant group \times brain site interaction, $F(10, 84) = 1.035, p = 0.422$. However, there was a significant main effect of brain site, $F(2, 84) = 62341, p < 0.001$, with a relatively higher level of FosB expression found in the PFC than in other brain areas.

4. Discussion

Our results show that repeated asenapine treatment in adolescence for only 5 days induced a long-term sensitized inhibition of avoidance responding and spontaneous motor activity that persisted into adulthood. These results are similar to what we previously reported on olanzapine and risperidone [13-16]. The only exception is that this sensitization effect in the PCP test was manifested mainly in the measurement of spontaneous motor activity and less in that of PCP-induced hyperlocomotion. In contrast to our expectation, we did not find any significant group differences on the protein levels of BDNF, D₂ and FosB in adult rats that were treated with asenapine or vehicle in adolescence. Therefore, although repeated adolescent asenapine administration induced a strong across-developmental sensitization effect in two validated behavioral tests of antipsychotic activity, this effect did not appear to be mediated by BDNF, D₂ and FosB.

Previous work on the avoidance-disruptive effect of asenapine has focused on its acute effect [33]. We recently showed that with repeated asenapine administration, its avoidance-disruptive effect intensified across test sessions in adult rats [18, 19]. For example, asenapine at 0.05 mg/kg did not show a significant disruptive effect on avoidance until the 3rd test day. The present study found a similar effect in adolescent rats with the significant

disruption occurred from the 2nd day on. These findings clearly demonstrated that repeated asenapine exposure altered drug sensitivity and this alteration may be stronger in adolescent rats than adult rats. Although the long-lasting nature of asenapine sensitization has been demonstrated before by us in adult rats, showing that asenapine sensitization could last up to 50 days since the last drug treatment [19], the present finding that it could be maintained throughout the adolescent development period into adulthood further solidified this feature, as it suggests that this drug memory effect is resistant to potential alterations due to brain maturation. Because this property of asenapine sensitization is no different from those of olanzapine and risperidone, it may be a general property of antipsychotic drugs except clozapine (clozapine causes a tolerance).

In the present study, we also examined the asenapine sensitization effect in the PCP-induced hyperlocomotion test in adolescent rats, which, to our knowledge, has not been explored before. However, the significant sensitization effect was only found in the asenapine challenge test in the first 30-min test period after ASE 0.10 mg/kg injection, a period that measures the inhibition of asenapine on spontaneous motor activity. No significant sensitization effect was detected in the 60-min test period after PCP challenge. This lack of sensitization in the PCP-induced hyperlocomotion is in contrast to our adolescent olanzapine study, in which we found the sensitization effect in both the spontaneous motor activity and PCP-induced hyperlocomotion [16]. It is also different from our recent adult rat study in which we demonstrated a dose-dependent asenapine sensitization in the PCP-induced hyperlocomotion [18]. The exact causes of these discrepancies are not clear, but may be due to differences in drug property (asenapine vs. olanzapine) or other experimental factors (e.g. 32 days vs. 3 days interval between repeated testing and challenge or PCP drug dose 2.00 vs. 3.20 mg/kg). Given that all antipsychotic drugs suppress both spontaneous motor activity and PCP-induced hyperlocomotion, and both effects putatively reflect their antipsychotic activity [38], it is safe to conclude that adolescent asenapine treatment causes a long-lasting increase in drug sensitivity in adult rats previously exposed to asenapine in adolescence. This conclusion is also consistent with our finding from the CAR test.

In our previous adolescent olanzapine, risperidone and clozapine studies, we noted that there was a rebound of spontaneous motor activity on the re-habituation days when all rats were tested drug-free and presumably in a drug withdrawal state [16, 39]. Rats treated with these drugs were more active in the 30-min period in the test boxes than those previously treated with vehicle. In the present study, we did not observe any rebound effect associated with adolescent asenapine treatment. The exact reason is not clear, as asenapine shares similar affinity and potency for blocking dopamine, serotonin, α -adrenergic and histamine receptors [25, 40]. It could be due to the unique combination of its action on these receptors, such as its preferential actions on 5-HT_{2A}, 5-HT_{2C}, 5-HT_{2B}, 5-HT_{1A}, 5-HT_{1B}, 5-HT₆ and 5-HT₇; D₂ and D₃, α _{2A}, α _{2B}, α _{2C} and H₁. As suggested before, this rebound effect resembles to some extent the antipsychotic withdrawal-induced behavioral hypersensitivity, possibly reflecting drug-induced increase in dopamine neurotransmission via D₂ (especially D₂^{high}) receptors [41-45], the lack of this effect suggests that adolescent asenapine treatment may not alter D₂ receptor levels, a finding consistent with our Western blotting data.

Our previous work demonstrated that asenapine at 0.05 mg/kg actually increased PCP-induced hyperlocomotion, an effect opposite to those of 0.10 and 0.20 mg/kg asenapine in adult rats [18]. This same effect was noted in adolescent rats (Figure 5B and 6C). This enhanced effect on PCP-induced hyperlocomotion may be related to its putative procognitive effect at low doses or its potential antidepressant effect. This notion is based on reports that asenapine at low doses are shown to improve certain cognitive deficits (e.g. reversal learning, cognitive flexibility, object recognition, etc.) induced by PCP or brain lesions [46-48]; and several commonly used antidepressants also increase the PCP-induced hyperlocomotion [49]. Future research should address the clinical relevance of this behavioral effect of asenapine.

In searching for the molecular basis of asenapine sensitization, we focused our attention on D₂, BDNF and FosB for the following reasons. First, all antipsychotic drugs antagonize dopamine D₂ receptors [50] and repeated treatment with certain antipsychotics is shown to increase D₂ expression in the hippocampus and striatum in both adolescent and adult rats [4]. Second, previous work from our lab has shown that risperidone-induced sensitization in the CAR test is related to drug-induced increase in D₂ sensitivity as assessed in the quinpirole (a D_{2/3} agonist)-induced hyperlocomotion test [19]. Pretreatment of quinpirole is also shown to attenuate olanzapine sensitization in the CAR test in adult rats [23]. Third, BDNF is known for its role in brain development and drug-induced neuroplasticity [51-53]. Antipsychotic drugs, especially upon repeated chronic administration, can also alter the brain levels of BDNF [27, 54, 55] (but see [56]) and prevent the stress-induced decrease in the levels of BDNF [27, 54, 56-59]. One recent study suggests that adolescent treatment of lurasidone prevented the reduction of prefrontal BDNF expression in adult rats that were exposed to prenatal stress [60]. Fourth, it is well established that repeated antipsychotic treatment causes an increase in FosB in several forebrain areas (e.g. striatum and prefrontal cortex) and this effect may be dependent on antagonism of D₂ dopamine receptors [29]. Based on these findings, it is reasonable to expect that repeated asenapine treatment in adolescence would induce long-lasting changes in D₂, BDNF and FosB levels in the brain regions implicated in the clinical actions of antipsychotic drugs. However, we did not find any significant group differences in all 3 proteins in adult rats that were exposed to asenapine and those to saline during adolescence. At least three reasons may explain for these negative findings. First, the asenapine treatment might have been too short to induce significant changes. It is possible that 5 days of asenapine was not enough to induce long-lasting changes in D₂, BDNF and FosB that could be detected in adulthood. In fact, much of previous antipsychotic work on these molecules utilized longer treatment regimens (>21 days) [4, 5]. The second reason is that asenapine challenge could potentially alter D₂, BDNF and FosB expression acutely, thus masking the prior adolescent asenapine treatment-induced changes in these proteins. Third, the doses tested might not be optimal to induce robust changes that last throughout the development and the method (Western blotting) may not be sensitive enough. The lack of strong group differences on these proteins seems to suggest that asenapine sensitization from adolescence to adulthood is not likely mediated by the persistent and region-specific changes in D₂, BDNF and FosB. Our finding on BDNF is also consistent with our recent study showing that repeated risperidone treatment in adolescence did not alter BDNF protein levels in the prefrontal cortex, hippocampus and

striatum in the PCP-induced hyperlocomotion test [39]. This conclusion may be premature as more studies are clearly needed to verify our findings.

If D₂, BDNF and FosB are not involved in the mediation of asenapine sensitization, what molecular action of asenapine could explain its long-lasting effect? At this point, we could only speculate. Because asenapine has relatively higher affinities for 5-HT_{2C}, 5-HT_{2A}, 5-HT_{2B}, 5-HT₇, 5-HT₆, α_{2B}, and D₃ receptors relative to D₂ receptor, it is possible that these targets are involved in mediating the clinical actions of asenapine, and possibly its sensitization effect. This possibility is enhanced by the findings that chronic asenapine treatment decreases 5-HT_{2A} receptor binding [61, 62] and dose-dependently increases extracellular dopamine release in the medial prefrontal cortex [33, 63]. Future research needs to examine the asenapine-induced changes in these proteins and identify their roles in asenapine sensitization.

Adolescence (human: 10-19 years old; rats: 35-60 days old) is a period in which the brain and various psychological functions undergo dramatic transitions [64]. It is also the time when symptoms of a variety of severe mental disorders often manifest. Extensive studies have shown that exposure to drugs of abuse during this period increases vulnerability to drug addiction in adulthood and is detrimental to many psychological functions in the long run [65]. The present study, together with several others, reveal that exposure to psychotherapeutic drugs could also alter drug sensitivity and change psychological functions. Some effects are desirable [9-12], while others are not [5, 8]. One important contribution of preclinical antipsychotic research is its ability to comprehensively reveal various behavioral and brain effects due to adolescent antipsychotic treatment. Such knowledge can then be translated into clinical practice to help guide drug dosing for patients with or without prior drug experience.

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Highlights

1. Five days of asenapine treatment in adolescence caused a sensitization in adulthood;
2. Asenapine sensitization was demonstrated in a conditioned avoidance response test;
3. Asenapine sensitization was demonstrated in a phencyclidine-induced hyperlocomotion test;
4. Adolescent asenapine treatment did not cause a long-term change in the levels of BDNF, D₂ receptor and FosB.

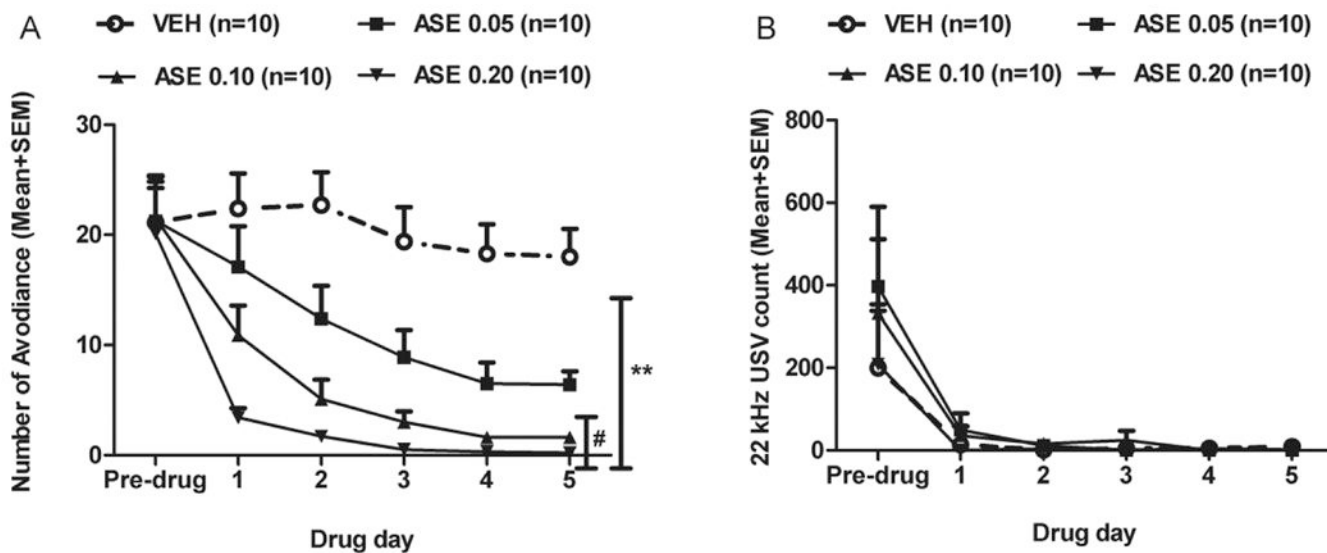


Figure 1.

Repeated asenapine treatment increased the suppression of avoidance response but did not affect 22 kHz USV at adolescence. Number of avoidance responses (**A**) and 22 kHz USV count (**B**) made by the rats from the ASE (0.05 mg/kg), ASE (0.10 mg/kg), ASE (0.20 mg/kg) and vehicle groups on the last training (pre-drug) day and throughout the 5 drug test days are expressed as mean + SEM. $**p < 0.001$, three asenapine groups relative to the VEH group; $\#p < 0.05$, ASE 0.10 and ASE 0.20 groups relative to the ASE 0.05 group respectively.

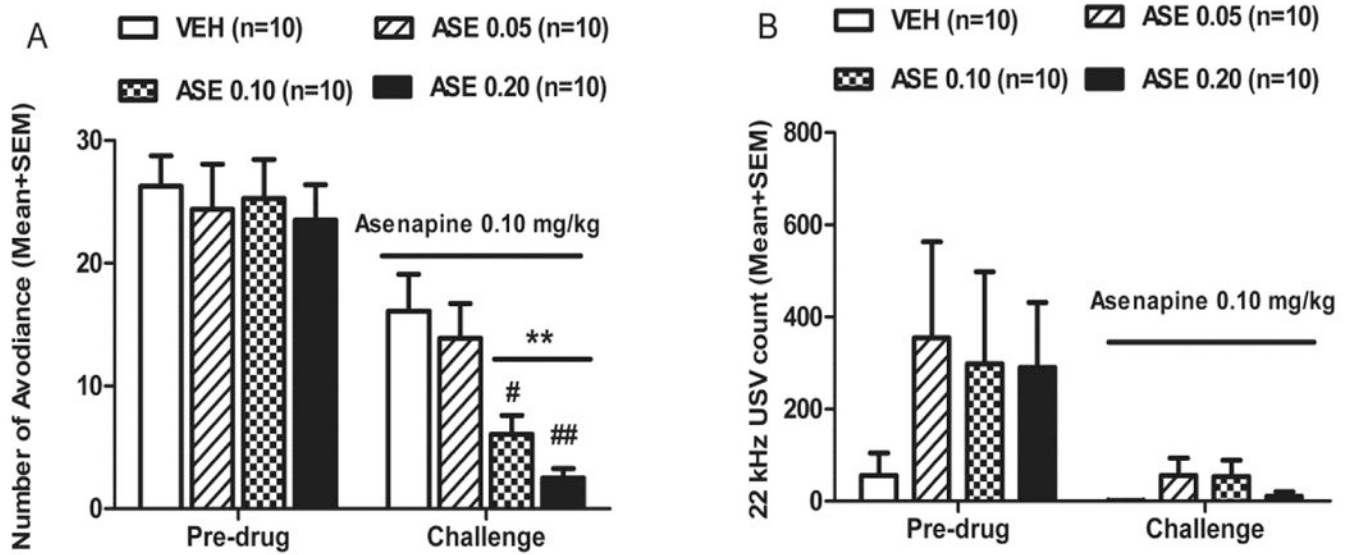


Figure 2.

Prior asenapine treatment increased sensitivity to asenapine re-exposure in the challenge test in adulthood. Number of avoidance responses (**A**) and 22 kHz USV counts (**B**) on the 7th re-training (pre-drug) day and the ASE 0.10 mg/kg challenge test day, are expressed as mean + SEM. ** $p < 0.004$ relative to VEH group; # $p < 0.05$, ## $p = 0.001$ relative to ASE 0.05 group, respectively.

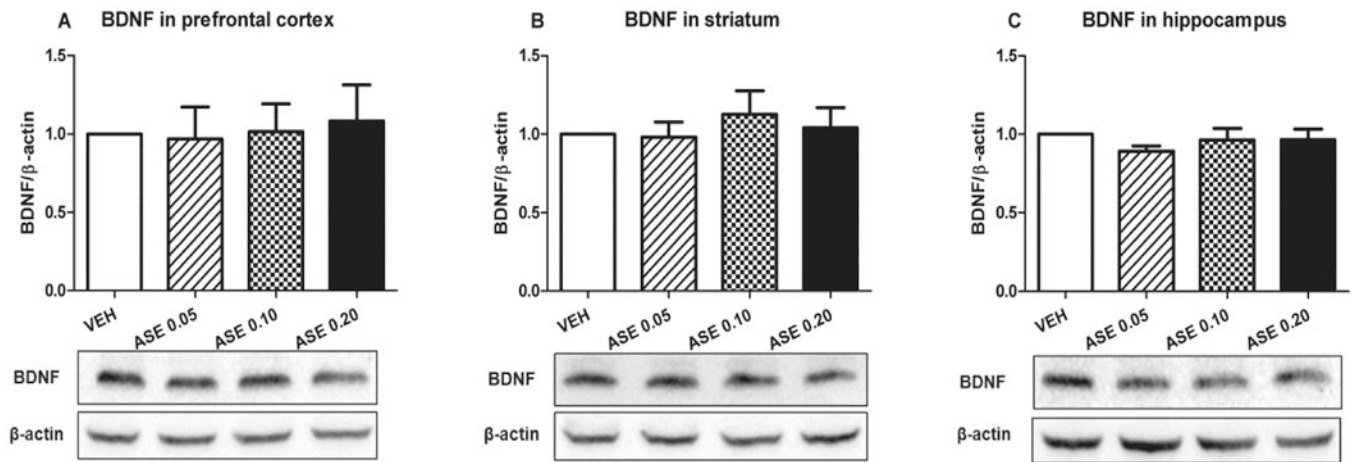


Figure 3.

BDNF protein levels and representative blots in the prefrontal cortex (PFC) (A), striatum (B) and hippocampus (C) in the 4 groups of rats previously treated with vehicle and ASE 0.05, 0.10 or 0.20 mg/kg for 5 days and challenged with ASE 0.10 mg/kg on PND 76. Group data (Mean + SEM, n = 6-8) are expressed as the ratio of mean values in the VEH group.

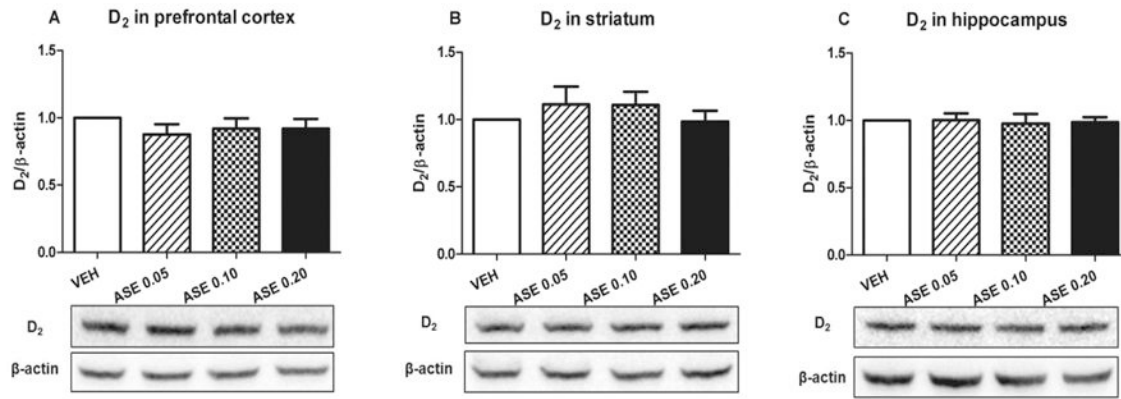


Figure 4.

D₂ receptor protein levels and representative blots in the prefrontal cortex (PFC) (A), striatum (B) and hippocampus (C) in the 4 groups of rats previously treated with vehicle or ASE 0.05, 0.10 or 0.20 mg/kg for 5 days and challenged with ASE 0.10 mg/kg on ~P 76. Group data (Mean + SEM, n = 8) are expressed as the ratio of mean values in the VEH group.

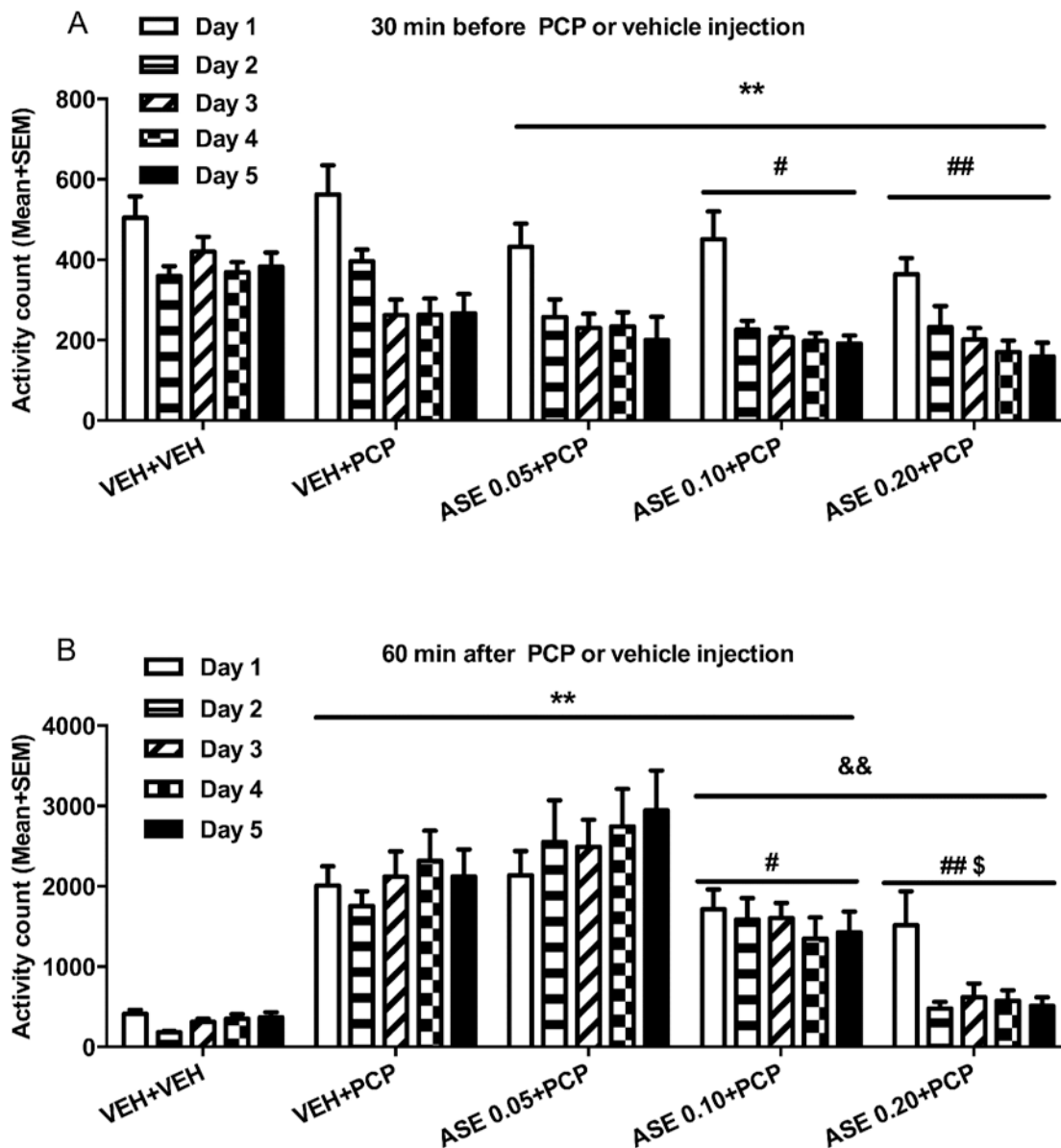


Figure 5.

Asenapine treatment suppressed PCP-induced hyperlocomotion throughout the 5 test days in adolescence. Locomotor activity was measured for 30 min before PCP (2.00 mg/kg) or vehicle injection (**A**) and for 60 min after PCP (2.00 mg/kg) or vehicle injection (**B**) are expressed as mean + SEM for each group (VEH: $n = 16$; others: $n = 8$ /group). ASE (0.05, 0.10 and 0.20 mg/kg) was injected 30 min before the vehicle or PCP (2.00 mg/kg) injection. $**p < 0.001$ relative to VEH+VEH group; $\#p < 0.05$, $##p < 0.010$ relative to VEH+PCP group; $\&\&p < 0.003$ relative to ASE 0.05+PCP group; $\$p = 0.003$ relative to ASE 0.10+PCP group.

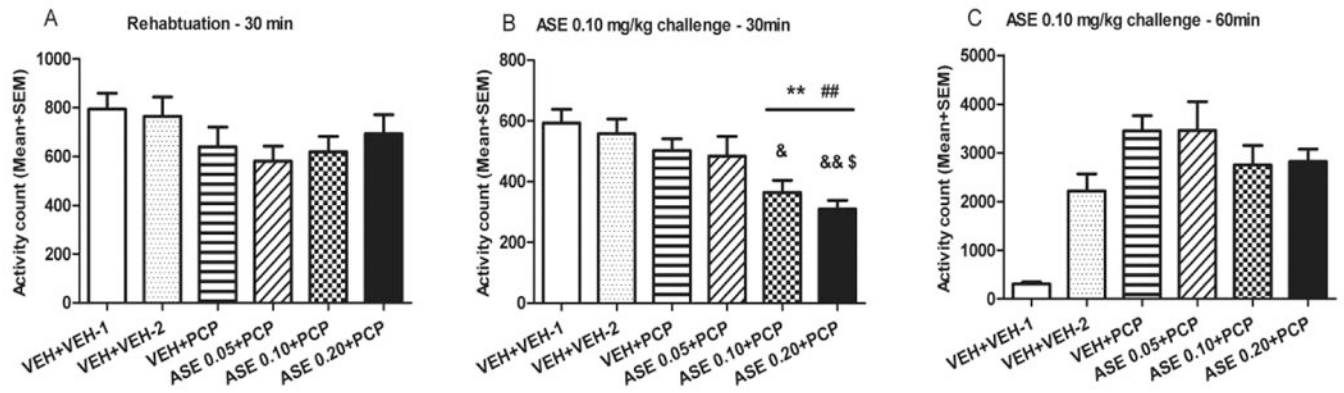


Figure 6.

Adolescent asenapine treatment increased the inhibition of spontaneous motor activity asenapine re-exposure in adulthood. Locomotor activity was measured for 30 min on the re-habituation day (~P 75) and is expressed as mean + SEM (**A**). Locomotor activity was measured for 30 min before PCP (2.00 mg/kg) or vehicle injection (**B**) and for 60 min after PCP (2.00 mg/kg) or vehicle injection (**C**). ASE 0.10 mg/kg was injected 30 min before the PCP (2.00 mg/kg) or vehicle injection. $**p < 0.001$ relative to VEH+VEH-1 group; $###p < 0.004$ relative to VEH+VEH-2 group; $&p < 0.05$, $&&p < 0.005$ relative to VEH+PCP group; $\$p = 0.011$, relative to ASE 0.05+PCP group.

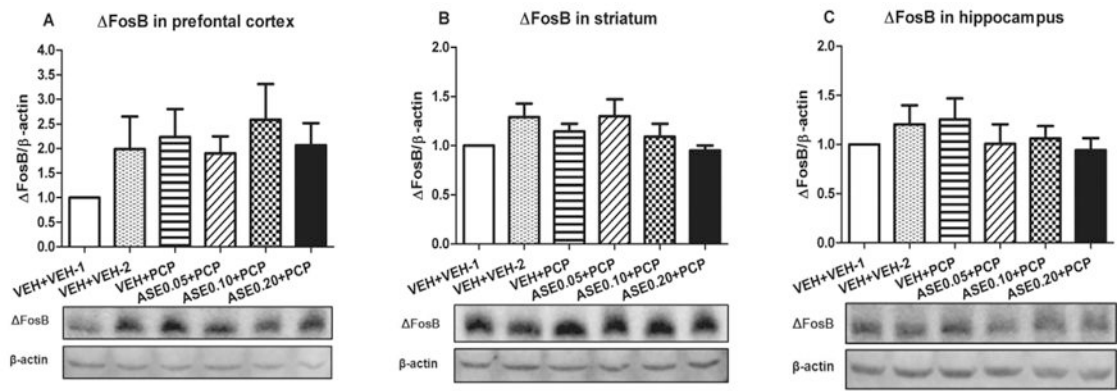


Figure 7.

FosB protein levels and representative blots in the prefrontal cortex (PFC) (A), striatum (B) and hippocampus (C) in the 6 groups of rats previously treated with vehicle, ASE 0.05, 0.10 or 0.20 mg/kg and PCP 2.00 mg/kg for 5 days and challenged with vehicle+vehicle (VEH+VEH-1 group) or ASE 0.10 mg/kg followed by PCP 2.00 mg/kg on ~P 76. Group data (Mean + SEM, n = 8) are expressed as the ratio of mean values in the VEH+VEH-1 group.

Table 1
Timeline of events in Experiment 1 (CAR model)

Days of study	Approximate age (days)	Manipulation
1-2	PND 31-32	Habituation to CAR boxes (30 min/day)
3-12	PND 33-42	10 Days of CAR training (CS-US)
13-17	PND 43-47	5 Days of drug testing (CS-only)
18-37	PND 48-67	Rest
38	PND 68	Habituation to CAR boxes (30 min/day)
39-45	PND 69-75	7 days of CAR retraining (CS-US)
46	PND 76	Challenge test (CS-only): ASE 0.10 mg/kg
47	PND 77	Collection of brain samples

ASE: asenapine; VEH: vehicle; PND: postnatal day.

Table 2
Timeline of events in Experiment 2 (PCP model)

Days of study	Approximate age (years)	Manipulation
1-2	PND 41-42	Habituation to Locomotor boxes (30 min/day)
3-7	PND 43-47	5 Days of drug testing (30+60 min): VEH/ASE (0.05, 0.10, 0.20 mg/kg) + VEH or PCP 2.00 mg/kg
8-34	PND 48-74	Rest
35	PND 75	Habituation to Locomotor boxes (30 min/day)
36	PND 76	Challenge test (30+60 min): ASE 0.10 mg/kg + VEH or PCP 2.00mg/kg
37	PND 77	Collection of brain samples

ASE: asenapine; PCP: phencyclidine; VEH: vehicle; PND: postnatal day.