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Inhibition of GSK3 attenuates amphetamine-induced hyperactivity and sensitization in the mouse

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Abstract

Glycogen synthase kinase 3 (GSK3) is implicated in mediating dopamine-dependent behaviors. Previous studies have demonstrated the ability of amphetamine, which increases extracellular dopamine levels and influences behavior, to regulate the activity of GSK3. This study used valproic acid and the selective GSK3 inhibitor, SB 216763, to examine the role of GSK3 in amphetamine-induced hyperactivity and the development of sensitized stereotypic behavior. Pretreatment with valproic acid (50–300 mg/kg, i.p.) or SB 216763 (2.5–5 mg/kg, i.p.) prior to amphetamine (2 mg/kg, i.p.) significantly reduced amphetamine-induced ambulation and stereotypy. To assess the development of sensitization to the stereotypic effects of amphetamine, mice were pretreated daily with valproic acid (300 mg/kg) or SB 216763 (5 mg/kg) prior to amphetamine (2 mg/kg) for five days. Upon amphetamine challenge (1 mg/kg) seven days later, mice pretreated with valproate or SB 216763 showed a significant attenuation of amphetamine-induced sensitization of stereotypy. To determine whether regulation of GSK3 activity was associated with attenuation of acute amphetamine-induced hyperactivity by valproic acid, valproate (300 mg/kg) or vehicle was injected prior to amphetamine (2 mg/kg) or saline and brain tissue obtained. Analysis of the levels of phospho-GSK3 α and β by immunoblot indicated that valproate increased phosphorylation of ser²¹-GSK3 α in the frontal cortex, as well as ser⁹-GSK3 β in the frontal cortex and caudate putamen of amphetamine-injected mice. These data support a role for GSK3 in acute amphetamine-induced hyperactivity and the development of sensitization to amphetamine-induced stereotypy.

Keywords

GSK3; amphetamine; hyperactivity; sensitization

1. Introduction

Amphetamine is a highly addictive psychostimulant that increases extracellular dopamine levels by inducing dopamine release and reversing the dopamine transporter [1, 2].

Dopamine plays a major role in the rewarding effects of natural reinforcers; however, enhanced dopaminergic transmission is also pertinent to the rewarding and stimulant effects of drugs of abuse [3, 4]. Acute administration of psychostimulants such as amphetamine

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produces an increase in locomotor and stereotypic activities in rodent models. Repeated exposure to psychostimulants can generate an augmentation of these behaviors known as sensitization. The development of amphetamine-induced sensitization is marked by the observation of intense stereotypic behaviors, rather than locomotion [5]. Behavioral sensitization persists after exposure to amphetamine is discontinued [6], thus, it has been hypothesized that the neural underpinnings of behavioral sensitization contribute to addictive behavior and chronic drug use [7].

Recent evidence suggests a role for the intracellular molecule, glycogen synthase kinase 3 (GSK3)¹, in dopaminergic signaling and related behaviors. The enzymatic activity of GSK3 is tightly regulated by upstream kinases such as Akt. Akt participates in dopamine D2 receptor mediated signaling via formation of a complex with β -arrestin 2 and protein phosphatase 2A following receptor activation (reviewed in [8]). Inactivation of GSK3 occurs via phosphorylation on serine²¹ (α -isoform) and serine⁹ (β -isoform), while enhanced activity can result from phosphorylation of tyrosine²⁷⁹-GSK3 α or tyrosine²¹⁶-GSK3 β . In the active state, GSK3 phosphorylates a number of metabolic, signaling, and structural proteins. Aberrant activity of GSK3 has been implicated in many diseases of the central nervous system [9]. GSK3 phosphorylation is altered by exposure to psychostimulants. Acute administration of amphetamine or cocaine decreases levels of phospho-ser⁹-GSK3 β in the dorsal striatum of mice [10, 11], and similar results are observed in the nucleus accumbens core following chronic cocaine and methamphetamine injections [12, 13]. In addition, inhibitors of GSK3 have been shown to decrease horizontal and stereotypic activities in dopamine transporter knockout mice, while amphetamine elicits a blunted locomotor response in GSK3 heterozygote mice [10]. Selective inhibitors of GSK3 also attenuate cocaine-induced hyperactivity in the mouse [11], as well as the development and expression of behavioral sensitization following repeated cocaine or methamphetamine injections [11–13].

Based on evidence implicating GSK3 in dopamine-mediated signaling and behavior, we examined its contribution to amphetamine-induced activity. Specifically, we investigated the effect of valproic acid, a non-selective inhibitor of GSK3, on amphetamine-induced hyperactivity and the development of behavioral sensitization. We also studied the effect of valproic acid on regulation of GSK3 phosphorylation in the caudate putamen, nucleus accumbens, and frontal cortex of amphetamine-treated mice. Finally, we examined the effect of SB 216763, a selective GSK3 inhibitor, on amphetamine-induced hyperactivity and sensitization.

2. Materials and methods

2.1. Animals

Male CD-1 mice (8 weeks old) were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed five per cage (28 × 18 × 14 cm) on a 12-hour light/dark cycle with *ad libitum* access to food and water. Animals were allowed to acclimate to the animal facility for seven days prior to behavioral testing, and were weighed and handled daily. All animal testing was conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Temple University.

¹Abbreviation: GSK3, glycogen synthase kinase 3

2.2. Drugs

D-amphetamine was generously supplied by the National Institute on Drug Abuse (Bethesda, MD) and was dissolved in sterile saline (0.9% NaCl). Sodium valproate was purchased from Sigma Aldrich (St. Louis, MO) and dissolved in sterile saline. Sterile saline was also used for as the vehicle for control injections. The selective inhibitor of GSK3, SB 216763 (Tocris Bioscience; Ellisville, MO), was dissolved in 3.6% DMSO and 89.4% Tocrisolve 100 (Tocris Bioscience). The same vehicle was used for control injections.

2.3. Behavioral Assessment

Behavioral activity was evaluated in automated monitors, each consisting of 16 infrared light emitters and sensors mounted on a frame within which a standard plastic animal cage was placed (45 × 20 × 20 cm) (AccuScan Instruments, Inc., Columbus, OH). The number of photocell beam breaks was recorded by a computer equipped with the Digiscan DMicro system. Activity was divided into ambulation and stereotypy. Ambulatory activity was defined as the number of consecutive beam breaks. Stereotypy was defined by the number of repetitive breaks of the same beam. This automated measure of stereotypy cannot be used to determine the specific activity the mouse was engaged in (ie. sniffing, rearing, grooming), but has been shown to be a reliable measure of stereotypic activity [14, 15].

2.4. Experimental Procedures

2.4.1. Hyperactivity with Valproic Acid Administration—Naïve adult CD-1 mice were placed in the activity monitors for 30 minutes to acclimate, followed by injection of vehicle or valproic acid (50–300 mg/kg, i.p.). Animals received an injection of saline or *D*-amphetamine (2 mg/kg, i.p.) 30 minutes later. Activity was measured for 60 minutes following amphetamine administration.

2.4.2. Immunoblotting—Naïve mice were pretreated with valproic acid (300 mg/kg, i.p.) or saline followed by amphetamine (2 mg/kg, i.p.) or saline 30 minutes later. Sixty minutes following amphetamine injections, the caudate putamen, nucleus accumbens, and frontal cortex were removed by gross dissection. Brain tissues were sonicated in 1% SDS with 1mM NaF and 1mM NaVO₄, boiled for 5 minutes, aliquotted, and stored at –80°C until assayed. Protein concentrations were determined by a modified Lowry assay [16]. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis (7.5% Tris-HCl BioRad Ready-gels, Hercules, CA) and transferred to nitrocellulose membranes for 95 minutes. Membranes were blocked for 1 hour in 5% nonfat dry milk and Tween-TBS solution prior to incubation overnight at 4°C in phospho-GSK3α/β (1: 2,000, Cell Signaling, Beverly, MA) and total GSK3α/β (1: 5,000, Santa Cruz, Santa Cruz, CA) primary antibodies. Membranes were also incubated in anti-tubulin antibody (1: 300,000, Sigma, St. Louis, MO) at room temperature for 90 minutes to correct for protein loading or transfer differences. Membranes were subsequently washed in Tween-TBS and incubated for 1 hour at room temperature with infrared anti-rabbit or anti-mouse secondary antibodies (1: 10,000, Licor Biosciences, Lincoln, NE). Nitrocellulose membranes were imaged and analyzed using the Odyssey infrared imaging system (Licor Biosciences, Lincoln, NE).

2.4.3. Sensitization with Valproic Acid Administration—Mice were pretreated with vehicle or valproic acid (300 mg/kg, i.p.) 30 minutes prior to an injection of saline or *D*-amphetamine (2 mg/kg, i.p.) once daily for five days. Stereotypic activity was recorded for 30 minutes following each daily injection. Following drug administration on day 5, mice were left untreated for six days. On day 12, all mice were challenged with *D*-amphetamine (1 mg/kg, i.p.) in the absence of valproate, and stereotypic activity was measured for 30 minutes.

2.4.4. Hyperactivity with SB 216763 Administration—Naïve mice were pretreated with vehicle or SB 216763 (2.5–5 mg/kg, i.p.) 20 minutes prior to an injection of saline or *D*-amphetamine (2 mg/kg, i.p.). Activity was measured for 60 minutes following amphetamine administration.

2.4.5. Sensitization with SB 216763 Administration—Mice were pretreated with vehicle or SB 216763 (5 mg/kg, i.p.) 20 minutes prior to an injection of saline or *D*-amphetamine (2 mg/kg, i.p.) once daily for five days. All animals were challenged with *D*-amphetamine (1 mg/kg, i.p.) on day 12 and stereotypy was measured for 30 minutes.

2.5. Statistical Analysis

Time course data reflecting acute amphetamine-induced hyperactivity were analyzed by a two-way repeated measures ANOVA with time and treatment group as main factors. Cumulative hyperactivity counts and the quantification of immunoblots were analyzed by two-way ANOVA with pretreatment (valproic acid, SB216763, or vehicle) and amphetamine administration as main factors. Significant ANOVAs were followed by Bonferroni post-hoc analysis for multiple comparisons (GraphPad Prism 4.0).

3. Results

3.1. Attenuation of acute amphetamine-induced activity by valproic acid

Mice were pretreated with valproic acid (300 mg/kg) or vehicle, followed by amphetamine (2 mg/kg, i.p.) or saline, and hyperactivity was measured for 60 minutes. The time course of ambulatory activity is represented in Fig. 1A. A two-way repeated measures ANOVA with time and treatment factors revealed significant differences between groups (Interaction: $F(33,440) = 2.682$, $p < 0.0001$; Time: $F(11,440) = 1.414$, $p > 0.05$; Treatment: $F(3,440) = 3.440$, $p < 0.001$). Bonferroni post-tests indicated that amphetamine significantly increased ambulatory activity compared to vehicle-saline controls (veh-amph vs. veh-sal). Pretreatment with valproic acid (300 mg/kg) significantly reduced amphetamine-induced ambulation compared to vehicle-amphetamine treated animals (VPA300-amph vs. veh-amph).

Cumulative ambulatory activity counts over 60 minutes for three doses of valproic acid are shown in Fig. 1B. Two-way ANOVA of the data showed significant interaction, pretreatment, and treatment effects (Interaction: $F(3,80) = 2.757$, $p < 0.05$; Pretreatment: $F(3,80) = 3.758$, $p < 0.05$; Treatment: $F(1,80) = 12.64$, $p < 0.001$). Post-hoc analysis revealed that amphetamine significantly increased ambulatory activity compared to saline injected controls (veh-amph vs. veh-sal, $p < 0.01$). Amphetamine-induced ambulation was significantly attenuated by 300 mg/kg of valproic acid (VPA300-amph vs. veh-amph, $p < 0.001$). Ambulatory activity was not significantly affected by lower doses of valproic acid (VPA50-amph vs. veh-amph, VPA150-amph vs. veh-amph, $p > 0.05$). Valproate did not alter basal ambulatory activity at any dose tested (VPA50-sal vs. veh-sal, VPA150-sal vs. veh-sal, VPA300-sal vs. veh-sal, $p > 0.05$).

Stereotypic activity recorded following amphetamine (2 mg/kg) or saline injection is shown in Fig. 1C for mice pretreated with valproic acid (300 mg/kg) or vehicle. Two-way repeated measures ANOVA demonstrated significant differences between treatment groups (Interaction: $F(33,440) = 3.705$, $p < 0.0001$; Time: $F(11,440) = 2.983$, $p < 0.001$; Treatment: $F(440) = 7.896$, $p < 0.0001$). Bonferroni post-hoc analysis revealed that amphetamine produced a significant increase in stereotypic behaviors compared to saline-injected controls (veh-amph vs. veh-sal). Pretreatment with valproic acid (300 mg/kg) significantly decreased stereotypic activity in amphetamine-treated mice (VPA300-amph vs. veh-amph).

Cumulative stereotypic activity over 60 minutes for three doses of valproic acid is represented in Fig. 1D. Two-way ANOVA of the results revealed significant interaction, pretreatment, and treatment effects (Interaction: $F(3,80) = 2.857, p < 0.05$; Pretreatment: $F(3,80) = 4.258, p < 0.01$; Treatment: $F(1,80) = 14.23, p < 0.001$). Mice injected with vehicle-amphetamine exhibited a significant increase in stereotypy compared to vehicle-saline injected mice (veh-amph vs. veh-sal, $p < 0.001$). Pretreatment with valproic acid dose-dependently attenuated amphetamine-induced stereotypy (VPA50-amph vs. veh-amph, $p < 0.05$; VPA150-amph vs. veh-amph, $p < 0.01$; VPA300-amph vs. veh-amph, $p < 0.001$). Valproic acid alone had no effect on stereotypy (VPA50-sal vs. veh-sal, VPA150-sal vs. veh-sal, VPA300-sal vs. veh-sal, $p > 0.05$).

3.2. Regulation of phosphorylation of GSK3 in brains from amphetamine-treated mice

Mice were pretreated with valproic acid (300 mg/kg, i.p.) or vehicle 30 minutes prior to amphetamine (2 mg/kg, i.p.), and brain tissue was obtained 60 minutes later. Protein extracts from the caudate putamen, nucleus accumbens, and frontal cortex were analyzed by immunoblot for levels of phosphorylated and total GSK3 α and β and tubulin (Fig. 2). Analysis by two-way ANOVA showed that levels of phosphorylated ser²¹-GSK3 α (ratio of pGSK3: GSK3) in the caudate putamen were similar among experimental groups (Interaction: $F(1, 36) = 3.454, p > 0.05$; Pretreatment: $F(1, 36) = 0.895, p > 0.05$; Treatment: $F(1, 36) = 2.164, p > 0.05$) (Fig. 2A). Significant interaction and pretreatment effects were detected for levels of phosphorylated ser⁹-GSK3 β in the caudate putamen (Interaction: $F(1, 36) = 5.225, p < 0.05$; Pretreatment: $F(1, 36) = 3.685, p < 0.05$; Treatment: $F(1, 36) = 6.380, p > 0.05$) (Fig. 2B). Post-hoc analysis showed significant elevations in phosphorylated ser⁹-GSK3 β in mice injected with VPA-amphetamine compared to vehicle-amphetamine (veh-amph vs. VPA-amph, $p < 0.01$) or valproate-saline (veh-amph vs. VPA-sal, $p < 0.05$).

In the nucleus accumbens, levels of p-ser²¹-GSK3 α (Interaction: $F(1, 34) = 3.025, p > 0.05$; Pretreatment: $F(1, 34) = 0.542, p > 0.05$; Treatment: $F(1, 34) = 2.636, p > 0.05$) (Fig. 2C) and p-ser⁹-GSK3 β were not significantly different between groups (Interaction: $F(1, 34) = 1.286, p > 0.05$; Pretreatment: $F(1, 34) = 0.080, p > 0.05$; Treatment: $F(1, 34) = 0.120, p > 0.05$) (Fig. 2D).

In the frontal cortex, significant interaction effects were detected for levels of phosphorylated ser²¹-GSK3 α (Interaction: $F(1, 36) = 10.14, p < 0.01$; Pretreatment: $F(1, 36) = 1.309, p > 0.05$; Treatment: $F(1, 36) = 0.0001, p > 0.05$) (Fig. 2E), and post-hoc analysis showed significantly higher levels of GSK3 α in the frontal cortex of mice pretreated with valproate prior to amphetamine compared to vehicle prior to amphetamine (veh-amph vs. VPA-amph, $p < 0.01$). Significant interaction effects were also found for levels of phosphorylated ser⁹-GSK3 β in the frontal cortex (Interaction: $F(1, 36) = 0.021, p < 0.05$; Pretreatment: $F(1, 36) = 0.077, p > 0.05$; Treatment: $F(1, 36) = 0.488, p > 0.05$) (Fig. 2F). Post-hoc analysis revealed significantly higher levels of phosphorylated ser⁹-GSK3 β in the frontal cortex of mice administered valproate-amphetamine compared to vehicle-amphetamine (veh-amph vs. VPA-amph, $p < 0.05$).

Total levels of GSK3 α and GSK3 β in each of the three brain regions tested were similar in all experimental groups (GSK3: tubulin; data not shown). Representative Western blots for phosphorylated and total ser²¹-GSK3 α and ser⁹-GSK3 β in each of the three brain regions analyzed are shown for mice administered VPA-amphetamine and vehicle-amphetamine in Fig. 3. Because GSK3 is inactivated by phosphorylation on ser²¹ (α) or ser⁹ (β), these results demonstrate that valproic acid inhibited GSK3 α and GSK3 β activity in the frontal cortex, as well as GSK3 β activity in the caudate putamen of amphetamine-treated animals.

3.3. Valproic acid attenuated amphetamine-induced behavioral sensitization

To test the effect of valproic acid on the development of amphetamine-induced sensitization of stereotypy, mice were pretreated daily with valproic acid (300 mg/kg, i.p.) or vehicle prior to amphetamine (2 mg/kg, i.p.) or saline for 5 days, and stereotypy was measured (Fig. 4). Following a drug-free period of six days, all mice were challenged with amphetamine (1 mg/kg) in the absence of valproate on day 12, and stereotypy was measured (Fig. 4B). Cumulative stereotypic activity measured for 30 minutes following amphetamine injections on days 1–5 was analyzed to investigate the development of sensitization to stereotypy within treatment groups (Fig. 4A). A two-way ANOVA indicated significant interaction, treatment group, and treatment day effects (Interaction: $F(12,168) = 6.581$, $p < 0.0001$; Treatment group: $F(3,168) = 13.65$, $p < 0.0001$; Treatment day: $F(4,168) = 10.82$, $p < 0.0001$). Bonferroni post-tests revealed that mice injected with daily vehicle-amphetamine exhibited significantly greater stereotypy counts on days 2–5 compared to day 1 (day 1 vs. day 2, $p < 0.01$; day 1 vs. days 3,4,5, $p < 0.001$), suggesting the development of amphetamine-induced sensitization. Mice administered valproic acid prior to daily amphetamine also showed a significant increase in stereotypy counts on days 4–5 compared to day 1 (day 1 vs. days 4,5, $p < 0.001$). Stereotypic activity counts measured on days 2–5 were not significantly different from day 1 in mice administered daily vehicle-saline or valproate-saline. Stereotypic activity was significantly lower each day in valproate-pretreated mice compared to those pretreated with vehicle prior to amphetamine (veh-amph vs. VPA300-amph, day 1, $p < 0.01$; days 2–5, $p < 0.001$).

The expression of a sensitized response to an amphetamine challenge was assessed between groups on day 12 (Fig. 4B). Two-way ANOVA of the data revealed significant interaction, pretreatment, and treatment effects (Interaction: $F(1,42) = 8.73$, $p < 0.05$; Pretreatment: $F(1,42) = 6.956$, $p < 0.05$; Treatment: $F(1,42) = 11.46$, $p < 0.01$). Bonferroni post-hoc analysis showed significantly higher stereotypy counts in mice injected daily with vehicle-amphetamine compared to vehicle-saline controls (veh-amph vs. veh-sal, $p < 0.001$), demonstrating a sensitized response to the stereotypic effects of amphetamine. In addition, daily pretreatment with valproic acid significantly attenuated amphetamine-induced sensitization of stereotypy (VPA300-amph vs. veh-amph, $p < 0.001$).

3.4. Attenuation of acute amphetamine-induced activity by a selective GSK3 inhibitor

Since valproic acid attenuated amphetamine-induced hyperactivity and the development of sensitization, and altered phosphorylation of GSK3 in mice injected with amphetamine, the effect of a selective inhibitor of GSK3 was also tested. Mice were pretreated with SB 216763 (2.5–5 mg/kg, i.p.) or vehicle 20 minutes prior to an injection of amphetamine (2 mg/kg, i.p.) or saline. The time course of changes in amphetamine-induced ambulatory activity produced by SB 216763 is shown in Fig. 5A. A two-way repeated measures ANOVA of the ambulatory time course indicated significant differences between treatment groups (Interaction: $F(33,462) = 2.004$, $p < 0.01$; Time: $F(11,462) = 9.653$, $p < 0.0001$; Treatment: $F(3,462) = 15.60$, $p < 0.0001$). Bonferroni post-tests showed that amphetamine significantly increased ambulatory activity (veh-amph vs. veh-sal). Pretreatment with SB 216763 (5 mg/kg) significantly decreased ambulatory behavior in amphetamine-treated mice compared to vehicle pretreatment (SB 5.0-amph vs. veh-amph). However, mice administered SB 216763 prior to amphetamine showed significantly higher ambulatory counts than saline-injected controls (SB 5.0-amph vs. veh-sal), suggesting only a partial reversal of amphetamine-induced ambulation.

Cumulative ambulatory activity counts over 60 minutes following two doses of SB 216763 and amphetamine or saline are shown in Fig. 5B. Two-way ANOVA of the ambulatory data revealed significant pretreatment and treatment effects (Interaction: $F(2,60) = 2.992$, $p > 0.05$;

Pretreatment: $F(2,60) = 6.762, p < 0.001$; Treatment: $F(1,60) = 47.94, p < 0.0001$). Post-hoc analysis showed that ambulatory activity in mice administered vehicle-amphetamine was significantly higher than that in vehicle-saline treated controls (veh-amph vs. veh-sal, $p < 0.001$). Amphetamine administration also generated significantly greater ambulatory counts in mice pretreated with SB 216763 compared to saline treated controls (SB 2.5-amph vs. SB 2.5-sal, SB 5.0-amph vs. SB 5.0-sal, $p < 0.05$). Amphetamine-induced ambulation was significantly attenuated by 2.5 mg/kg and 5 mg/kg of SB 216763 (SB 2.5-amph vs. veh-amph, $p < 0.01$; SB 5.0-amph vs. veh-amph, $p < 0.001$). Baseline ambulatory activity counts were not significantly affected by SB 216763 (SB 2.5-sal vs. veh-sal, SB 5.0-sal vs. veh-sal, $p > 0.05$).

The time course of stereotypic activity in mice pretreated with SB 216763 (5 mg/kg) or vehicle prior to amphetamine (2 mg/kg) or saline is shown in Fig. 5C. Two-way ANOVA demonstrated that there were significant differences between treatment groups (Interaction: $F(33,462) = 2.256, p < 0.0001$; Time: $F(11,462) = 10.61, p < 0.0001$; Treatment: $F(3,462) = 16.92, p < 0.0001$). Bonferroni post-hoc analysis showed an increase in stereotypy in mice injected with amphetamine (veh-amph vs. veh-sal), and pretreatment with SB 216763 (5 mg/kg) significantly reduced this effect (SB 5.0-amph vs. veh-amph). Mice administered SB 216763 prior to amphetamine had significantly higher stereotypy counts than saline-injected controls (SB 5.0-amph vs. veh-sal).

Cumulative stereotypy counts over the 60 minutes following amphetamine (2 mg/kg) or saline injection are shown in Fig. 5D. Analysis by a two-way ANOVA revealed significant interaction and treatment effects (Interaction: $F(2,60) = 3.644, p < 0.05$; Pretreatment: $F(2,60) = 3.106, p > 0.05$; Treatment: $F(1,60) = 54.34, p < 0.0001$). Bonferroni post-hoc analysis showed a significant increase in stereotypy in mice administered vehicle-amphetamine compared to those injected with vehicle-saline (veh-amph vs. veh-sal, $p < 0.001$). Animals pretreated with SB 216763 (2.5–5 mg/kg) prior to amphetamine also exhibited significantly more stereotypy counts than their respective saline injected controls (SB 2.5-amph vs. SB 2.5-sal, $p < 0.01$; SB 5.0-amph vs. SB 5.0-sal, $p < 0.05$). Pretreatment with SB 216763 significantly reduced amphetamine-induced stereotypy compared to vehicle-amphetamine controls (SB 2.5-amph vs. veh-amph, $p < 0.05$; SB 5.0-amph vs. veh-amph, $p < 0.01$). SB 216763 alone had no effect on stereotypic activity (SB 2.5-sal vs. veh-sal, SB 5.0-sal vs. veh-sal, $p > 0.05$).

3.5. SB 216763 attenuated amphetamine-induced behavioral sensitization

In order to investigate the role of GSK3 in the development of amphetamine-induced sensitization of stereotypy, mice were pretreated daily with SB 216763 (5 mg/kg, i.p.) or vehicle prior to amphetamine (2 mg/kg, i.p.) or saline for 5 days. Mice remained drug free for six days and were challenged with amphetamine (1 mg/kg) in the absence of SB 216763 on day 12. Cumulative stereotypy counts measured following amphetamine injections on days 1–5 are shown in Fig. 6A. Two-way ANOVA revealed significant interaction, treatment group, and treatment day effects (Interaction: $F(12,128) = 6.745, p < 0.0001$; Treatment group: $F(3,128) = 14.14, p < 0.0001$; Treatment day: $F(4,128) = 19.00, p < 0.0001$). Bonferroni post-tests indicated that mice injected daily with vehicle-amphetamine showed significantly higher stereotypy counts on days 2–5 compared to day 1 (day 1 vs. day 2, $p < 0.01$; day 1 vs. days 3,4,5, $p < 0.001$). Mice injected daily with SB 216763 prior to amphetamine showed a significant increase in stereotypy counts on days 3–5 compared to day 1 (day 1 vs. day 3, $p < 0.05$; day 1 vs. days 4,5, $p < 0.001$). Stereotypic activity counts measured on days 2–5 were unaltered compared to day 1 in mice administered daily vehicle or SB 216763 prior to saline. These results suggest that mice administered daily amphetamine developed significant sensitization to stereotypy as assessed within groups, whereas mice injected daily with saline did not. In addition, stereotypic activity was

significantly lower in mice administered SB 216763 prior to amphetamine compared to those pretreated with vehicle on days 2–5 (veh-amph vs. SB 5.0-amph, day 2, $p<0.05$; day 3, $p<0.01$; days 4–5, $p<0.001$).

Stereotypic activities following an amphetamine challenge on day 12 were analyzed to examine the expression of sensitization between treatment groups, and are shown in Fig. 6B. Two-way ANOVA indicated significant interaction and treatment effects (Interaction: $F(1,32) = 4.476$, $p<0.05$; Pretreatment: $F(1,32) = 1.928$, $p>0.05$; Treatment: $F(1,32) = 6.017$, $p<0.05$). Bonferroni post-hoc analysis revealed a significant increase in stereotypic activity in response to the amphetamine challenge in mice injected daily with vehicle-amphetamine compared to those injected with vehicle-saline (veh-amph vs. veh-sal, $p<0.01$). Mice pretreated daily with SB 216763 prior to amphetamine exhibited significantly lower stereotypic activity compared to vehicle-pretreated mice (SB 5.0-amph vs. veh-amph, $p<0.05$), indicating an attenuation of amphetamine-induced sensitization.

4. Discussion

The findings presented here demonstrate that GSK3 makes a functional contribution to the acute motor-activating and sensitizing effects of amphetamine. Valproic acid dose-dependently reduced amphetamine-induced ambulation and stereotypy, while reducing the activity of GSK3 (increasing phosphorylation on GSK3) in the caudate putamen and frontal cortex of amphetamine-treated mice. In addition, selective inhibition of GSK3 by SB 216763 reduced ambulatory and stereotypic behaviors produced by amphetamine. Daily pretreatment with valproic acid or SB 216763 attenuated sensitization to the stereotypic effects of amphetamine.

Psychostimulant-induced hyperactivity has largely been attributed to enhancement of dopaminergic transmission within components of the mesolimbic and nigrostriatal dopamine pathways. Dopaminergic cell bodies of the mesolimbic system originate in the ventral tegmental area and project to the nucleus accumbens and prefrontal cortex, while those of the nigrostriatal pathway reside in the substantia nigra and project to the caudate putamen [17–20]. Acute exposure to amphetamine dose-dependently increases extracellular dopamine levels in the caudate putamen, nucleus accumbens, and prefrontal cortex [21], which is necessary for its motor-stimulating properties. Studies suggest that amphetamine-induced locomotor and stereotypic activities are primarily mediated by dopamine in the nucleus accumbens and caudate putamen, respectively. Depletion of dopamine via 6-hydroxydopamine lesions within the nucleus accumbens prevents the characteristic increase in amphetamine-induced locomotion [22]. In contrast, bilateral lesions of the caudate putamen with 6-OHDA inhibit stereotypic activities generated by amphetamine [23]. The contribution of dopaminergic innervation in the frontal cortex to motor behaviors associated with amphetamine is less clear [24, 25]. However, due to the therapeutic actions of amphetamines in the treatment of ADHD, many studies implicate a role for the frontal cortex in the action of this stimulant (reviewed in [26]).

Valproic acid is a widely used therapeutic agent for the treatment of epilepsy and bipolar disorder. Several mechanisms of action have been implicated in its anticonvulsant and mood-stabilizing properties. These include enhancement of GABAergic activity, direct histone deacetylase inhibition, and inhibition of GSK3 [27]. Previous studies demonstrate that valproic acid can inhibit dopamine-dependent behaviors [10]. Data from the present study suggest that the ability of valproic acid to inhibit amphetamine-induced behavior is associated with its ability to modulate GSK3 activity in the caudate putamen and frontal cortex. Specifically, our results demonstrate a dose-dependent decrease in amphetamine-induced hyperactivity following pretreatment with valproic acid. We also showed that

administration of valproate to amphetamine-treated mice increased phosphorylation of ser⁹-GSK3 β in the caudate putamen, as well as phospho-ser²¹ and ser⁹ in the frontal cortex, suggesting an inhibition of the activity of the kinase in these regions. Phosphorylation of GSK3 was not altered in any brain region by vehicle-amphetamine administration compared to vehicle-saline. In a previous study, a significant decrease in striatal phosphorylated GSK3 is reported at 90 minutes following an amphetamine injection, but not at 60 minutes [10]. Our results are consistent with these findings, as brain tissue was obtained 60 minutes following amphetamine administration in the present study.

Our results support the hypothesis that valproate attenuates amphetamine-induced hyperactivity via regulation of GSK3. However, because valproate is not selective for GSK3, we chose to further investigate whether GSK3 plays a functional role in acute amphetamine-induced behavior by utilizing the selective inhibitor, SB 216763. Inhibition of GSK3 with SB 216763 (2.5–5 mg/kg) prior to acute amphetamine administration attenuated the characteristic increases in ambulatory and stereotypic activities. These results agree with others emphasizing the importance of GSK3 in behavior resulting from enhanced dopaminergic transmission [10, 11, 28]. Specifically, various selective GSK3 inhibitors including SB 216763, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD), alsterpaullone, and indirubin-3-monoxime, reduce hyperactivity in dopamine transporter knockout mice [10]. Administration of SB 216763 (1–7.5 mg/kg) also attenuates hyperactivity produced by cocaine, as well as SKF-82958, a selective D1 receptor agonist [11, 28]. Of note, 2.5–7.5 mg/kg of SB 216763 completely attenuated cocaine-induced activity [11], but only partially attenuated hyperactivity produced by SKF-82958 [28], similar to the present findings with amphetamine. Transgenic mice overexpressing GSK3 β exhibit enhanced locomotor activity compared to wild-type controls, as do mutant mice rendered resistant to GSK3 inactivation by protein kinase B (Akt) [29, 30]. These findings, as well as the present experimental results, convey the importance of GSK3 in mediating behaviors produced by acute exposure to psychostimulants in mice.

In addition to investigating the function of GSK3 in the acute effects of amphetamine, we sought to determine whether this kinase plays a role in the development of behavioral sensitization. Sensitization is defined as an augmented response following repeated, intermittent exposure to a drug. Sensitization of both locomotor and stereotypic activity can be generated by repeated exposure to psychostimulants (reviewed in [7]), however, sensitization to amphetamine-like stimulants manifests as intensified stereotypic behavior which competes with locomotion [5]. Data from the present study demonstrate that pretreatment with valproic acid prior to daily amphetamine attenuated amphetamine-induced sensitization of stereotypy. These data are in conjunction with studies evaluating the effect of valproic acid on sensitization to other psychostimulants. For example, pretreatment with valproic acid inhibits the development and expression of sensitization to methylphenidate in mice [31, 32], as well as methamphetamine and cocaine [33].

As described above, valproic acid has several actions including regulation of GABAergic neurotransmission, histone deacetylase inhibition, and inhibition of GSK3. Valproic acid has been shown to increase GABA levels by several metabolic processes such as decreasing degradation by GABA transaminase, increasing the availability of the precursor α -ketoglutarate, and enhancing the activity of glutamate decarboxylase, an enzyme required for the production of GABA (for review [34]). Enhancement of GABA tone could attenuate psychostimulant-induced sensitization. Administration of the GABA_B receptor agonist, baclofen, prevents the development and expression of behavioral sensitization to amphetamine and cocaine [35–37]. Although valproic acid also inhibits histone deacetylases, current literature does not support the involvement of this mechanism in the inhibition of psychostimulant-mediated behavior. Exposure to psychostimulants increases

histone acetylation, a process generally associated with increases in gene transcription (for review, see [38]). Not surprisingly, histone deacetylase inhibitors can potentiate psychostimulant-induced behaviors. For example, administration of sodium butyrate enhances both induction and expression of cocaine-induced locomotor sensitization [39]. Thus, it is unlikely that valproate inhibits the development of the sensitized response to amphetamine via histone deacetylase inhibition. An additional mechanism potentially underlying the attenuation of sensitization to amphetamine by valproic acid is inhibition of GSK3. Although our data demonstrate that valproic acid inhibited sensitization to amphetamine, it was unclear whether this effect was a result of GSK3 inhibition due to valproic acid's non-selective properties. Therefore, we further investigated the role of this kinase in amphetamine-induced sensitization to stereotypy using SB 216763. Our results indicate that inhibition of GSK3 prior to daily amphetamine also attenuates sensitization, as sensitized stereotypy was blunted upon an amphetamine challenge. These results are consistent with previous reports demonstrating an essential role for GSK3 in the development of cocaine and methamphetamine-induced locomotor sensitization [11–13], suggesting that activation of GSK3 is an important component of the development of sensitization to psychostimulants.

5. Conclusions

The results of the present study suggest that activation of GSK3 is important for the acute and sensitized stimulant effects of amphetamine. Further investigation of the physiological role of GSK3 in the behavioral and neurochemical adaptations produced by psychostimulants may provide pertinent insight regarding the mechanisms of neuroplasticity produced by drugs of abuse.

Highlights

- Amphetamine-induced hyperactivity was attenuated by valproate or SB 216763
- Valproic acid increased GSK3 phosphorylation in amphetamine-treated mice
- Valproic acid and SB 216763 attenuated the development of amphetamine-induced sensitization
- We conclude that GSK3 activation is important for amphetamine-induced hyperactivity and the development of sensitization to stereotypy

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Fig. 1. Valproate attenuated acute amphetamine-induced behavioral activity

(A) The time course of ambulatory activity is shown for mice injected with vehicle or valproic acid (300 mg/kg) prior to amphetamine (2 mg/kg) or saline. Pretreatment with valproic acid significantly attenuated amphetamine-induced ambulation compared to saline-injected controls ($***p<0.001$, $**p<0.01$, $*p<0.05$). (B) Cumulative ambulatory activity for 60 minutes post-amphetamine or saline is shown for mice pretreated with valproate (5–300 mg/kg) or vehicle. Amphetamine significantly increased ambulatory activity counts compared to mice treated with saline or valproate (50 mg/kg) ($###p<0.001$, $#p<0.05$). Valproic acid (300 mg/kg) significantly decreased amphetamine-induced locomotion versus vehicle ($***p<0.001$). (C) The time course of changes in stereotypic activity is shown for mice pretreated with valproic acid (300 mg/kg) or vehicle prior to amphetamine (2 mg/kg) or saline. Valproate significantly reduced the number of stereotypy counts observed in amphetamine-injected mice ($***p<0.001$, $**p<0.01$). (D) Stereotypy counts recorded from mice pretreated with valproate (50–300 mg/kg) or vehicle prior to amphetamine (2 mg/kg) or saline injection are shown. Amphetamine produced significantly higher stereotypy counts than saline controls ($###p<0.001$). Valproic acid dose-dependently decreased amphetamine-induced stereotypy ($***p<0.001$, $**p<0.01$, $*p<0.05$). Data points represent the mean + SEM ($n=11$ /group).

Fig. 2. Regulation of GSK3 by valproic acid in amphetamine-treated mice

Brain tissue was obtained 60 minutes following amphetamine (2 mg/kg) or saline administration from mice pretreated with valproic acid (300 mg/kg) or vehicle. Immunoblots of protein extracts obtained from caudate putamen, nucleus accumbens, and frontal cortex were analyzed. (A) Pretreatment with valproic acid did not significantly alter levels of phosphorylated GSK3α in the caudate putamen. (B) Phosphorylated GSK3β in the caudate putamen was significantly higher in mice pretreated with valproic acid compared to vehicle prior to amphetamine (** $p < 0.01$) and saline (# $p < 0.05$). (C) Administration of valproic acid prior to amphetamine did not significantly alter phosphorylation of GSK3α or (D) GSK3β in the nucleus accumbens. (E) Pretreatment with valproic acid significantly increased levels

of phosphorylated GSK3 α in amphetamine-injected mice compared to pretreatment with vehicle (** $p<0.01$). (F) Levels of phosphorylated GSK3 β in the frontal cortex were significantly greater in mice pretreated with valproic acid prior to amphetamine (* $p<0.05$). Bars represent the mean + SEM (n=9–10/group).

Fig. 3. Levels of phosphorylated and total GSK3 protein

Representative protein bands on immunoblots for phosphorylated ser²¹-GSK3α (51 kD), phosphorylated ser⁹-GSK3β (48 kD), and total GSK3α and GSK3β in the caudate putamen (A), nucleus accumbens (B), and frontal cortex (C) of mice pretreated with valproic acid (300 mg/kg) or vehicle prior to amphetamine (2 mg/kg) or saline. Pretreatment with valproic acid significantly increased levels of phosphorylated GSK3β in amphetamine treated mice (A, right), but did not alter levels of phosphorylated GSK3α (A, left). Levels of p-ser²¹-GSK3α and p-ser⁹-GSK3β remained unchanged in the nucleus accumbens following pretreatment with valproate or vehicle in mice injected with amphetamine (B). Levels of

phosphorylated GSK3 α (C, left) and GSK3 β (C, right) were significantly increased in the frontal cortex of mice pretreated with valproic acid prior to amphetamine.

Fig. 4. Valproic acid attenuated sensitization of stereotypic activity

(A) Stereotypic activity measured for 30 minutes following amphetamine (2 mg/kg) or saline injections on days 1–5. Stereotypy was significantly increased on days 2–5 in mice injected with daily vehicle-amphetamine ($**p<0.01$, $***p<0.001$), and on days 4–5 in mice administered daily valproateamphetamine ($***p<0.001$), compared to day 1. Stereotypy in mice that received daily vehicle-saline and valproate-saline was unchanged throughout days 1–5. (B) Cumulative stereotypy counts measured for 30 minutes following the amphetamine challenge (1 mg/kg) on day 12 are shown. Mice administered daily vehicle-amphetamine showed significant sensitization of stereotypy compared to vehicle-saline treated controls when challenged with amphetamine ($###p<0.001$). Pretreatment with valproic acid (300 mg/kg) prior to daily amphetamine (2 mg/kg) attenuated sensitization of stereotypy compared to vehicle-amphetamine treated controls ($***p<0.001$). Bars represent the mean + SEM ($n=10$ – 12 /group).

Fig. 5. SB 216763 reduced acute amphetamine-induced hyperactivity

(A) The time course of ambulatory activity over 60 minutes post-amphetamine (2 mg/kg) or saline administration is shown for mice pretreated with SB 216763 (5 mg/kg) or vehicle. SB 216763 significantly decreased amphetamine-induced locomotor activity ($***p<0.001$, $**p<0.01$, $*p<0.05$). Ambulatory counts were significantly higher in mice pretreated with SB 216763 prior to amphetamine compared to saline ($##p<0.01$, $#p<0.05$). (B) Total ambulatory counts measured for 60 minutes in mice pretreated with SB 216763 (2.5–5 mg/kg) or vehicle prior to amphetamine (2 mg/kg) or saline. Amphetamine significantly increased ambulatory counts compared to mice injected with saline ($###p<0.001$, $#p<0.05$). SB 216763 (2.5–5 mg/kg) significantly attenuated amphetamine-induced ambulation ($***p<0.001$, $**p<0.01$). (C) The time course of stereotypic activity is shown for mice injected with SB 216763 (5.0 mg/kg) or vehicle prior to amphetamine (2 mg/kg) or saline. Administration of SB 216763 significantly decreased amphetamine-induced stereotypic activity compared to saline-injected controls ($**p<0.01$, $*p<0.05$). Stereotypy counts were significantly higher in mice pretreated with SB 216763 prior to amphetamine compared to saline ($#p<0.05$). (D) Cumulative stereotypic activity recorded following amphetamine or saline injection is shown for mice pretreated with SB 216763 (2.5–5 mg/kg) or vehicle. Stereotypy counts were significantly higher in mice injected with amphetamine compared to saline controls ($###p<0.001$, $##p<0.01$, $#p<0.05$). Pretreatment with SB 216763 (2.5–5 mg/

kg) significantly attenuated amphetamine-induced stereotypic activity (** $p<0.01$, * $p<0.05$). Data points represent the mean + SEM (n=10–12/group).

Fig. 6. SB 216763 attenuated sensitization of stereotypic activity

(A) Stereotypic activity measured for 30 minutes following daily amphetamine (2 mg/kg) or saline injections. Stereotypic activity counts were significantly increased in mice injected daily with vehicle-amphetamine on days 2–5 compared to day 1 (** $p < 0.01$, *** $p < 0.001$). Stereotypy was also increased on days 3–5 in mice administered daily SB 216763 (5 mg/kg) prior to daily amphetamine (* $p < 0.05$, *** $p < 0.001$). Stereotypy counts were unchanged in mice given daily saline injections (vehicle-saline, SB 216763-saline). (B) Stereotypy counts were measured for 30 minutes following an amphetamine challenge (1 mg/kg) on day 12. Mice treated daily with vehicle-amphetamine (2 mg/kg) exhibited significant sensitization of stereotypy compared to vehicle-saline injected controls (## $p < 0.01$). Daily pretreatment with SB 216763 (5 mg/kg) prior to amphetamine significantly attenuated sensitization of stereotypy compared to vehicle-amphetamine treated controls (* $p < 0.05$). Bars represent the mean + SEM ($n = 8-12$ /group).