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Effects of sodium benzoate on prepulse inhibition deficits and hyperlocomotion in mice after administration of phencyclidine

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Running title: Sodium benzoate in the phencyclidine model

ABSTRACTS

Objective: A recent clinical study demonstrated that sodium benzoate (SB), a prototype competitive D-amino acid oxidase (DAAO) inhibitor, was effective in the treatment of several symptoms such as positive and negative symptoms, and cognitive impairment, in medicated patients with schizophrenia. The objective of the study was undertaken to examine the effects of SB on behavioral abnormalities, such as prepulse inhibition (PPI) deficits and hyperlocomotion in mice, after a single administration of the *N*-methyl-D-aspartate (NMDA) receptor antagonist, phencyclidine (PCP).

Methods: Effects of SB on behavioral abnormalities (PPI deficits and hyperlocomotion) in mice after PCP administration were examined. Furthermore, effects of SB on tissue levels of amino acids were also examined.

Results: A single oral dose of SB (100, 300 or 1000 mg/kg) attenuated PPI deficits in mice after administration of PCP (3.0 mg/kg, s.c.), in a dose dependent manner. In contrast, L-701,324 (10 mg/kg), an antagonist at glycine-site of the NMDA receptor, did not affect the effect of SB (1000 mg/kg) on PCP-induced PPI deficits. Furthermore, a single oral dose of SB (1000 mg/kg) significantly attenuated the hyperlocomotion in mice after administration of PCP (3.0 mg/kg, s.c.). However, a single oral dose of SB (1000 mg/kg) caused no changes to D-serine levels in plasma or the frontal cortex, hippocampus and striatum of these animals.

Conclusion: This study suggests that SB induced antipsychotic effects in the PCP model of schizophrenia, even though it did not increase D-serine levels in the brain.

Key words: D-amino acid oxidase; D-serine; Phencyclidine; Sodium benzoate.

Significance outcomes

- Pretreatment with sodium benzoate (SB), a prototype D-amino acid oxidase (DAAO) inhibitor, attenuated prepulse inhibition deficits and hyperlocomotion in mice after a single administration of phencyclidine.
- However, a single administration of SB did not affect D-serine levels in the blood and brain.

Limitations of the study

- In this study, we did not measure D-serine levels in the cerebellum where DAAO is rich.
- The effects of SB in other models of schizophrenia should be examined.
- The effects of chronic administration of SB on levels of amino acids in the brain should be examined.

Introduction

Multiple lines of evidence suggest that dysfunctional glutamatergic neurotransmission via *N*-methyl-D-aspartate (NMDA) receptors are involved in the pathophysiology of schizophrenia [1-9]. The NMDA receptor antagonists, phencyclidine (PCP), and ketamine induce schizophrenia-like symptoms, including positive and negative symptoms, and cognitive impairment in healthy subjects [1, 10-12]. This resulted in the frequent use of PCP to generate animal models of schizophrenia [13-22].

Accumulating evidence suggests that disturbed NMDA receptor neurotransmission, due to decreased D-serine levels, may be a causative factor in the pathophysiology of schizophrenia [6, 7, 23-25]. These findings include firstly, lower levels of D-serine in the blood, cerebrospinal fluid (CSF), and postmortem brain tissue from patients with schizophrenia, relative to normal controls [26-30]. Secondly, treatment with D-serine reduces positive, negative and cognitive symptoms in patients with schizophrenia [31-35]. In addition, a recent meta-analysis supports the findings that D-serine is effective in the treatment of schizophrenia [36]. Thirdly, mRNA expression and the activity of D-amino acid oxidase (DAAO), the enzyme which metabolizes D-serine, is increased in postmortem brains from patients with schizophrenia [37, 38]. Fourthly, the G72 gene, located at chromosome 13q, shows significant association with schizophrenia [39, 40]. This gene has been designated a DAAO activator, since the G72 protein interacts physically with DAAO [39]. Subsequent meta-analysis found highly significant association between nucleotide variations in the G72/G30 region and schizophrenia [41].

Klein and Kamin [42] first reported on SB as a prototype competitive DAAO inhibitor (Ki ≈ 16 μM), as early as the 1940s [43]. More recently, Lane et al. [44]

performed a randomized, double-blind, placebo-controlled study using SB in stabilized patients with schizophrenia. Given at a dose of 1 g/day for 6 weeks, SB produced a 21% improvement in Positive and Negative Syndrome Scale (PANSS) total scores and large effect sizes in the PANSS total and subscales, Scales for the Assessment of Negative Symptoms (SANSS) - 20 items, Global Assessment of Function (GAF), Quality of Life (QOL) Scale, and Clinical Global Impression (CGI), as well as improved neurocognition [44]. Additionally, SB was well tolerated without significant adverse effects. However, there are no reports demonstrating the antipsychotic effects of SB in animal models of schizophrenia, even though SB could be a potential therapeutic drug for this disorder.

In the present study, we examined whether SB attenuated prepulse inhibition (PPI) deficits and hyperlocomotion in mice, after the administration of PCP. In addition, we measured levels of D-serine in blood and brain regions after oral administration of SB. We also measured levels of other the amino acids, L-serine, glycine, glutamate, glutamine and γ-aminobutyric acid (GABA), since they are involved in the glutamine – glutamate – GABA cycle [9, 45, 46].

Materials and methods

Animals

Male ddY mice (8 weeks old) weighing 25 – 30 g were purchased from SLC Japan (Hamamatsu, Shizuoka, Japan). The mice were housed in clear polycarbonate cages (22.5×33.8×14.0 cm) in groups of 5 or 6 per cage under a controlled 12/12-h light–dark cycle (lights on from 7:00 AM to 7:00 PM), with room temperature at 23 ± 1°C and humidity at 55 ± 5%. The mice were given free access to water and food pellets. The

experimental procedure was approved by the Animal Care and Use Committee of Chiba University.

Drugs

Sodium benzoate (SB; Wako Pure Chemical Co., Tokyo, Japan) was dissolved in 0.5% carboxymethy cellulose (CMC) (Wako Pure Chemical Co., Tokyo, Japan). PCP hydrochloride was synthesized in our laboratory, and the dose (3.0 mg/kg) of PCP was expressed as a hydrochloride salt [22]. L-701,324 (Sigma-Aldrich Co., Ltd., St Louis, MO, USA) was dissolved in 20% polyethylene glycol (PEG300: Wako Pure Chemical Co., Tokyo, Japan) with pH adjusted to 10 with 1M NaOH. Other drugs were purchased from commercial sources.

Effect of SB on PPI deficits after a single administration of PCP

The mice were tested for their acoustic startle reactivity (ASR) in a startle chamber (SR-LAB; San Diego Instruments, San Diego, CA, USA) using the standard methods described previously [22, 47-50]. The test sessions were begun after an initial 10-min acclimation period in the chamber. The mice were subjected to one of six trials: (1) pulse alone, as a 40 ms broadband burst; a pulse (40 ms broadband burst) preceded by 100 ms with a 20 ms prepulse that was (2) 4 dB, (3) 8 dB, (4) 12 dB, or (5) 16 dB over background (65 dB); and (6) background only (no stimulus). The amount of prepulse inhibition (PPI) was expressed as the percentage decrease in the amplitude of the startle reactivity caused by presentation of the prepulse (% PPI). SB (30, 100 or 1000 mg/kg), or vehicle (0.5 % CMC) (10 ml/kg) was administered orally 60 min (including the 10-min acclimation period) before the machine records, and PCP (3.0 mg/kg) or saline

(10 ml/kg) was administered subcutaneously (s.c.) 10 min (including the 10-min acclimation period) before. The PPI test lasted 20 min in total.

Effect of SB and L-701,324 on PPI deficits after a single administration of PCP

In order to study the role of glycine-site of the NMDA receptor, we examined the effects of L-701,324, an antagonist of glycine-site of the NMDA receptor, on the effect of SB on PCP-induced PPI deficits in mice. Thirty minutes after oral administration of SB (1000 mg/kg), or vehicle (0.5 % CMC) (10 ml/kg), L-701,324 (10 mg/kg) or vehicle (20% PEG) was administered intraperitoneally (i.p.) 30 minute later. Thirty minute after injection of L-701,324 (or vehicle), PCP (3.0 mg/kg) or saline (10 ml/kg) was administered s.c.. The PPI test was performed as described above.

Effect of SB on hyperlocomotion after a single administration of PCP

After habituation (30 min) in the cage, SB or vehicle was injected into mice (each group n = 8 – 12). One hour after a single oral administration of SB (1000 mg/kg) or vehicle (10 ml/kg, 0.5% CMC), PCP (3.0 mg/kg) or vehicle (physiological saline; 10 ml/kg) was administered s.c. into the mice. Locomotor activity was measured using an animal movement analysis system (SCANET MV-40, Melquest, Toyama, Japan). The system consisted of a rectangular enclosure (560 x 560 mm). The side walls (height, 60 mm) of the enclosure were equipped with 144 pairs of photosensors located at 6-mm intervals at a height of 30 mm from the bottom edge. An animal was placed in the observation cage 30 minutes (habituation) before injection of vehicle or SB. Vehicle or PCP was injected 60 minutes after oral injection of vehicle or SB, and the locomotion activity was measured for 60 minutes after injection of vehicle or PCP. A pair of photosensors was

scanned every 0.1 second to detect the animal's movements. The intersection of paired photosensors (10 mm apart) in the enclosure was counted as one unit of locomotor activity. Data collected for total 150 minutes were used in this study. The sum of locomotion in mice for 60 minutes after the PCP administration was used for data analysis.

Measurement of amino acids by high performance liquid chromatography (HPLC)

One hour after a single oral administration of SB (1000 mg/kg), mice were sacrificed by decapitation after collection of blood sample. The brain was removed and the frontal cortex, hippocampus, and striatum were dissected on ice. Plasma and brain tissues were frozen on dry ice and stored at -80 °C until measurement.

Briefly, plasma (20 μL) was homogenized in 180 μL of methanol (HPLC grade) on ice. The homogenates were centrifuged at 3,000 x g for 6 min at 4°C, and 20 μL of supernatant was evaporated to dryness at 40°C. To the residue, 20 μl of H_2O (HPLC grade), 20 μL of 0.1 M borate buffer (pH 8.0) and 60 μL of 50 mM 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F; Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) in CH_3CN (HPLC grade) were added. The reaction mixture was then heated at 60°C for 2 min, and immediately supplemented with 100 μL of H_2O/CH_3CN (90/10) containing 0.1 % trifluoroacetic acid to stop the reaction. Brain tissues were homogenized in 1.5 mL of methanol (HPLC grade) on ice. The homogenates were centrifuged at 3000g for 6 min at 4 °C, and 20 μL of supernatant was evaporated to dryness at 40 °C. To the residue, 20 μL of H_2O (HPLC grade), 20 μL of 0.1 M borate buffer (pH 8.0), and 60 μL of 50 mM NBD-F in CH_3CN (HPLC grade) were added. The reaction mixture was then heated to 60 °C for 2 min, and immediately supplemented with 100 μL of H_2O/acetonitrile (90/10) containing 0.1% trifluoroacetic acid (TFA) to

stop the reaction. Levels of amino acids (D-serine, L-serine, glycine, glutamine, glutamate, GABA) were measured using high performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan), as previously reported [51-53]. Fluorescence detection was performed at 530 nm with an excitation wavelength of 470 nm.

Statistical Analysis

The data are presented as the mean ± standard error of the mean (S.E.M.). The PPI data were analyzed by multivariate analysis of variance (MANOVA), followed by *post-hoc* Fisher's Least Significance Difference (LSD) test. The data of hyperlocomotion were analyzed by one-way analysis of variance (ANOVA), followed by *post-hoc* Fisher LSD test. The data of amino acids were analyzed using Student t-test. Significance for the results was set at $P < 0.05$.

Results

Figure 1 shows the effects of SB (100, 300 or 1000 mg/kg) on PCP (3.0 mg/kg)-induced PPI deficits in mice. The MANOVA analysis of all PPI data revealed that there was a significant effect [Wilks lambda = 0.346, $P < 0.001$]. Subsequent ANOVA analysis revealed the significant differences ($P < 0.001$) at all dB groups (69, 73, 77, and 81 dB). A *post-hoc* analysis indicated a significant ($P < 0.001$) difference in PPI deficits between the vehicle + vehicle group and vehicle + PCP (3.0 mg/kg) group at all dB groups (Figure 1). Pretreatment with SB (100, 300 or 1000 mg/kg) attenuated PCP-induced PPI deficits, in a dose dependent manner. High dose (1000 mg/kg) of SB significantly ($P < 0.001$) attenuated PCP-induced PPI deficits at all dB groups (Figure 1).

Moderate dose (300 mg/kg) of SB significantly ($P < 0.05$ at 69 – 77 dB groups, $P < 0.001$ at 81 dB group) attenuated PCP-induced PPI deficits at all dB groups (Figure 1). In contrast, PPI in mice after administration of SB (1000 mg/kg) alone was similar to control mice (Figure 1).

In order to study the role of glycine-site of the NMDA receptor, we examined the effect of L-701,324, an antagonist at glycine-site of the NMDA receptor, on effect of SB on PCP-induced PPI deficits. Figure 2 shows the effects of SB (1000 mg/kg) and L-701, 324 (10 mg/kg) on PCP (3.0 mg/kg)-induced PPI deficits in mice. The MANOVA analysis of all PPI data revealed that there was a significant effect [Wilks lambda = 0.193, $P < 0.001$]. A *post-hoc* analysis indicated a significant ($P < 0.001$) difference in PPI deficits between the vehicle + vehicle group and vehicle + PCP (3.0 mg/kg) group at all dB groups (Figure 2). Pretreatment with SB (1000 mg/kg) significantly attenuated PCP-induced PPI deficits. However, L-701,324 (10 mg/kg) did not affect the effect of SB on PCP-induced PPI deficits (Figure 2). Furthermore, L-701,324 (10 mg/kg) did not affect PCP-induced PPI deficits in mice (Figure 2).

A single administration of PCP (3.0 mg/kg, s.c.) markedly increased locomotion in mice. One-way ANOVA revealed significant differences among the four groups [$F(3, 35) = 6.17$, $P = 0.002$]. Pretreatment with SB (1000 mg/kg) significantly ($P < 0.01$) attenuated PCP-induced hyperlocomotion in mice (Figure 2). In contrast, administration of SB (1000 mg/kg) alone did not affect spontaneous locomotion in mice.

A single oral administration of SB (1000 mg/kg) did not alter plasma levels of D-serine, L-serine and glycine. In contrast, SB significantly decreased plasma levels of glutamine whereas SB significantly increased plasma levels of glutamate (Table 1). Furthermore, SB significantly increased the ratio of L-serine to glycine in plasma,

suggesting that SB may affect L-serine – glycine cycle (Table 2). Moreover, SB significantly decreased the ratio of glutamine to glutamate in plasma, suggesting that SB may affect glutamine – glutamate cycle (Table 2).

A single oral administration of SB (1000 mg/kg) did not alter tissue levels of D-serine and other amino acids except L-serine levels in striatum (Table 1). However, SB significantly increased the ratio of D-serine to L-serine in the striatum, but not frontal cortex and hippocampus. Furthermore, SB significantly decreased the ratio of glutamine to glutamate in the striatum, but not frontal cortex and hippocampus. These findings suggest that SB may affect D-serine – L-serine cycle and glutamine – glutamate cycle in the striatum (Table 2).

Discussion

In this study, we found that SB attenuated PPI deficits and hyperlocomotion in mice after administration of PCP. Furthermore, L-701,324 did not affect the effect of SB on PCP-induced PPI deficits, suggesting that activation at glycine-site of the NMDA receptor may not be involved in the mechanism of action of SB. This is the first report to demonstrate that SB is effective in the PCP model of schizophrenia. However, SB (1000 mg/kg) did not increase the tissue levels of D-serine in the mouse brain, indicating that D-serine in the brain may not be involved in the acute therapeutic action of SB in this model. In contrast, a single dose of SB significantly increased the ratio of D-serine to L-serine in the striatum, suggesting that SB may affect the D-serine – L-serine cycle. Therefore, it is likely that repeated administration of SB increases D-serine levels in the brain although a further study is needed to confirm this.

Although DAAO inhibitors were proposed as a new therapeutic drug for schizophrenia, their clinical use has been largely unsuccessful [54, 55]. Ferraris et al.

[43] reported 5-chloro-benzo[d]isoxazol-3-ol (CBIO; IC_{50} = 1680 nM) as being a more potent DAAO inhibitor than SB (Ki ≈ 16 μM). In a subsequent report, we found that a single oral dose of CBIO (30 mg/kg) did not increase levels of D-serine in the plasma or frontal cortex, and that CBIO alone did not improve the NMDA receptor antagonist dizocilpine-induced PPI deficits in mice [48]. In addition, we found that a low dose of D-serine (30 mg/kg) did not improve dizocilpine-induced PPI deficits in mice, although this dose significantly increased plasma levels of D-serine [48]. Taken together, it is likely that the extensive inhibition of DAAO in the periphery and brain has a limited effect on brain or extracellular levels of D-serine, and that the behavioral effects of DAAO inhibitors may be very weak. In contrast, we found that co-administration of CBIO with D-serine (or D-alanine) increased brain levels of D-serine (or D-alanine) compared with D-serine (or D-alanine) alone, and that CBIO potentiated the effects of D-serine (or D-alanine) on dizocilpine-induced PPI deficits in mice [43, 48, 49]. Therefore, we proposed that combination therapy of D-serine (or D-alanine) with a DAAO inhibitor could reduce doses of D-serine (or D-alanine) in humans, particularly since the clinical doses of D-serine (or D-alanine) are quite high (30-60 mg/kg) [43, 48, 49].

DAAO shows very low activity in adult forebrains, with high activity in the adult cerebellum. Therefore, it is possible that this increase in cerebellar D-serine levels by DAAO inhibition, may in part confer antipsychotic effects, by augmenting D-serine-mediated regulation of NMDA receptors in the cerebellum [9, 56], although we did not measure these levels in the present study. Recent reports show that SB up-regulated brain-derived neurotrophic factor (BDNF) in mice [9, 57]. This implies that the therapeutic effect of SB may be mediated through increased BDNF levels, since

the TrkB agonist, 7,8-dihydroxyflavone attenuated the behavioral abnormalities of hyperlocomotion and PPI deficits in mice after administration of the stimulant methamphetamine [50, 58].

Glutamine – glutamate cycle in the glia – neuron communication is involved in the glutamatergic neurotransmission in the brain [6, 45, 46]. In this study, we found that SB significantly increased the ratio of glutamine to glutamate, a marker for glutamine – glutamate cycle, in plasma and striatum. These findings suggest that SB can affect glutamine – glutamate cycle in the striatum and plasma, resulting in the regulation of the NMDA receptor.

Accumulating evidence suggests a role for inflammation and oxidative stress in the pathophysiology of schizophrenia [59-63]. SB is thought to have a potent anti-inflammatory effect via modulation of the mevalonate pathway and $p21^{ras}$ [64]. In addition, SB up-regulates the neuroprotective protein, DJ-1, a Parkinson disease protein, also via modulation of the mevalonate pathway [65]. Previously, we reported that potent anti-inflammatory and antioxidants, including minocycline and sulforaphane, attenuate behavioral abnormalities in mice after administration of PCP or methamphetamine [21, 22, 47, 66, 67]. Taken together, it is possible that SB mediates its therapeutic action through anti-inflammatory and antioxidant pathways. Further detailed studies on molecular targets of SB are needed.

Conclusions

Our study suggests that SB shows potential antipsychotic activity in animal models of schizophrenia. It is possible that SB could be used for the effective and safe treatment of schizophrenia, particularly since SB is generally recognized as a safe food preservative.

In addition, the use of amino acids, including D-serine as biomarkers for treatment efficacy will be an interesting future development.

Author contributions

A.M for substantial contributions to conception and design, acquisition of data, analysis and interpretation of the data, drafting the article, final approval of the version to be published; Y.F. for substantial contributions to conception and design, final approval of the version to be published; M. I. for substantial contributions to conception and design, final approval of the version to be published; K.H. for substantial contributions to conception and design, acquisition of data, analysis and interpretation of the data, drafting the article, final approval of the version to be published.

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Ethical Standards

The experimental procedure was approved by the Animal Care and Use Committee of Chiba University. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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Figure legends

Figure 1. Effect of SB on PCP-induced PPI deficits in mice

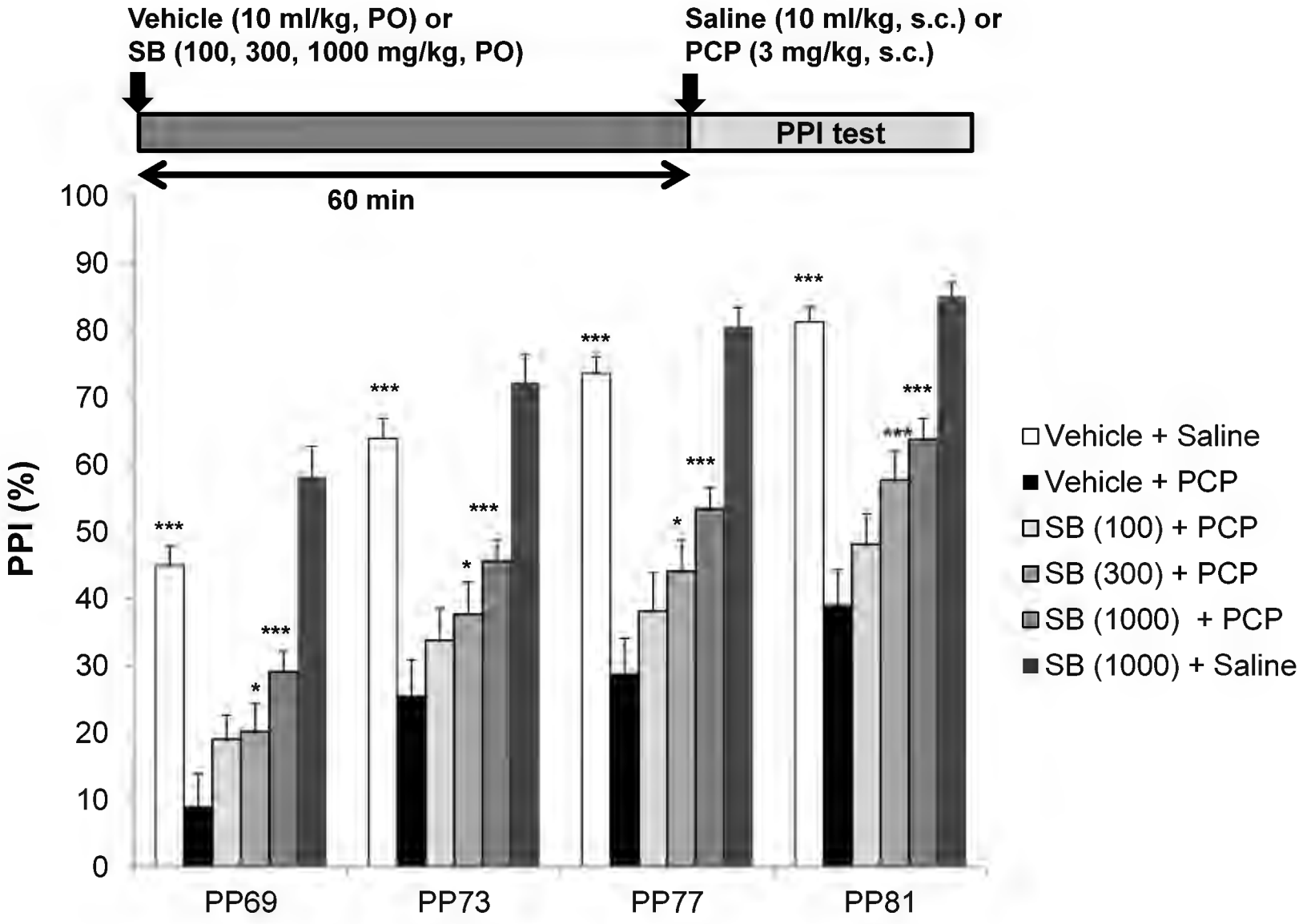
One hour after a single oral administration of vehicle (10 ml/kg), or SB (100, 300 or 1000 mg/kg), PCP (3 mg/kg) or saline (10 ml/kg) was administered s.c. to the mice. Each value is the mean ± S.E.M. (n = 17 - 21 per group). *P < 0.05, ***P < 0.001 as compared with the vehicle + PCP treated group.

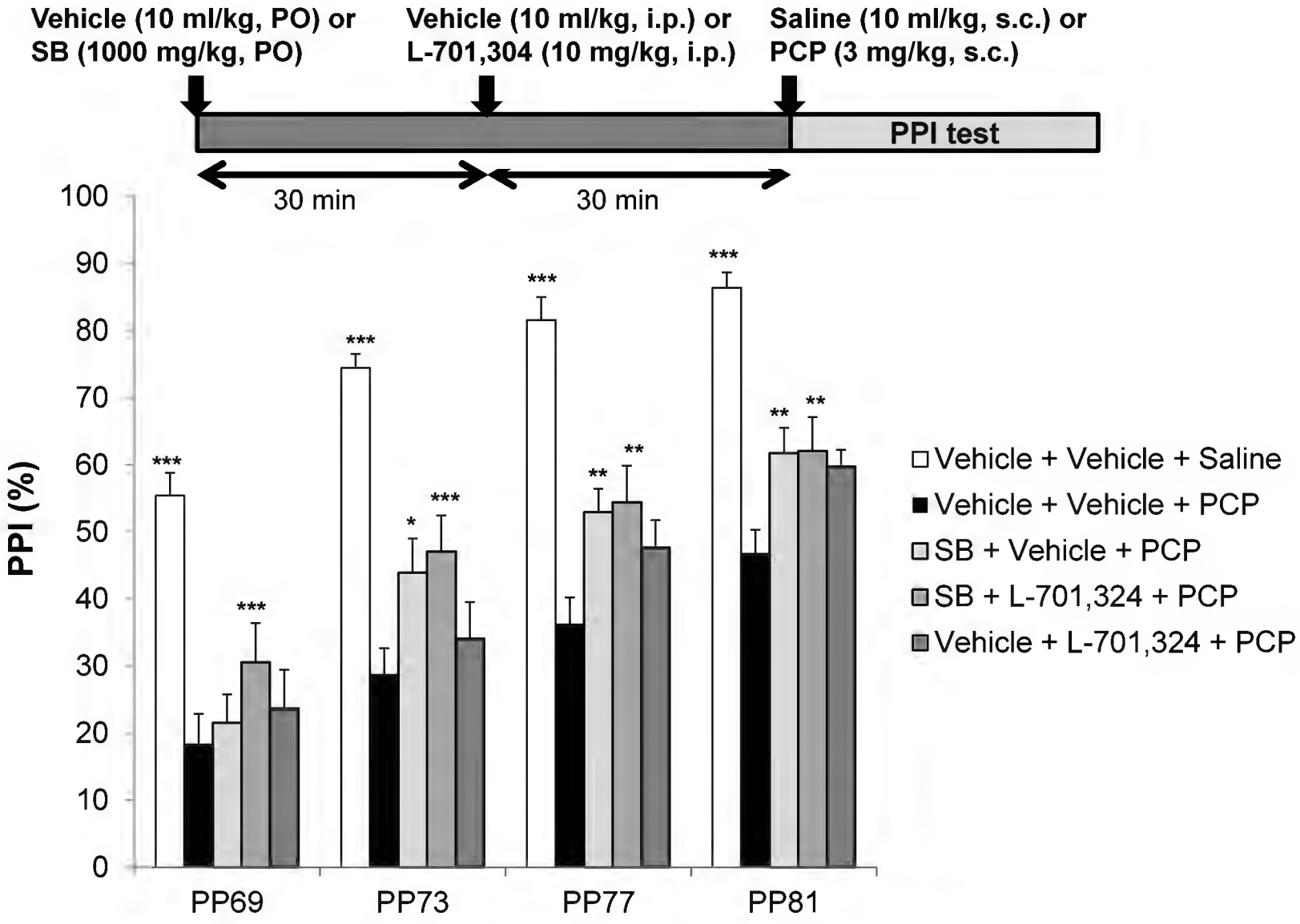
Figure 2. Effects of SB and L-701,324 on PCP-induced PPI deficits in mice

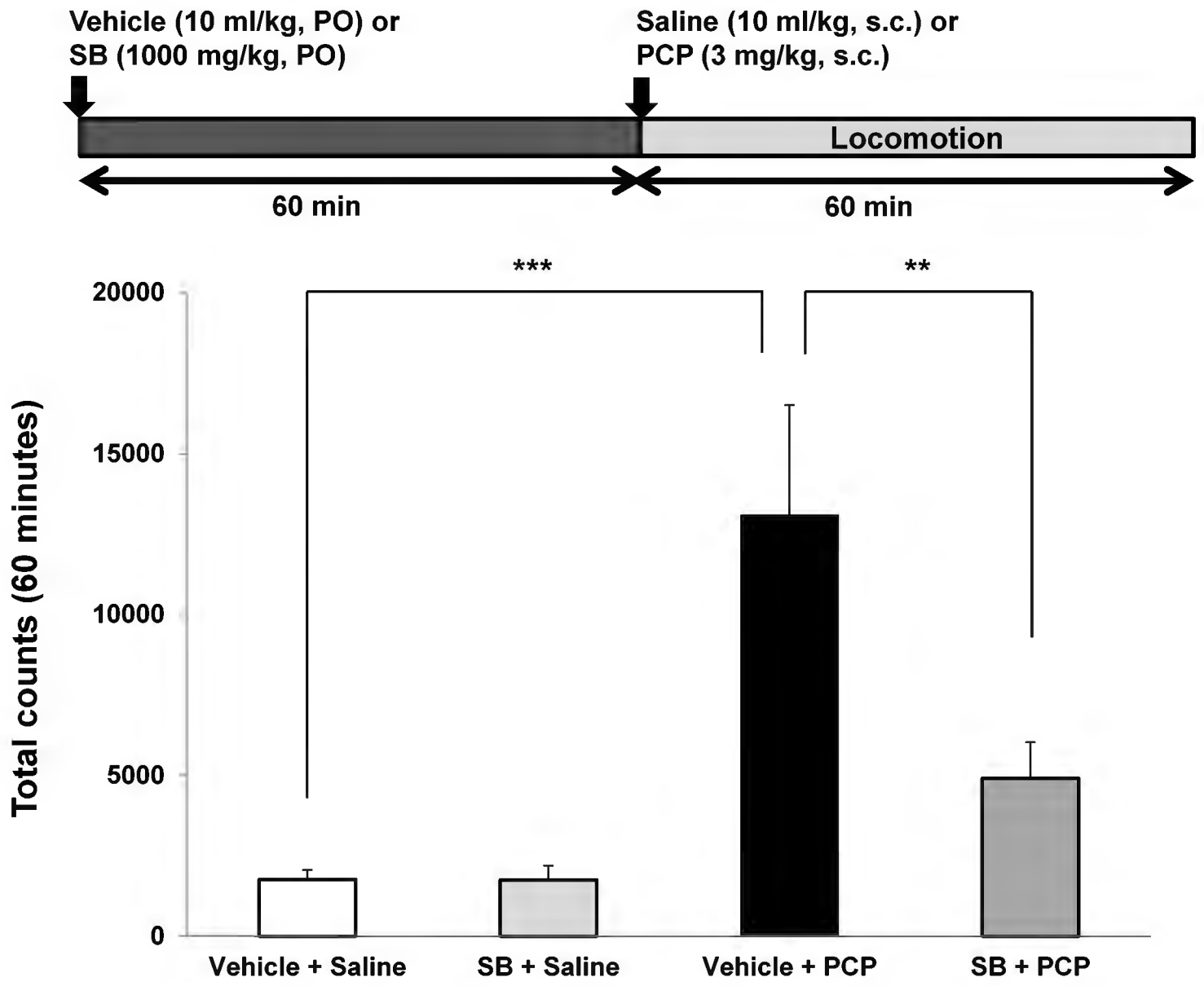
Thirty minutes after a single oral administration of vehicle (10 ml/kg) or SB (1000 mg/kg), L-701,304 (10 mg/kg) or vehicle (10 ml/kg) was administered i.p. to the mice. Thirty minutes after i.p. injection of L-701,304 (or vehicle), PCP (3 mg/kg) or saline (10 ml/kg) was administered s.c. to the mice. Each value is the mean ± S.E.M. (n = 8 - 11 per group). *P < 0.05, **P < 0.01, ***P < 0.001 as compared with the vehicle + PCP treated group.

Figure 3. Effect of SB on PCP-induced hyperlocomotion in mice

One hour after a single oral administration of vehicle (10 ml/kg) or SB (1000 mg/kg), PCP (3.0 mg/kg) or saline (10 ml/kg) was administered s.c. into the mice. Behavior (locomotion) in the mice was evaluated for 1 hour after administration of PCP. Each value is the mean ± S.E.M. (n = 8 - 12 per group). **P < 0.01, ***P < 0.001 as compared with the vehicle + PCP group


Vehicle (10 ml/kg, PO) or
SB (100, 300, 1000 mg/kg, PO)
Saline (10 ml/kg, s.c.) or
PCP (3 mg/kg, s.c.)
PPI test
60 min
PPI (%)
100
90
80
70
60
50
40
30
20
10
0
PP69
PP73
PP77
PP81
Vehicle + Saline
Vehicle + PCP
SB (100) + PCP
SB (300) + PCP
SB (1000) + PCP
SB (1000) + Saline


Vehicle (10 ml/kg, PO) or
SB (1000 mg/kg, PO)
Vehicle (10 ml/kg, i.p.) or
L-701,304 (10 mg/kg, i.p.)
Saline (10 ml/kg, s.c.) or
PCP (3 mg/kg, s.c.)
PPI test
30 min
30 min
PPI (%)
100
90
80
70
60
50
40
30
20
10
0
PP69
PP73
PP77
PP81
Vehicle + Vehicle + Saline
Vehicle + Vehicle + PCP
SB + Vehicle + PCP
SB + L-701,324 + PCP
Vehicle + L-701,324 + PCP


Vehicle (10 ml/kg, PO) or
SB (1000 mg/kg, PO)
Saline (10 ml/kg, s.c.) or
PCP (3 mg/kg, s.c.)
Locomotion
60 min
60 min
***
**
Total counts (60 minutes)
20000
15000
10000
5000
0
Vehicle + Saline
SB + Saline
Vehicle + PCP
SB + PCP