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Short communications

Potential role of tyrosine hydroxylase in the loss of psychostimulant effect of amphetamine under conditions of impaired dopamine transporter activity

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ABSTRACT

Amphetamine and methylphenidate are known to have stimulatory effect in healthy subjects but not in humans with attention deficit hyperactivity disorder and in rodents with impaired dopamine transporter (DAT) function. This phenomenon is called the paradoxical calming effect of psychostimulants. It has been previously demonstrated that psychostimulants may regulate the enzymatic activity of tyrosine hydroxylase (TH). Hence, the objective of the present study was to determine whether the lack of activity-stimulating effects of amphetamine in hyperactive rats is associated with changes in TH activity. To model hyperactivity in rats, acute administration of DAT inhibitor GBR12909 was used. Changes in TH activity, assessed as L-DOPA accumulation and TH phosphorylation levels, were measured in amphetamine treated rats with or without pretreatment with GBR12909. Our results showed that amphetamine treatment alone increased locomotor activity in rats, whereas pretreatment of rats with GBR12909 counteracted this effect, a finding consistent with the paradoxical calming effect. GBR12909, while having no effect on its own, blocked amphetamine-induced elevation of TH activity in dorsal striatum and nucleus accumbens, measured as increased tissue L-DOPA concentration. However, the phosphorylation levels of TH were not affected by treatment with amphetamine, GBR12909 or the combination of both. Our findings indicate that other mechanisms than phosphorylation-regulated TH activity changes are responsible for the paradoxical calming effect of amphetamine under conditions of impaired DAT activity.

Activity-stimulating effects of amphetamines are lost in patients with attention deficit hyperactivity disorder (ADHD). In these patients, psychostimulants are known to reverse hyperactivity (inattention, impulsiveness and motor restlessness) and improve attentional focus [1]. Similar effect is measured in animals with impaired dopamine transporter (DAT) function [2,3]. Although involvement of serotonergic neurotransmission has been suggested [2], the paradoxical calming effect of amphetamines in ADHD patients, and in animals with impaired DAT function, is poorly understood in terms of the underlying mechanism.

Several alterations in dopaminergic neurotransmission have been described in rodents with loss of functional DAT. Microdialysis studies demonstrated that under baseline conditions extracellular subcortical dopamine (DA) levels were augmented in animals lacking functional DAT compared to wild-type animals [3]. Acute amphetamine administration have been shown to stimulate DA release in the dorsal striatum and nucleus accumbens (NAc) of wild-type animals [4] but had no effect on extracellular levels of DA in animals with impaired DAT function [3,5]. Studies using fast scan cyclic voltammetry showed that electrically stimulated striatal dopamine release is decreased by about 75% in DAT knock-out mice [6]. While these studies point to increased extracellular DA levels associated with a blunting of evoked DA release in DAT deficient animals, the underlying mechanism is unclear. Disrupted regulation of tyrosine hydroxylase (TH) activity could be one mechanism involved in disturbed presynaptic dopaminergic terminal function in DAT deficient animals. Indeed, using in vivo assays of TH activity, Jones et al.[6] demonstrated elevated DA synthesis rate in DAT knock-out mice.

In the presynaptic dopaminergic terminal, TH plays a crucial role in DA turnover. It converts tyrosine to DA precursor L-DOPA. It is a rate-limiting step in the whole DA biosynthesis process [7]. Psychostimulant drugs have been reported to modulate TH activity in the dopaminergic nerve terminals. For instance, acute administration of cocaine was shown to inhibit catecholamine biosynthesis by altering the phosphorylation state of TH [8]. To the contrary, amphetamine was reported to increase the activity of TH in rats with intact DAT [9]. Moreover, inhibition of TH with α-methyl-*p*-tyrosine has been shown to reduce locomotor-stimulating effect of amphetamine [10], suggesting

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that increased TH activity may be involved in behavioral response to amphetamine.

To date, the effect of amphetamine on the activity of TH in hyperactive animals with reduced DAT activity has not been assessed. In the present study, we explored whether the amphetamine-induced activation of TH in rats, measured by the in vivo accumulation of L-DOPA, was lost by pre-treatment with the DAT inhibitor GBR12909. Acute administration of DAT inhibitor GBR12909 was used to model hyperactivity in rats. To address the question of a potential involvement of TH activity in the loss of psychostimulant effects of amphetamine under conditions of impaired DAT function, we investigated the change of TH phosphorylation state at pSer19, pSer31 and pSer40 in amphetamine treated rats with or without pretreatment with GBR12909.

All experiments were conducted with male Sprague Dawley rats (Janvier, Le Genest-St-Isle, France). All experiments were approved by the AbbVie's Animal Welfare Office (Ludwigshafen, Germany) as well as institutional Committees on Animal Care and Use, and by the Regierungspräsidium Karlsruhe, and were performed in accordance with the European and German national guidelines as well as the recommendations and policies of the United States National Institutes of Health Principles of Laboratory Animal Care. All animal housing and experiments were conducted in facilities with full accreditation by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

GBR12909 (Sigma-Aldrich) was dissolved in cremophor (Sigma-Aldrich, Germany) and then diluted with 0.9% saline to the final cremophor concentration 20%. D-Amphetamine (Sigma-Aldrich, Germany) and NSD-1015 (Fluka, Germany) was dissolved in sterile 0.9% saline. All substances were freshly prepared and injected as a volume of 1 mL/kg intraperitoneally.

Locomotor activity studies were conducted in four identical arenas made from grez plastic (40 × 40 cm, Tru Scan Activity Monitoring System, Coulbourn Instruments). For the test, the animal was placed in the center of the box and the experimenter then left the room while a camera above the apparatus recorded the animal's movements. For locomotor activity measurements, animals were divided into four groups (n = 8 per group). One group was administered vehicle, whereas the other groups received either 30 mg/kg of GBR12909 or 2 mg/kg of D-amphetamine or combination of both 30 mg/kg of GBR12909 and 2 mg/kg D-amphetamine. GBR12909 was administered 60 min before the test, and D-amphetamine was given immediately before the test. Distance travelled (cm) was recorded by video tracking software (Noldus Ethovision) for a period of 60 min.

TH activity was evaluated by measuring tissue L-DOPA accumulation and TH phosphorylation in the dorsal striatum and NAc. Similarly to locomotor activity measurements, one group of rats was injected with vehicle, whereas others received either 30 mg/kg of GBR12909 or 2 mg/kg of D-amphetamine or combination of both (n = 6 per group). 15 min after administration of drugs, aromatic-amino-acid-decarboxylase inhibitor NSD-1015 (100 mg/kg) was administered to all animals in order to avoid conversion of L-DOPA to DA. 30 min after NSD-1015 administration animals were sacrificed by cervical dislocation under isoflurane anesthesia. The defined brain regions were dissected on an ice-cold plate, rapidly frozen in the liquid nitrogen and stored at −80 °C until analysis.

For the HPLC analysis brain tissue was homogenized in 8× of Prep-Buffer (Tris buffered saline (TBS) 10, Protease Inhibitor Complete and Phosphatase inhibitor cocktail) by 5–10 s ultrasonication (UP50H Ultrasonic Processor HIELSCHER). 30 µL of the homogenate from each animal group was mixed with 30 µL of 0.2 M perchloric acid and stored for 20 min on ice. Afterwards, samples were centrifuged for 30 min at 16 000 rpm at 4 °C and 30 µL of the supernatants were collected in HPLC vials. Samples were analyzed by reverse phase HPLC (column packed with C18 silica; particle size – 3.5 µm) with electrochemical detection (dual channel carbon working electrode versus Ag/AgCl reference electrode).

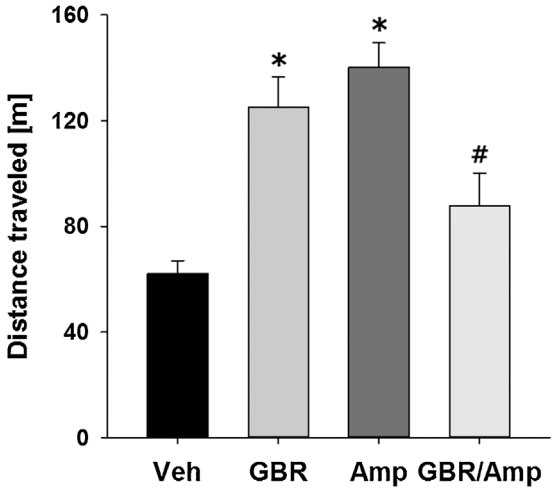

Fig. 1. Effect of administration of either vehicle or compounds: 30 mg/kg of GBR12909 (GBR), 2 mg/kg of amphetamine (Amp) and combination of both 30 mg/kg of GBR12909 and 2 mg/kg of amphetamine on total distance travelled (m) in the 60 min open field test (n = 8 per treatment condition) in male Sprague-Dawley rats. All data are expressed as means ± SEM. * indicates significant differences from the control vehicle group, # indicates significant differences from both GBR12909 and amphetamine treatment groups, $p < 0.05$.

For Western blot analysis 20 µg/µL of protein from each animal group was mixed with the PAGE loading buffer [E-PAGE™ Loading buffer, NuPAGE® Sample Reducing Agent (10×)] and distilled water, and electrophoresis was run on Invitrogen™ electrophoresis device (E-BASE™). Immunodetection was performed with following three primary antibodies: pSer19, pSer31 and pSer40 (Class: Polyclonal, Host: Rabbit; dilution: 1:1000; Thermo Scientific). The detection of the primary antibodies was performed using WesternBreeze® Chemiluminescent (Invitrogen™) and Quantity One, version 4.6.9 software (BioRad).

One-way ANOVA was used for data analysis derived from locomotor activity, L-DOPA accumulation and TH phosphorylation measurements. Whenever indicated by ANOVA results, post-hoc Student Newman Keul's tests were performed. The chosen level of significance was $p < 0.05$.

Analysis of the locomotor activity data demonstrated that total distance travelled was significantly different between treatment groups [$F(3,28) = 13.0$, $p < 0.001$] (Fig. 1). Post hoc analysis revealed that both 30 mg/kg of GBR12909 and 2 mg/kg of D-amphetamine treatment significantly increased rat locomotor activity compared to the vehicle treated control group. However, administration of D-amphetamine in GBR12909 pretreated rats significantly reduced hyperactivity, so it was no longer different from that seen in the vehicle treated group.

One-way ANOVA demonstrated that administration of GBR12909 alone had no effect on TH activity with respect to L-DOPA accumulation. To the contrary, administration of D-amphetamine significantly increased tissue L-DOPA levels in the dorsal striatum [$F(3,20) = 8.1$, $p < 0.001$] and NAc [$F(3,20) = 5.5$, $p < 0.01$] when compared to that seen after vehicle administration (Fig. 2). However, pretreatment of rats with GBR12909 prevented d-amphetamine-induced increase in L-DOPA accumulation in both dorsal striatum and NAc, showing that GBR12909 fully antagonized stimulatory effect of D-amphetamine on L-DOPA synthesis.

Measurements of TH phosphorylation levels showed that L-DOPA accumulation, seen after D-amphetamine administration in the above described experiment, was not accompanied by any changes in the TH phosphorylation state in the dorsal striatum and NAc (Fig. 3). Hence, levels of pSer19, pSer31 and pSer40 were similar in all treatment groups (vehicle, d-amphetamine, GBR12909 and combination of both D-amphetamine and GBR12909) in the dorsal striatum ($p = 0.43$, $p = 0.97$ and $p = 0.57$ for pSer19, pSer31 and pSer40, respectively)