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Dopamine transporter-dependent and -independent actions of trace amine β-phenylethylamine

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Abstract

β-Phenylethylamine (β-PEA) is an endogenous amine that is found in trace amounts in the brain. It is believed that the locomotor-stimulating action of β-PEA, much like amphetamine, depends on its ability to increase extracellular dopamine (DA) concentrations owing to reversal of the direction of dopamine transporter (DAT)-mediated DA transport. β-PEA can also bind directly to the recently identified G protein-coupled receptors, but the physiological significance of this interaction is unclear. To assess the mechanism by which β-PEA mediates its effects, we compared the neurochemical and behavioral effects of this amine in wild type (WT), heterozygous and 'null' DAT mutant mice. In microdialysis studies, β-PEA, administered either systemically or locally via intrastriatal infusion, produced a pronounced outflow of striatal DA in WT mice whereas no increase was detected in mice lacking the DAT (DAT-KO mice). Similarly, in fast-scan voltammetry studies β-PEA did not alter DA release and clearance rate in striatal slices from DAT-KO mice. In behavioral studies β-PEA produced a robust but transient increase in locomotor activity in WT and heterozygous mice. In DAT-KO mice, whose locomotor activity and stereotypy are increased in a novel environment, β-PEA (10–100 mg/kg) exerted a potent inhibitory action. At high doses, β-PEA induced stereotypies in WT and heterozygous mice; some manifestations of stereotypy were also observed in the DAT-KO mice. These data demonstrate that the DAT is required for the striatal DA-releasing and hyperlocomotor actions of β-PEA. The inhibitory action on hyperactivity and certain stereotypies induced by β-PEA in DAT-KO mice indicate that targets other than the DAT are responsible for these effects.

Keywords: attention deficit hyperactivity disorder, microdialysis, psychostimulant, trace amines, voltammetry.

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In addition to well known classical biogenic amine neurotransmitters, there is a group of endogenous trace amines [such as β-phenylethylamine (β-PEA), octopamine, tyramine and tryptamine] that are important in invertebrates, and are found at low concentrations in peripheral and brain tissues of vertebrates (Usdin and Sandler 1976; Saavedra 1989). It is likely that trace amines are older phylogenetically than classical biogenic amines, with whom they share structural properties and metabolic pathways (Boulton 1983; Paterson *et al*. 1990), but their physiological roles and mechanisms of action in vertebrates remain unclear (Borowsky *et al*. 2001; Bunzow *et al*. 2001; Kim and Von Zastrow 2001; Premont *et al*. 2001; Branchek and Blackburn 2003).

β-PEA is structurally related to dopamine (DA) as well as several psychotropic molecules, including the psychostimulant drug amphetamine (Saavedra 1989; Paterson *et al*. 1990; Janssen *et al*. 1999). It is distributed heterogeneously throughout mammalian brain, and the

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Abbreviations used: ADHD, attention deficit hyperactivity disorder; DA, dopamine; DAT, dopamine transporter; DAT-HET, dopamine transporter heterozygous mice; DAT-KO, dopamine transporter knockout mice; DOPAC, 3,4-dihydroxyphenylacetic acid; FSCV, fast-scan cyclic voltammetry; GPCR, G protein-coupled receptor; 5-HT, serotonin; HVA, homovanillic acid; MAO, monoamine oxidase; β-PEA, β-phenylethylamine; WT, wild type.

highest levels are found in the nigrostriatal and mesolimbic regions, such as the caudate–putamen, olfactory tubercles and nucleus accumbens (Paterson *et al*. 1990). In the striatum, β-PEA is synthesized by decarboxylation of L-phenylalanine by aromatic L-amino acid decarboxylase in neurons that also contain tyrosine hydroxylase, with a rate of synthesis similar to that of DA (Paterson *et al*. 1990). However, β-PEA is very rapidly metabolized (half-life 0.4 min), mainly by monoamine oxidase (MAO) type B (MAO-B), to phenylacetic acid, resulting in striatal tissue β-PEA concentrations that are approximately three orders of magnitude lower than DA levels (Saavedra 1989; Paterson *et al*. 1990). The role of β-PEA in mammalian physiology is unknown; however, changes in β-PEA metabolism have been demonstrated in various human disorders including phenylketonuria, migraine, schizophrenia, attention deficit hyperactivity disorder (ADHD) and depression (Sandler *et al*. 1980; Baker *et al*. 1991, 1993; O'Reilly and Davis 1994; Kusaga *et al*. 2002; Branchek and Blackburn 2003).

In rodents, β-PEA produces a robust behavioral phenotype consisting of short-term stimulation of locomotor activity and stereotypy as well as a plethora of peripherally mediated effects (Dourish 1982; Boulton 1982; Saavedra 1989; Lapin 1996). Neurochemical characterization of this psychostimulant action leads to two major hypotheses. It has been noted that β-PEA can inhibit uptake and/or induce efflux of DA, as well as norepinephrine and serotonin (5-HT) (Horn and Snyder 1972; Raiteri *et al*. 1977; Dyck 1983; Bailey *et al*. 1987). It has been suggested that β-PEA can act as an 'endogenous amphetamine' (Janssen *et al*. 1999) by inducing the efflux of presynaptically stored monoamines in the extracellular space via reversal of the direction of plasma membrane monoamine transporter-mediated transport (Stamford *et al*. 1986; Parker and Cubeddu 1988). Alternatively, radioligand binding studies have suggested that β-PEA can act through specific trace amine receptors (Hauger *et al*. 1982; Greenshaw 1989; Paterson *et al*. 1990). Accordingly, 15 members of a distinct family of G protein-coupled receptors (GPCRs) that bind various trace amines including β-PEA have been identified recently (Borowsky *et al*. 2001; Bunzow *et al*. 2001; Kim and Von Zastrow 2001; Premont *et al*. 2001). Thus, delineation of indirect (transporter mediated) and direct (GPCR mediated) effects of β-PEA is an important issue.

Mice lacking the DAT (DAT knockout, DAT-KO) represent a unique *in vivo* model for evaluating the role of DAT in the effects of psychoactive compounds (Giros *et al*. 1996; Gainetdinov *et al*. 2002). Lack of the DAT in these mice results in disrupted DA clearance and persistently raised levels of striatal extracellular DA (Jones *et al*. 1998a). This constitutive functional hyperdopaminergia manifests itself behaviorally as pronounced hyperactivity, perseverations and multiple other behavioral abnormalities (Giros *et al*. 1996; Gainetdinov *et al*. 1999; Gainetdinov and Caron 2003; Barr *et al*. 2004; Morice *et al*. 2004; Powell *et al*. 2004). Importantly, amphetamine and other psychostimulants can paradoxically inhibit hyperactivity of DAT-KO mice (Gainetdinov *et al*. 1999; Spielewoy *et al*. 2001; Morice *et al*. 2004; Powell *et al*. 2004) recapitulating the therapeutic action of psychostimulants in ADHD (Gainetdinov *et al*. 1999; Gainetdinov and Caron 2003). To assess the mechanism by which β-PEA mediates its action, we have analyzed neurochemical and behavioral effects of this trace amine in DAT mutant mice.

Materials and methods

Animals

The DAT-KO mice were generated as described previously (Giros *et al*. 1996). Offspring from heterozygote crossings remained in the same cage until weaning. Mice were genotyped by PCR analysis of DNA extracted from tail tissue. They were separated into different cages according to sex and genotype, and were maintained under standard housing conditions. Food and water were provided *ad libitum*. Animal care was in accordance with the *Guide for Care and Use of Laboratory Animals* (National Institutes of Health publication 865–23, Bethesda, MD, USA) and approved by the Institutional Animal Care and Use Committee. Three to four-month-old littermate wild-type (WT), heterozygote (DAT-HET) and homozygote (DAT-KO) mixed background (C57BL/6J × 129/SVJ) mice of both sexes were used in these experiments. In all behavioral experiments, WT littermates served as controls for mutant mice and all the genotypes were evaluated simultaneously.

Drug administration

β-PEA (Sigma RBI, St Louis, MO, USA) or saline (0.9% NaCl) was administered i.p. in a volume of 10 mL/kg. β-PEA was dissolved in saline for systemic administration or artificial CSF for intrastriatal infusion.

In vivo microdialysis

To perform *in vivo* microdialysis experiments, mice were anesthetized and placed in a stereotaxic frame as described previously (Gainetdinov *et al*. 1997). Dialysis probes (2 mm membrane length, 0.24 mm external diameter, Cuprophane, 6 kDa cut-off, CMA-11; CMA/Microdialysis, Solna, Sweden) were implanted into the right striatum. Owing to significant differences in animal size (Giros *et al*. 1996; Bosse *et al*. 1997), the stereotaxic coordinates for implantation of microdialysis probes were (in mm): anterior-posterior (AP) 0.0, dorsal-ventral (DV)− 4.4, lateral (L) 2.5 for wild type and DAT-HET mice, and AP 0.0, DV − 3.2, L 1.8 for DAT-KO mice, relative to bregma (Franklin and Paxinos 1996). Placement of the probe was verified by subsequent histological examination. After surgery the animals were returned to their home cages with free access to food and water. At 24 h after surgery, the dialysis probe was connected to a syringe pump and perfused at 1 μL/min with artificial CSF (NaCl 147 mM, KCl 2.7 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 0.85 mM; CMA/Microdialysis). After equilibration for at least 1 h, the perfusate was collected every 20 min into tubing containing 1 μL 2 M perchloric acid. At least four controls samples were taken before β-PEA was either administered systemically (50 mg/kg, i.p.) or locally infused into the striatum via the microdialysis probe (1–100 μM). Perfusate

samples were assayed for DA using HPLC with electrochemical detection. DA was separated on a microbore Unijet C18 reverse-phase column (C-18, 5 μm, 1 × 150 mm; BAS, West Lafayette, IN, USA) with a mobile phase consisting of 0.03 M citrate–phosphate buffer with 2.1 mM octyl sodium sulfate, 0.1 mM EDTA, 10 mM NaCl and 17% methanol (pH 3.6) at a flow rate of 90 μL/min and detected by a 3-mm glass carbon electrode (Unijet; BAS) set at +0.8 V. The injection volume was 5 μL. The sensitivity of the method permitted detection of approximately 3 fmol DA.

Cyclic voltammetry in brain slices

Mice were killed by decapitation and the brains were rapidly removed and cooled in ice-cold, pre-oxygenated (95% O_2/5% CO_2), modified buffer. The tissue was then sectioned into coronal slices 400 μm thick, containing the caudate–putamen, with a vibrating tissue slicer (Leica VT1000S; Leica Instruments, Germany). Slices were kept in a reservoir of oxygenated Krebs' buffer at room temperature (20°C) until required. Thirty minutes before each experiment, a brain slice was transferred to a 'Scottish-type' submersion recording chamber, perfused at 1 μL/min with 34°C oxygenated Krebs' buffer, and allowed to equilibrate. The Krebs' buffer contained NaCl 126 mM, KCl 2.5 mM, NaH_2PO_4 1.2 mM, $CaCl_2$ 2.4 mM, $MgCl_2$ 1.2 mM, $NaHCO_3$ 25 mM, glucose 11 mM, HEPES 20 mM and L-ascorbic acid 0.4 mM, pH 7.4. DA was evoked by a single, rectangular, electrical pulse (300 μA, 2 ms/phase, biphasic), applied every 15 min. DA was detected using fast-scan cyclic voltammetry (FSCV) as described earlier (Jones *et al*. 1998a,b; Budygin *et al*. 2001, 2002).

Once a stable DA response to electrical stimulation was observed for three successive stimulations, β-PEA was applied to the striatum via the superfusate. Each slice served as its own pre-condition control. For each experimental group, slices were obtained from at least four animals. Background-subtracted cyclic voltammograms were constructed by subtracting the background current obtained before release (baseline) from the current measured after release. In each case, DA was the substance detected and was identified by its characteristic cyclic voltammogram. The oxidation current for DA was converted to concentration by electrode calibration with 10 μM DA at the end of the experiment. Clearance rate of evoked DA (rate of disappearance of evoked DA signal) in μM/s was measured from the peak amplitude (overflow amplitude) of the post-drug response to baseline on both pre-drug and post-drug curves (Budygin *et al*. 2001).

Behavioral methods

Locomotor activity of littermate WT, DAT-HET and DAT-KO mice was measured in an Omnitech CCDigiscan (Accuscan Instruments, Inc., Columbus, OH, USA) activity monitor under bright illumination. All behavioral experiments were performed between 10.00 and 17.00 hours. Activity was measured at 5-min intervals. To evaluate the effects of β-PEA on locomotor behavior, mice were habituated to activity monitor chambers (20 × 20 cm^2) for 30 min before administration of drug or vehicle i.p., and locomotor activity was monitored for the following 60 min. Under these conditions mice were used on a single occasion only.

Ethologically based assessment of stereotyped behaviors was carried out in a manner similar to that described previously (Mcnamara *et al*. 2002). After habituation to a locomotor activity chamber for 30 min, each animal was injected with β-PEA or saline and evaluated for a 30-s period once every 5 min using a conventional 0–6-point stereotypy scale: 0, asleep or inactive; 1, episodes of normal activity; 2, discontinuous activity with bursts of prominent sniffing and rearing; 3, continuous stereotypy such as sniffing or rearing along a path; 4, stereotyped sniffing or rearing fixed in one location; 5, stereotyped behavior with bursts of licking or gnawing; and 6, continuous licking or gnawing. To evaluate the effect of the treatment on grooming behavior, the number of 30-s periods when stereotyped grooming was observed was counted. This procedure was repeated on the same mice on three additional occasions with different doses of the drug or saline; all genotypes were evaluated simultaneously. Under these conditions mice were used repeatedly, with each animal allocated randomly to one of various treatment groups, separated with drug-free interval of at least 1 week. All the assessments made by an observer who was unaware of genotype and treatment of each animal.

Data analysis

Data are presented as mean ± SEM and were analyzed using two-tailed Student's *t*-test or one-way ANOVA. Owing to large differences between genotypes in most behavioral variables, all statistical analysis of dose–effect was performed within genotypes only using one-way ANOVA followed by Dunnet's multiple comparison test.

Results

Effect of systemic administration and local intrastriatal infusion of β-PEA on striatal extracellular DA levels

A potent action of β-PEA on efflux and uptake of DA, norepinephrine and 5-HT has been demonstrated *in vitro* using synaptosomes and brain slice preparations as well as *in vivo* using push–pull cannula and microdialysis approaches (Raiteri *et al*. 1977; Dyck 1983; Philips 1986; Bailey *et al*. 1987; Parker and Cubeddu 1988; Nakamura *et al*. 1998). In the present study, an *in vivo* microdialysis approach was used to evaluate the effect of β-PEA on extracellular levels of DA in the striatum of freely moving WT and DAT-KO mice. It is worth noting that disruption of DAT-mediated transport in mutant mice significantly changes basal extracellular concentrations of DA in the striatum, with a two-fold increase observed in DAT-HET mice and a five-fold increase in DAT-KO mice (Jones *et al*. 1998a). Figure 1 shows that systemic administration of β-PEA at the most effective dose for stimulating locomotion in mice (50 mg/kg, i.p.; Lapin 1996) produced a transient 6.5-fold increase in extracellular DA levels in WT mice, but not in DAT-KO mice. In DAT-HET mice, which express 50% of the DAT (Giros *et al*. 1996), the same dose of β-PEA induced a less pronounced increase in extracellular DA (up to 181 ± 9% of basal levels, $n = 5$; data not shown), demonstrating a gene dosage dependence of this effect. Furthermore, this treatment did not alter the dialysate levels of major DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in either genotype (data not shown).

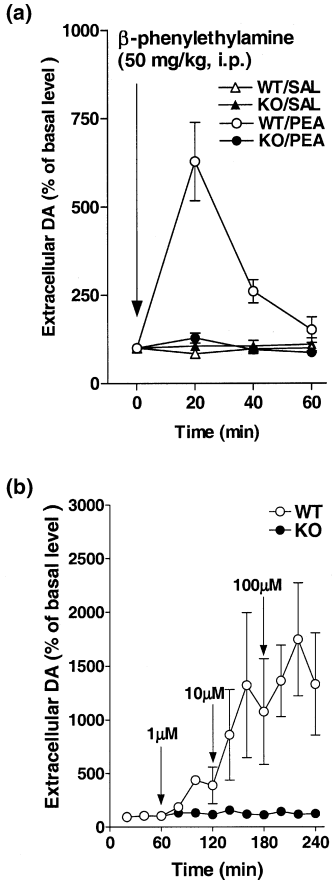

Fig. 1 Effect of systemic administration (a) and intrastriatal infusion (b) of β-PEA (PEA) or saline (SAL) on extracellular striatal levels of DA in WT and DAT-KO (KO) mice. Microdialysis was used to measure extracellular DA levels in freely moving mice. Data represent mean ± SEM values obtained from 4–6 mice per group. (a) Systemic administration of β-PEA (50 mg/kg, i.p.) induced a significant increase in extracellular DA in WT mice (analysis of area under curve values for 60-min periods after drug or saline administration revealed significant effect of β-PEA vs. respective saline-treated WT mice; $p < 0.001$, Student's *t*-test) but not in DAT-KO mice. (b) Effect of local intrastriatal infusion of β-PEA (1, 10 and 100 μM) on striatal extracellular DA in WT and DAT-KO mice. Analysis of area under curve values (data not shown) for 60-min periods before and after each concentration of drug infused revealed a significant effect of each concentration of β-PEA over pre-drug values in WT mice ($p < 0.001$, one-way ANOVA followed by Dunnet's multiple comparison test), but no significant effect was observed in mutant mice. The basal extracellular levels of DA in DAT-KO mice were higher than those in WT mice [pre-drug concentrations of DA in dialysates: WT, 92 ± 14 fmol per 20 μL ($n = 14$); DAT-KO, 302 ± 64 fmol per 20 μL ($n = 15$)].

It is believed that the major site of the DA-releasing action of β-PEA responsible for its behavioral activation is localized in striatal and mesolimbic DA terminals (Boulton *et al.* 1990). In a recent microdialysis investigation, it was observed that local infusion of β-PEA into the nucleus accumbens produced a dramatic increase in the level of extracellular DA (Nakamura *et al.* 1998). Therefore, to investigate directly the effect of β-PEA on striatal DA dynamics, increasing concentrations of β-PEA (1–100 μM) were applied intrastriatally via a microdialysis probe. In the striatum of WT mice, β-PEA produced a pronounced concentration-dependent increase in extracellular DA, whereas DAT-KO mice showed no significant change. Taken together, data obtained after both systemic and local administration of β-PEA indicate that the DAT is required for the DA-releasing effect of β-PEA in the striatum.

Effect of β-PEA on DA overflow amplitudes and clearance rates in striatal slices from WT and DAT-KO mice

It is believed that the DA-releasing action of amphetamine and related phenylethylamines involves several critical steps, including penetration into the dopaminergic neuron via the DAT, but also via diffusion, and displacement of DA from intracellular vesicular stores into the cytoplasm with subsequent reversal of the direction of DAT-mediated transport of DA, resulting in accumulation of DA in the extracellular space (Liang and Rutledge 1982; Parker and Cubeddu 1988; Zaczek *et al.* 1991a,b; Seiden *et al.* 1993; Sulzer *et al.* 1995; Amara and Sonders 1998; Jones *et al.* 1998a,b). In fact, voltammetric investigations have demonstrated that β-PEA, much like amphetamine, produces a potent decrease in the amplitude of stimulated DA release, reflecting the vesicular DA-depleting action of β-PEA (Stamford *et al.* 1986) and/or D2 autoreceptor activation (Schmitz *et al.* 2001). Furthermore, reversal of the direction of DAT-mediated transport might result in a concurrent inhibition of the rate of DA clearance from the extracellular space, a process that is is detectable by FSCV (Kuhr *et al.* 1985; Jones *et al.* 1998b; Wu *et al.* 2001). Therefore, FSCV was used to compare the effects of β-PEA on stimulated release and uptake rate in the presence and absence of the DAT (Figs 2 and 3). In accordance with previous investigations (Jones *et al.* 1998a,b), the clearance rate of evoked DA in striatal slices from DAT-KO mice was dramatically slower than that in WT mice (Fig. 2). At the same time the amplitude of stimulated DA release was three times lower in DAT-KO mice (1.19 ± 0.07 vs. 3.12 ± 0.15 μM). As shown previously (Stamford *et al.* 1986), β-PEA (50 and 100 μM) decreased the amplitude of DA release in normal animals, but no such decrease was observed in slices from DAT-KO mice (Figs 2 and 3a). Furthermore, the DA clearance rate was affected by β-PEA in striatal slices of WT mice, but no effect was found in the striatum of DAT-KO mice (Figs 2 and 3b). Some differences in effective concentrations of β-PEA observed in microdialysis and voltammetry experiments are probably related to the use of different brain preparations (slices in voltammetry experiments and intact tissue in microdialysis *in vivo* experiments). Together

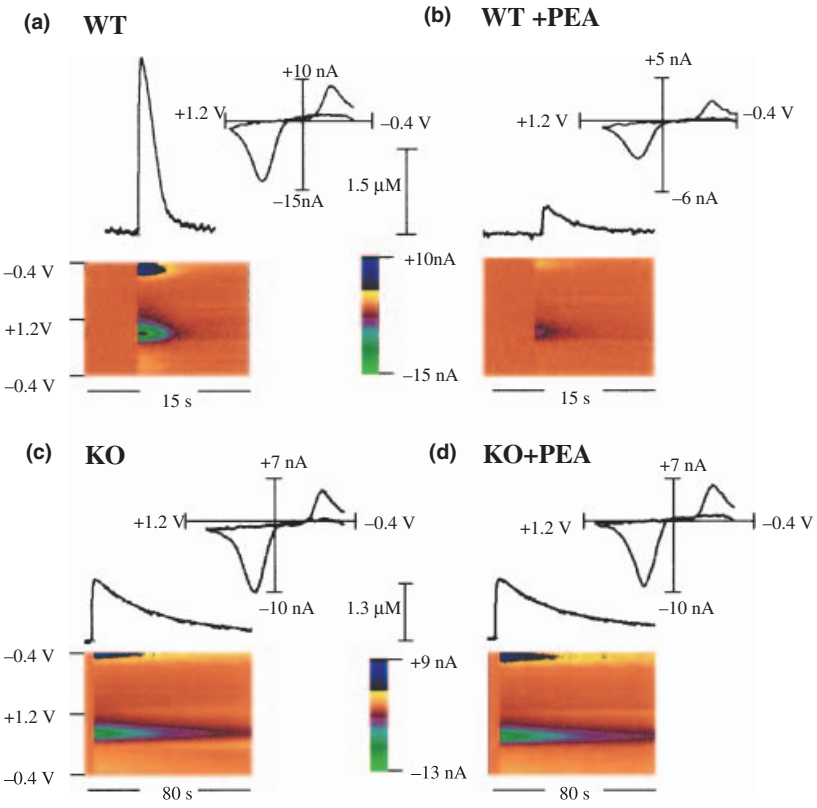

Fig. 2 DA overflow in response to single-pulse stimulation (300 µA, 2 ms per phase, biphasic pulse) measured by FSCV in striatal slices from WT (a, b) and DAT-KO (KO) (c, d) mice before (a, c) and during (b, d) β-PEA 100 µM bath application. The color plot topographically depicts the voltammetric data, with time on *x*-axis, applied scan potential on the *y*-axis (scan direction from bottom to top) and background-subtracted current measured on the z-axis in pseudocolor. Insets are background–subtracted cyclic voltammograms taken at the peak response. For further explanation of this type of data presentation, see Michael *et al.* (1998).

with results from the microdialysis experiments, these data highlight a critical role for the DAT in the dopaminergic effects of β-PEA.

Behavioral effects of β-PEA in WT and DAT mutant mice

The systemic administration of β-PEA produces a complex set of behavioral manifestations, including short-term hyperlocomotion, rearing, sniffing, headbobbing, headweaving, forepaw padding, gnawing, licking, abortive grooming, backward walking, wet dog shakes, head twitch, straub tail, seizures, salivation and labored breathing (Boulton 1982; Dourish 1982; Saavedra 1989; Lapin 1996). Because β-PEA is subject to fast metabolism by MAO-B (Usdin and Sandler 1976; Saavedra 1989; Janssen *et al*. 1999), these effects are highly dose and time dependent. For example, it is well known that β-PEA has a biphasic stimulatory action on locomotor activity of rodents (Boulton 1982; Dourish *et al*. 1982; Lapin 1996). Particularly, it has been demonstrated that at doses close to 50 mg/kg i.p. β-PEA produces short-term stimulatory effects on locomotion (forward locomotion), whereas at higher doses (75–100 mg/kg, i.p.), it produces predominately stereotyped behaviors (headbobbing, head weaving, rearing, sniffing, grooming, licking) as well as prominent peripheral effects (salivation, labored breathing).

To determine the contribution of DAT-mediated processes to behavioral responses of β-PEA, locomotor activity and stereotypy of WT, DAT-HET and DAT-KO mice were examined. β-PEA was administered 30 min after mice had been placed into locomotor activity chambers, and its effect on locomotion and stereotypy was analyzed during a 60-min post-drug treatment period. No analysis of vertical activity was performed because β-PEA produced a prominent straub tail in all genotypes and this effect contaminated measurement of vertical activity related to rearing behavior (data not shown).

In agreement with previous observations (Giros *et al*. 1996; Gainetdinov *et al*. 1999; Ralph *et al*. 2001; Spielewoy *et al*. 2001), saline-treated DAT-KO mice revealed spontaneous hyperactivity in a novel environment ($p < 0.01$ vs. WT or DAT-HET mice; one-way ANOVA followed by Dunnet's multiple comparison test), whereas DAT-HET mice did not differ from WT littermates following treatment with saline (Fig. 4). β-PEA at 50 mg/kg, a dose that is maximally effective in eliciting hyperactivity in mice (Lapin 1996), produced a significant short-term (10–15 min) locomotor stimulation in both WT and DAT-HET mice (Figs 4a and b). Paradoxically, the same treatment decreased locomotion in DAT-KO mice (Fig. 4c). Importantly, the decrease in locomotion in DAT-KO mice lasted for at least 60 min after treatment with β-PEA, whereas the stimulation observed in

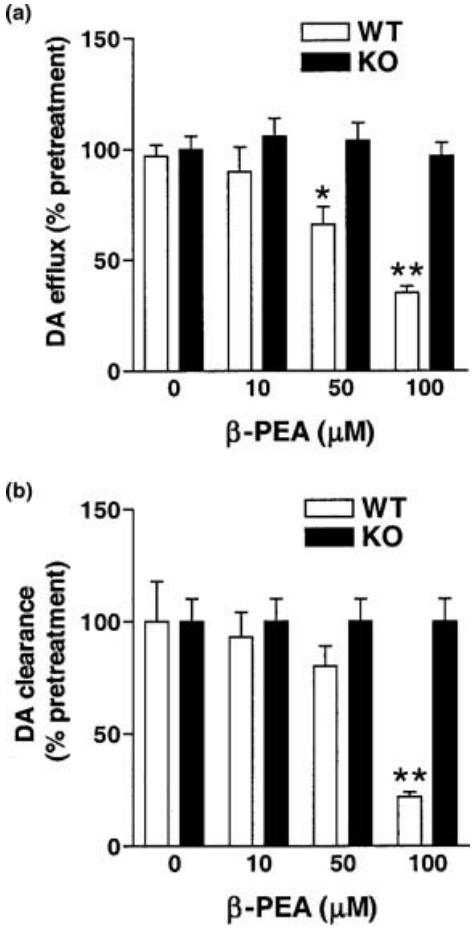

Fig. 3 Effect of β-PEA on DA overflow amplitudes (a) and DA clearance rates (b) in striatal slices from WT and DAT-KO (KO) mice. Stimulated DA efflux was measured by FSCV as the peak height of DA in μM, and clearance rate was measured as DA concentration change in μM/s. Data represent the mean ± SEM of values obtained from 4–5 mice per group. Significant values for DA efflux, expressed as percentage of pretreatment values, are as follows: 50 μM, 66 ± 8%; 100 μM, 35 ± 3% [$^{*}p < 0.05$; $^{**}p < 0.01$ vs. time-matched control value (3.12 ± 0.15 μM) in the WT; one-way ANOVA followed by Dunnet's multiple comparison test]. Significant values for DA clearance are as follows: 100 μM, 22 ± 2 [$^{**}p < 0.01$ vs. time-matched control value (0.54 ± 0.1 μM/s) in WT mice; one-way ANOVA followed by Dunnet's multiple comparison test]. β-PEA had no effect on DA efflux or clearance in DAT-KO mice.

WT and DAT-HET mice was very brief (Figs 4a–c). Analysis of the dose–response effect of β-PEA in WT mice (Fig 4d) showed that the maximal locomotor stimulation occurred at a dose 50 mg/kg, i.p. Lower doses (10 and 30 mg/kg, i.p.) had no significant effect on locomotor activity in these mice, whereas hyperactivity was less pronounced at higher doses (70 and 100 mg/kg, i.p.), most probably because stereotyped behavior competed with locomotor activation. In DAT-HET mice maximal increases in locomotor activity were observed with 70 mg/kg β-PEA (Fig. 4e). In striking contrast to results in both the WT and DAT-HET mice, all doses of β-PEA decreased the raised levels of activity in DAT-KO mice (Fig. 4f).

As described above, β-PEA is known to produce stereotyped behaviors which compete with locomotor activity. In fact, high doses of β-PEA (70 and 100 mg/kg, i.p.) produced robust stereotypies in WT and DAT-HET mice, such as headweaving, padding, sniffing, rearing, grooming and licking (visual observations). In DAT-KO mice both increased horizontal activity and stereotypical manifestations were markedly suppressed by β-PEA at doses up to 50 mg/kg, i.p.; however, higher doses induced not only inhibited activity but also induced some stereotypies, such as sniffing, grooming and headweaving (visual observations).

Analysis of computer-generated data on stereotypical behaviors revealed a significant decrease in stereotypy measurements in DAT-KO mice at all doses tested (data not shown) and failed to reveal an increase in certain stereotypical behaviors visually observed in WT mice at 70–100 mg/kg β-PEA. Because technical limitations may prevent reliable detection of stereotypies in a computerized locomotor activity monitor, an ethological, observer-based approach was applied to assess stereotypy (Mcnamara *et al*. 2002) in a separate group of mice. The effects of 50, 70 and 100 mg/kg β-PEA were assessed in all genotypes. Using this approach, it was observed that high doses of β-PEA (70 and 100 mg/kg) increased global stereotypy scores in both WT and DAT-HET mice to the same extent (Figs 5a and b). The dose–response curve for these stereotypy measurements was more complex in DAT-KO mice (Fig. 5c); at 50 mg/kg β-PEA, the global stereotypy score was decreased but no such reduction was observed at 70 and 100 mg β-PEA.

Because the global stereotypy score may not reflect the fact that certain stereotypical behaviors such as sniffing, grooming, headweaving are actually increased over those in saline-treated controls, one measure of stereotypy – stereotypical grooming – was selected for separate analysis. β-PEA increased the number of 5 min observational periods in which stereotypical grooming was observed in both WT and DAT-HET mice. Similarly, β-PEA increased stereotypical grooming in DAT-KO mice, although this effect was less pronounced than that in WT and DAT-HET mice (Figs 5d–f).

Discussion

It is well known that β-PEA can produce both psychostimulant-like and DA-releasing effects (Boulton 1982; Dourish 1982; Janssen *et al*. 1999; Bergman *et al*. 2001). Several studies have shown that β-PEA can inhibit uptake of DA as well as norepinephrine and 5-HT in brain synaptosomal preparations (Raiteri *et al*. 1977; Dyck 1983; Philips 1986; Bailey *et al*. 1987) and/or, much like amphetamine, enhance DA transmission by a complex interaction with the DAT and vesicular storage (Parker and Cubeddu 1988). Recent

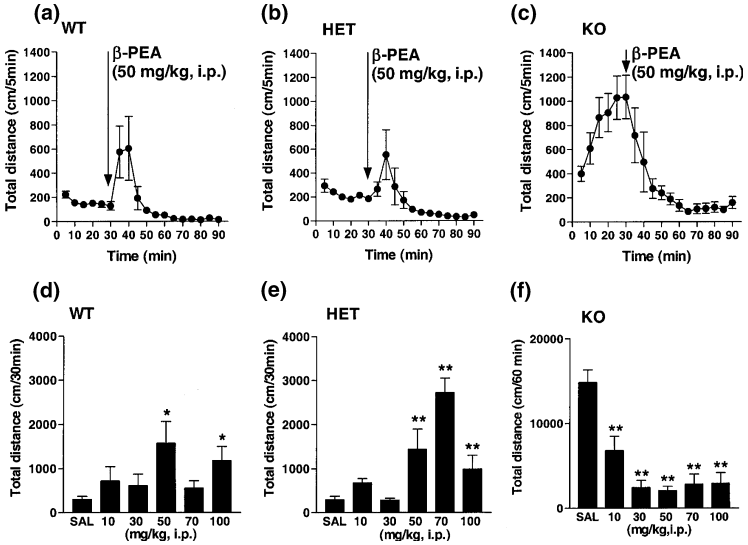

Fig. 4 Effect of β-PEA on locomotor activity in WT, DAT-HET and DAT-KO mice. (a–c) Time dependence of the effect of β-PEA (50 mg/kg, i.p.) on the locomotor activity of WT (a), DAT-HET (b) and DAT-KO (c) mice. Mice were placed in the open field apparatus for an initial period of 30 min and then injected with β-PEA (50 mg/kg, i.p.) and activity was recorded every 5 min during 1 h. Results are presented as the mean ± SEM of $n = 15$ for WT, $n = 8$ for DAT-HET and $n = 15$ for DAT-KO mice. In all genotypes analysis of total distance traveled for 30 min after drug administration revealed a significant difference from respective data points in saline-treated controls (data not shown). (d–f) Dose–response of the locomotor effect of β-PEA in WT (d), DAT-HET (e) and DAT-KO (f) mice. Mice were placed in the open field apparatus for an initial period of 30 min and then injected with saline or β-PEA (10, 30, 50, 70 and 100 mg/kg, i.p.). Activity was recorded every 5 min and presented as cumulative values for 30 min after drug or saline treatment for WT and DAT-HET mice. Owing to significant differences in the duration of effects, the data for DAT-KO are presented as cumulative values for 60 min. Results are the mean ± SEM of $n = 10$–15 for WT, $n = 8$–13 for DAT-HET and $n = 10$–15 for DAT-KO mice. $^{*}p < 0.05$, $^{**}p < 0.01$ versus respective saline-treated (SAL) control (one-way ANOVA followed by Dunnet's multiple comparison test).

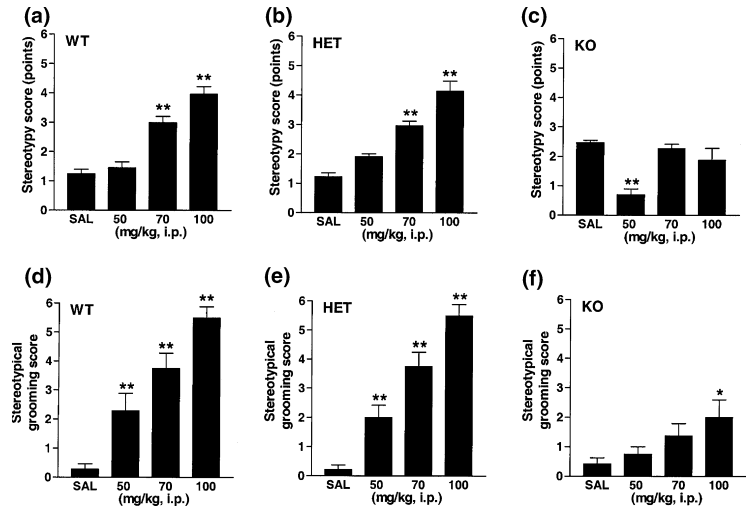

Fig. 5 Ethologic analysis of stereotyped behaviors after administration of β-PEA (50, 70 and 100 mg/kg, i.p.) in WT (a, d), DAT-HET (b, e) and DAT-KO (c, f) mice. Each mouse was placed in a locomotor activity chamber, injected with the drug 30 min later and observed visually for 30 min. (a–c) To determine the global stereotypy score each animal was evaluated over a 30-s period every 5 min using a conventional 0–6-point stereotypy scale. Stereotypical counts were averaged for each mouse for six periods. (d–e) To determine stereotypical grooming score the number of 5-min periods in which stereotyped grooming was prominent was counted. Each group consisted of $n = 8$ for all genotypes. $^{*}p < 0.05$, $^{**}p < 0.01$ versus respective saline-treated (SAL) control (one-way ANOVA followed by Dunnet's multiple comparison test).

evidence also indicates that β-PEA may act directly on its own GPCRs (Borowsky *et al*. 2001; Bunzow *et al*. 2001), a possibility that others have suggested previously (Greenshaw 1989; Paterson *et al*. 1990). The aim of our study was therefore to investigate the role of the DAT in the neurochemical and behavioral effects of β-PEA using mice lacking the DAT as a test system.

In a previous microdialysis investigation in rats, β-PEA, infused directly to the nucleus accumbens via a microdialysis probe, produced a potent increase in extracellular DA (Nakamura *et al*. 1998). Our microdialysis experiments confirmed these observations. Both systemic and intrastriatal administration of β-PEA markedly increased extracellular DA levels in the striatum of WT mice. However, this effect was not observed in DAT-KO mice, clearly demonstrating that DAT is required for this action. It is worth re-emphasizing that the measurements were made in the striatum, the brain region that has the highest density of DA terminals. Recent investigations have shown that in the nucleus accumbens of DAT-KO mice, in contrast to the striatum, both cocaine and amphetamine are able to increase extracellular levels of DA, most likely as a result of an indirect modulation of mesolimbic dopaminergic neurons via 5-HT mechanisms (Jones *et al*. 1998b; Rocha *et al*. 1998; Carboni *et al*. 2001; Budygin *et al*. 2002, Budygin *et al*. 2004; Gainetdinov *et al*. 2002; Mateo *et al*. 2004). Therefore, it is possible that DA dynamics in the nucleus accumbens and/or other brain regions of DAT-KO mice might be still affected by β-PEA. However, given the present focus on the mechanism of action of β-PEA on DA neuronal functions, the effects of β-PEA were examined only in the striatum where the contribution of DAT-unrelated mechanisms to the effects of psychostimulants on extracellular DA dynamics is negligible (Jones *et al*. 1998a; Rocha *et al*. 1998; Gainetdinov *et al*. 1999).

Hypothetically, exogenous β-PEA might increase the level of extracellular DA in the striatum by at least two mechanisms. First, as a lipophilic amine (Mack and Bonisch 1979) β-PEA could enter the nerve terminal either by diffusion across the plasma membrane or via DAT-mediated inward transport. Consequently, it could displace DA from vesicles and promote release from nerve terminals by a DAT-dependent reverse transport mechanism in a manner similar to amphetamine (Liang and Rutledge 1982; Stamford *et al*. 1986; Parker and Cubeddu 1988; Zaczek *et al*. 1991a,b; Seiden *et al*. 1993; Sulzer *et al*. 1995; Jones *et al*. 1998b). Alternatively, because trace amine receptors are expressed in primary monoaminergic brain areas (Borowsky *et al*. 2001; Bunzow *et al*. 2001), β-PEA may act directly on these receptors, potentially affecting monoaminergic neurons in a transporter-independent mechanism. Our findings strongly suggest that the contribution of trace amine receptors to this effect is negligible and supports the hypothesis that the DA-releasing action of β-PEA, like that of amphetamine (Jones *et al*. 1998b), is primarily depend on the DAT. In fact, it has been shown that the β-PEA-induced increase in extracellular DA (Nakamura *et al*. 1998), like that induced by amphetamine (Westerink *et al*. 1987), is not affected by co-perfusion of tetrodotoxin, confirming the action potential-independent nature of this outflow.

The mechanism of action of amphetamine and related drugs appears to be more complex than that of classical DAT inhibitors. Recent experiments in DAT-KO mice using microdialysis and FSCV (Jones *et al*. 1998b) have supported previous studies (Parker and Cubeddu 1988; Sulzer *et al*. 1995; Amara and Sonders 1998) demonstrating that amphetamine's ability to induce DA release is critically dependent on both vesicle depletion and reversal of DA transport. Particularly, it has been observed that amphetamine increases extracellular DA concentration simultaneously with a decrease in depolarization-evoked DA release, reflecting displacement of DA from vesicular storage by this drug (Jones *et al*. 1998b) and D2 DA autoreceptor activation (Schmitz *et al*. 2001). Previous voltammetric experiments with β-PEA have shown that this amine acts similarly to amphetamine by causing a large decrease in stimulated DA release in the rat striatum (Stamford *et al*. 1986). In the present voltammetric investigation, β-PEA was effective in decreasing both the amplitude of evoked DA efflux and DA clearance rate in the striatum of WT mice. However, the same treatment failed to change DA efflux or clearance in the striatum of DAT-KO mice, providing additional evidence that the primary mechanism of β-PEA action on DA neuron depends on an intact DAT.

There are some characteristics of the action of amphetamine in WT and DAT-KO mice (Jones *et al*. 1998b) that we were not able to demonstrate in the present voltammetric studies using β-PEA. In WT mice, a significant increase in baseline DA signal concomitant with a decrease in amplitude of evoked DA release was observed after amphetamine (Jones *et al*. 1998b). In DAT-KO mice, no increase in baseline voltammetric signal for DA was observed but the ability of amphetamine to displace vesicular DA seemed to be preserved (Jones *et al*. 1998b). In the present investigation, β-PEA did not affect the baseline signal in either WT or DAT-KO mice (data not shown), probably owing to the very short lifetime of this amine. Furthermore, β-PEA was not able to induce a decrease in amplitude of evoked DA release in DAT-KO mice. This may indicate that the predominant route of entry of exogenous β-PEA in the nerve terminal is DAT dependent; without the DAT β-PEA may not reach the intracellular concentration necessary for its vesicle-depleting action. Furthermore, these potentially small amounts of β-PEA entering the DA terminal via trans-membrane diffusion in the DAT-KO mice might undergo enzymatic degradation, whereas amphetamine is not known to be metabolized within DA neurons. It should also be noted that the lack of recycling mechanism in DAT-KO mice results in

markedly reduced intraneuronal DA storage (Jones *et al*. 1998a; Gainetdinov and Caron 2003) and disrupted autoreceptor function (Jones *et al*. 1999), which may also contribute to the lack of effect of β-PEA in mutant mice.

Another important difference between amphetamine and β-PEA was observed in microdialysis experiments. No effect of β-PEA on DA metabolite levels was found in either genotype, whereas amphetamine is known to significantly decrease extracellular levels of DA metabolites in both WT and DAT-KO mice (Jones *et al*. 1998b). It is believed that the amphetamine-induced decrease in DOPAC and HVA levels may reflect depletion of a newly synthesized cytoplasmic pool of DA, MAO inhibitory action and/or the effect of the amine on efflux of DA metabolites from the brain (Westerink and Kikkert 1986; Seiden *et al*. 1993; Jones *et al*. 1998b). The lack of effect of β-PEA on DA metabolites probably relates to the short lifetime of this amine, but may also reflect fundamental differences between β-PEA and amphetamine with respect to the transport of DA metabolites from the brain (Westerink and Kikkert 1986).

These mechanisms of action of exogenously applied β-PEA may also relate to endogenously synthesized β-PEA, although it is highly questionable whether endogenous β-PEA can reach the concentrations required for these effects under normal conditions (Paterson *et al*. 1990; Barroso and Rodriguez 1996). Endogenously synthesized β-PEA is known to be metabolized very rapidly (Paterson *et al*. 1990; Holschneider *et al*. 2001), but one cannot completely exclude the possibility that, under certain conditions, the concentration of this amine may substantially increase.

Several observations were made when the behavioral effects of β-PEA were monitored in DAT mutant mice. First, β-PEA produced a complex set of competing behaviors in WT mice which included psychomotor activation and various stereotypies as well as prominent peripherally mediated effects. Second, effects of β-PEA were only modestly altered in mice whose DAT levels were 50% of normal (DAT-HET). Furthermore, in mice lacking the DAT (DAT-KO), some characteristic behaviors induced by β-PEA in normal mice were absent whereas others remained relatively intact. Finally, some previously uncharacterized actions of β-PEA were revealed in DAT-KO mice. The most notable behaviors absent in DAT-KO mice were the brief hyperlocomotion and most of the stereotypical reactions observed in the WT and DAT-HET mice following β-PEA administration. At high doses, however, β-PEA still produced certain stereotypies in DAT-KO mice, including sniffing, abortive grooming and headweaving. Thus, these stereotypical behaviors induced by high doses of β-PEA are partially independent of the DAT and may reflect the action of this amine on another neurotransmitter system and/or trace amine receptors.

One unexpected finding of the present study is that a wide range of doses (10–100 mg/kg) of β-PEA markedly inhibited hyperactivity of DAT-KO mice. Although decreases in locomotor activity observed after higher doses of this amine (> 70 mg/kg) were accompanied by stereotypies and therefore might be potentially explained by the competititve nature of stereotypical behaviors, this does not appear to be the case for lower doses of β-PEA, which suppressed virtually all measures of activity in DAT-KO mice. Similarly, amphetamine at doses up to 10 mg/kg, i.p. potently inhibited both hyperlocomotion and stereotypy of DAT-KO mice (Gainetdinov *et al*. 1999; Spielewoy *et al*. 2001; Gainetdinov R. R., unpublished observations). Another intriguing feature of the inhibitory effect of β-PEA on hyperactivity is its relatively long-lasting nature, although it is not immediately clear how this rapidly metabolized amine can induce such a long-lasting action.

Previously, we and others reported that amphetamine and other psychostimulants exert similar inhibitory effects in DAT-KO mice apparently through modulation of the 5-HT system (Gainetdinov *et al*. 1999; Spielewoy *et al*. 2001; Morice *et al*. 2004; Powell *et al*. 2004). It is well known that DA-dependent locomotor activity can be modulated by 5-HT, and both 'stimulatory' and 'inhibitory' 5-HT actions have been described (Gainetdinov *et al*. 1999; Martin *et al*. 1998; Rocha *et al*. 2002; Barr *et al*. 2004). Among the 14 5-HT receptors known to date, primary candidates for the 'stimulatory' effect are 5-HT1B and 5-HT2A receptors, whereas 5-HT1A and 5HT2C receptors are probably 'inhibitory' (Martin *et al*. 1998). Furthermore, it is suggested that a balance between action of 5-HT on these 'stimulatory' and 'inhibitory' receptors may be critical for normal locomotor activity (Martin *et al*. 1998). Thus, DA-dependent locomotor hyperactivity can be suppressed either by activation of 'inhibitory' 5-HT receptors or blockade of 'stimulatory' receptors (Martin *et al*. 1998). In fact, hyperactivity of DAT-KO mice can be suppressed either by direct and indirect 5-HT agonists (Gainetdinov *et al*. 1999; Spielewoy *et al*. 2001; Powell *et al*. 2004) or 5-HT2A antagonist (Barr *et al*. 2004). Importantly, the 'paradoxical' inhibitory action of β-PEA observed in this study, as well as the effects of other psychostimulants in DAT-KO mice reported elsewhere (Gainetdinov *et al*. 1999; Spielewoy *et al*. 2001; Morice *et al*. 2004), have not been associated with a corresponding decrease in striatal extracellular DA (Jones *et al*. 1998b; Rocha *et al*. 1998; Gainetdinov *et al*. 1999). However, this effect was dependent on intact glutamatergic transmission suggesting an involvement of the frontostriatal glutamatergic pathway (Gainetdinov *et al*. 2001) that can control locomotion in a DA-independent manner (Martin *et al*. 1998; Mohn *et al*. 1999). Accordingly, recent evidence indicates that the 'inhibitory' 5-HT2C receptors in the frontal cortex are involved in the regulation of psychostimulant-induced hyperactivity (Rocha *et al*. 2002; Filip and Cunningham 2003). Because it is known that β-PEA can normally affect the extracellular dynamics of other monoamines in addition

to DA via similar transporter-mediated mechanisms (Sloviter *et al*. 1980; Dourish 1981; Parker and Cubeddu 1988; Paterson *et al*. 1990; Nakamura *et al*. 1998), it is possible that the action of raised levels of 5-HT on the 'inhibitory' 5-HT receptors in the frontal cortex may be critical for this effect. However, the possibility of involvement of trace amine receptors, particularly TA1, which shows affinity for β-PEA, amphetamine and other closely related amines (Borowsky *et al*. 2001; Bunzow *et al*. 2001), cannot be excluded. Intriguingly, it has been reported that systemic administration of a recently identified TA1 receptor agonist, 3-iodothyronamine, induces potent behavioral suppression in mice (Scanlan *et al*. 2004). Further detailed investigations will be needed to clarify this issue.

In summary, these results provide direct evidence that an intact and functional DAT is required for psychostimulant-like and striatal DA-releasing effects of β-PEA. Furthermore, the potent inhibitory effect of β-PEA on locomotion and persistence of some stereotypical behaviors in DAT-KO mice suggest that other neurotransmitter systems or a direct action of this trace amine on its own receptors are responsible for these effects.

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