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β -phenylethylamine Requires the Dopamine Transporter to Increase Extracellular Dopamine in *C. elegans* Dopaminergic Neurons

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Abstract

β -phenylethylamine (β PEA) is an endogenous amine that has been shown to increase the synaptic levels of dopamine (DA). A number of *in vitro* and behavioral studies suggest the dopamine transporter (DAT) plays a role in the effects generated by β PEA, however the mechanism through which β PEA affects DAT has not yet been elucidated. Here, we used *Caenorhabditis (C.) elegans* DAT (DAT-1) expressing LLC-pk1 cells and neuronal cultures to investigate whether the β PEA-induced increase of extracellular DA required DAT-1. Our data show that β PEA increases extracellular dopamine both in DAT-1 transfected cells and cultures of differentiated neurons. RTI-55, a cocaine homologue and DAT inhibitor, completely blocked the β PEA-induced effect in transfected cells. However in neuronal cultures, RTI-55 only partly inhibited the increase of extracellular DA generated by β PEA. These results suggest that β PEA requires DAT-1 and other, not yet identified proteins, to increase extracellular DA when tested in a native system. Furthermore, our results suggest that β PEA-induced increase of extracellular DA does not require functional monoamine vesicles as genetic ablation of the *C. elegans* homologue vesicular monoamine transporter, *cat-1*, did not compromise the ability of β PEA to increase extracellular DA. Finally, our electrophysiology data show that β PEA caused fast-rising and self-inactivating amperometric currents in a subset of wild-type DA neurons but not in neurons isolated from *dat-1* knockout animals. Taken together, these data demonstrate that in both DA neurons and heterogeneous cultures of differentiated *C. elegans* neurons, β PEA releases cytoplasmic DA through DAT-1 to ultimately increase the extracellular concentration of DA.

Keywords

dopamine transporter; β -phenylethylamine; dopamine release; electrophysiology; *C. elegans*

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Conflict of Interest

The authors declare no conflict of interest.

1. INTRODUCTION

β -phenylethylamine (β PEA) is an endogenous trace amine present in the central nervous system, however, its role in the mammalian physiology is still unknown. Previous studies demonstrated that β PEA is synthesized in neurons that also contain tyrosine hydroxylase and coexists with dopamine (DA) in the nigrostriatal brain regions (Juorio et al. 1991). In striatal tissue, β PEA synthesis occurs with a rate similar to that of DA, but since it is more efficiently metabolized by monoamine oxidase enzymes, striatal β PEA concentrations are about three orders of magnitude lower than DA levels (Paterson et al. 1990). These data suggest that it is crucial the DA neurons keep low concentrations of β PEA to guarantee a proper physiological activity. In fact, changes in urinary β PEA levels have been documented in various human disorders including schizophrenia, attention deficit hyperactive disorder (ADHD) and depression (Baker et al. 1991, O'Reilly and Davis 1994, Sandler et al. 1980). A direct interaction between dopaminergic neurons and β PEA has also been demonstrated in *in vivo* and *in vitro* experiments showing that β PEA induces DA release (Bailey et al. 1987, Ishida et al. 2005, Kuroki et al. 1990, Nakamura et al. 1998, Sotnikova et al. 2004, Yamada et al. 1998), and inhibits DA uptake (Liang and Rutledge 1982, Raiteri et al. 1976). Moreover, *in vivo* studies showed that physiological β PEA concentrations directly and transiently inhibit the firing rate of the DA neurons through the activation of the DA D2 autoreceptors (Ishida et al. 2005, Mercuri et al. 1997, Rodriguez and Barroso 1995). Interestingly, the firing inhibition caused by β PEA as well as β PEA-induced behaviors (Barroso and Rodriguez 1996) were not affected by pretreatment with the vesicular monoamine transporter (VMAT) blocker reserpine. These data suggested that β PEA stimulates the release of DA from a non-vesicular cytoplasmic pool. In this study, we investigated the effect of β PEA on extracellular levels of DA in *C. elegans* cultured neurons. We found that in isolated DA neurons, β PEA requires DAT to induce transient DA efflux. Furthermore, our data suggest that β PEA-induced DA efflux utilizes cytosolic DA since genetic ablation of VMAT did not affect the increase of extracellular DA induced by β PEA.

2. MATERIALS AND METHODS

2.1 *C. elegans* husbandry and transgenic animals

wild-type animals (N2) and knockout animals for DAT-1 (*dat-1(ok157)III*) and the *C. elegans* VMAT homologue CAT-1 (*cat-1(ok411)*) were obtained from the Caenorhabditis Genetic Center (University of Minnesota, Minneapolis, MN). All *C. elegans* strains were grown on bacteria lawns of NA22 and maintained at 22-24°C using standard methods (Brenner 1974). For amperometry recordings, we used the BY250 strain (gift from Dr. Blakely, Vanderbilt University) expressing cytosolic GFP under the control of the *dat-1*.

2.2 Extracellular [^{3} H]DA in DAT-1 transfected cells and *C. elegans* embryonic cultures

C. elegans embryonic cultures were prepared as previously described (Carvelli et al. 2004, Strange and Morrison 2006). Embryonic cells were seeded on 22x22 mm glass cover slips coated with peanut lectin (Sigma) and maintained with L15 media (GIBCO) enriched with 5% inactivated FBS and 100 units/ml of penicillin and streptomycin. Cells were assayed 2

days after been seeded. LLC-pk1 (pig epithelial kidney) cells were grown in DMEM medium enriched with 5% FBS, 2mM L-glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin. 100,000 cells were seeded into 12 well plates and after 18-24 hours were transfected with 0.5 μ g DAT-1 in the pEGFP vector using X-tremeGENE HP DNA transfection reagent (Roche). Control cells were transfected with pEGFP vector alone. 24-30 hours after transfection, cells were washed 2 times with KRH buffer (120 mM NaCl or MNDG⁺/4.7 mM KCl/1.2 mM KH₂PO₄/10 mM Hepes/2.2 CaCl₂/10 mM glucose) containing 100 μ M of ascorbic acid and tropolone (Sigma) and incubated for 30 minutes at room temperature with 20 nM [³H]DA (PerkinElmer). Cells were then washed 3 times with ascorbic acid/tropolone containing KRH buffer and treated with drugs (β PEA and/or RTI-55) or KRH (control) for 1 minute. Supernatant was collected from each well and counted for radioactivity. We used the same protocol to measure extracellular concentration of [³H]DA in cultured *C. elegans* neurons except 5 nM of [³H]DA was used and the buffer contained 145 mM NaCl or NMDG⁺, 5 mM KCl, 1 mM CaCl₂, 5 mM MgCl₂, 10 mM Hepes, and 20 mM D-glucose (pH 7.2 and 350 osmolarity).

2.3 Amperometric Recordings

4-5 days after *C. elegans* embryonic cells were seeded in peanut lectin coated glass dishes (MatTech Corporation, Cincinnati, OH), cells were washed twice with bath solution containing 145 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 5 mM MgCl₂, 10 mM HEPES, and 20 mM *d*-glucose (pH 7.2; 325 osmolarity adjusted with sucrose). DA fluxes were induced by applying 50 μ M β PEA and recorded using a carbon fiber electrodes connected to an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Electrodes were placed in proximity to an isolated dopaminergic neuron expressing cytosolic GFP. Amperometric currents were digitized with an Axon Digidata 1440A, collected with pClamp 10.2 (Molecular Devices), filtered with a low-pass Bessel filter set at 5 Hz, and analyzed using pClampfit 10.2 (Molecular Devices) and Origin 8.5 software (OriginLab Corp, Northampton, MA).

3. RESULTS AND DISCUSSION

3.1. β -phenylethylamine increases extracellular levels of DA in DAT-1 transfected cells and *C. elegans* cultured embryonic cells

Previous studies showed that β PEA induces DA release and/or inhibits DA uptake. We investigated whether β PEA increased extracellular DA in cultures of LLC-pk1 cells expressing *C. elegans* DAT (DAT-1). After preloading with 20 nM [³H]DA, cells were treated with 100 μ M β PEA for 1 minute. As shown in Figure 1, β PEA caused a statistically significant increase ($120 \pm 20\%$) of extracellular [³H]DA with respect to control treated samples (one-way ANOVA test; * $p = 0.0001$), whereas no change in extracellular [³H]DA was measured in non-transfected cells (data not shown). Previous reports demonstrated that DA uptake through DAT-1 is specifically antagonized by monoamine transporters inhibitors such as mazindol, nisoxetine and cocaine (Jayanthi et al. 1998). Here we tested whether RTI-55, a cocaine homologue, was also capable of blocking the β PEA-induced increase of extracellular [³H]DA in DAT-1 transfected cells. We found that 100 μ M RTI-55 completely blocked the effect of β PEA on the extracellular levels of [³H]DA (Figure 1, gray bar). In

fact, β PEA/RTI-55 treatment caused a decrease of extracellular [^{3} H]DA comparable to control-treated samples, $109 \pm 12\%$ and $100 \pm 2\%$, respectively. The extracellular levels of [^{3} H]DA following β PEA/RTI-55 treatment were statistically different than those measured after β PEA treatment (one-way ANOVA test, $O_p = 0.05$). To test whether RTI-55 itself had an effect on extracellular [^{3} H]DA, we treated DAT-1 transfected cells with RTI-55 alone (stripe bar, Figure 1) and found that $100 \mu\text{M}$ RTI-55 caused no effect on extracellular [^{3} H]DA with respect to controls, 108 ± 12 and 100% respectively. Taken together, these data demonstrate that β PEA causes an increase of extracellular DA in DAT-1 transfected cells which is selectively blocked by the DAT-1 inhibitor RTI-55.

Previously, it was shown that cultured *C. elegans* embryonic cells are instrumental in studying the activity of proteins in their native cells. *C. elegans* embryonic cells, which undergo terminal differentiation within a few days after being seeded, have been used to characterize protein function and regulation and to determine specific gene expression patterns (Strange and Morrison 2006, Carvelli et al. 2008). Particularly, we used this preparation to investigate the ability of DAT-1 to accumulate [^{3} H]DA in the dopaminergic neurons and showed that like the mammalian homologue, *C. elegans* DAT-1 efficiently accumulated DA, whereas genetic and pharmacological ablation of DAT-1 prevented [^{3} H]DA uptake (Carvelli et al. 2004). Importantly, our previous studies demonstrated that functional characterization of DAT-1 in differentiated neurons recapitulates the pharmacological properties observed in mammalian DAT expressed in heterologous systems. In this study, we used differentiated cultures of *C. elegans* embryonic cells to investigate the effects of β PEA on dopaminergic neurons. Three days after preparation, we preloaded *C. elegans* embryonic cells with 5 nM [^{3} H]DA and then applied $100 \mu\text{M}$ β PEA for 1 minute. Similarly to what we saw in DAT-1 transfected cells, β PEA significantly increased the amount of extracellular [^{3} H]DA (one-way ANOVA test, $*p = 0.0001$). We measured a $157 \pm 12\%$ increase in extracellular [^{3} H]DA following β PEA treatment with respect to control treated samples (Figure 2). When β PEA was applied together with $100 \mu\text{M}$ RTI-55 we measured an increase of $85 \pm 34\%$ in extracellular [^{3} H]DA with respect to control-treated samples (Figure 2, gray bar). Interestingly, this value was statistically different to both control- and β PEA-treated sample ($*p = 0.0001$ vs controls and $O_p = 0.001$ vs β PEA-treated samples). This result showed that in primary cultures RTI-55 partly blocked the β PEA-induced increase in extracellular DA, and RTI-55 applied alone did not change the extracellular amount of [^{3} H]DA with respect to controls (Figure 2, stripe bar), 126 ± 11 and 100% respectively.

In conclusion, these results demonstrate that β PEA caused an increase of extracellular DA in DAT-1 transfected cells and differentiated *C. elegans* neurons. Moreover, the ability of β PEA to increase extracellular DA was similar in DAT-1 transfected cells (157%) and neuronal cultures (120%), suggesting that DAT-1 accounts for the increase of extracellular DA measured after β PEA treatment. Interestingly though, in neuronal cultures the DAT-1 blocker RTI-55 only partly inhibited the β PEA-induced increase of extracellular DA. This result suggests that in native neurons DAT-1 and other unknown proteins are required by β PEA to elevate extracellular DA. Perhaps the DA D2 receptors as well as low affinity neurotransmitter transporters might be involved (Ishida et al. 2005, Mercuri et al. 1997,

Rodriguez and Barroso 1995, Horton et al. 2013), however, we can not exclude that a dose-related dependency of β PEA and/or RTI-55 in transfected cells versus neuronal culture underlies the different effect of RTI55 seen in these two preparations.

3.2. β -phenylethylamine-induced increase of extracellular dopamine does not involve vesicle-dependent dopamine release

Neurotransmitters like DA and serotonin are released in the synaptic cleft through 2 different mechanisms: a vesicle-dependent mechanism or a Ca^{2+} -independent and DAT mediated mechanism. In *C. elegans*, all major proteins involved in the dopaminergic system have been identified (Chase and Koelle 2007), including the vesicular monoamine transporter (VMAT) homologue CAT-1. Moreover, animals made knockout for cat-1 have been previously created and characterized (Duerr et al. 1999). We investigated whether the effects of β PEA seen in Figure 2 required intact DA vesicles. Thus, we measured the increase of extracellular [^3H]DA induced by β PEA in cultures of differentiated cells obtained from *cat-1* knockout animals. We found that β PEA significantly (* $p = 0.01$; t-test) augmented the extracellular amount of [^3H]DA of about $110 \pm 32\%$ with respect to control treated cells (Figure 3A). The comparison of β PEA effects between *cat-1* knockout and wild type neurons did not show a statistically significant difference, 110 ± 32 and 137 ± 12 respectively (Figure 3B). These data demonstrated that genetic ablation of cat-1 did not compromise the ability of β PEA to increase extracellular DA in native neuronal cultures, suggesting that CAT-1 (VMAT) does not play a significant role in the β PEA-induced increase of extracellular DA. Therefore, these data suggest that the elevated levels of extracellular DA measured during β PEA treatment are mediated by DAT-1.

3.3. β PEA generates amperometric currents in a subset of *C. elegans* DA neurons

Previously, we demonstrated that amphetamine promoted DA release through DAT-1 (efflux) in *C. elegans* dopaminergic neurons (Carvelli et al. 2010). Since our data (Figure 3) demonstrated that β PEA-induced increase of [^3H]DA does not occur through vesicle release, we investigated whether β PEA induces DA efflux by measuring amperometric currents. Microamperometry is a technique which allows detecting DA by measuring the electrons released during its oxidizing reaction. When DA touches a carbon fiber electrode held at a voltage of +700 mV, each DA molecule releases 2 electrons that are detected by the same carbon fiber electrode. Thus, DA efflux stimulated by β PEA can be detected by a carbon fiber electrode as a measure of DA oxidation (Carvelli 2010, Khoshbouei et al. 2003, Schroeder et al. 1994). When *C. elegans* DA neurons engineered to express cytosolic GFP (Figure 4A) were perfused with 50 μM β PEA, we observed fast-rising and outward amperometric currents (0.26 ± 0.02 pA) in 44% of DA neurons tested (Figure 4B and E). This result demonstrated that β PEA transiently increases the extracellular levels of DA in proximity of a single dopaminergic neuron. However, 33 dopaminergic neurons of 59 total neurons tested exhibited no effect following β PEA application (Figure 4C). Interestingly, the amperometric currents, which reached their maximal levels within 11 ± 0.5 seconds after β PEA perfusion, spontaneously returned to basal levels after 24 ± 3 seconds even though β PEA was still present in the recording chamber (Figure 4B and F). To test whether DAT-1 was required in β PEA-induced currents, we performed recordings in DA neurons lacking expression of DAT-1. β PEA did not cause significant amperometric currents (0.016 ± 0.004

pA) in any of the *dat-1* knockout neurons (N=20) we tested (Figure 3D and E). This result demonstrated that the increase of extracellular DA measured during β PEA treatment required a functional DAT-1 and suggested that β PEA needs DAT-1 to increase extracellular DA.

Taken together, these data demonstrate that in cultures of *C. elegans* DA neurons β PEA generates amperometric currents. Moreover, they show that about 40% of dopaminergic neurons did not respond to β PEA treatment suggesting, as previously shown for mammalian DA neurons, that physiological differences might exist among different subtypes of *C. elegans* DA neurons (Grenhoff et al. 1988, Murata et al. 2009, Li et al. 2012, Weihe et al. 2006). Importantly, our data support that the amperometric currents induced by β PEA are mediated by DAT-1. In fact, in DA neurons lacking expression of *dat-1*, β PEA did not generate amperometric currents.

4. CONCLUSION

In the brain, the amount of extracellular DA is controlled by a tight balance between DA release and uptake events. *In vitro* and *in vivo* studies have showed that β PEA enhances the extracellular levels of DA by inducing DA release and/or preventing DA uptake through mammalian DAT. Here we found that in *C. elegans* neuronal cultures, the β PEA-induced increase of extracellular DA was partly inhibited by the DAT blocker RTI-55, whereas RTI-55 did not have any effect when applied alone (Figure 2). Moreover, our data demonstrate that β PEA-induced release of DA requires DAT. This conclusion was supported by two major observations: I) the increase of extracellular DA induced by β PEA in *cat-1* (VMAT) knockout neurons was comparable to that measured in wild-type neurons (Figure 3B), and II) transient amperometric DA currents generated by β PEA were lost in neurons lacking expression of DAT-1 (Figure 4). Interestingly, our data show that in DAT-1 transfected cells, RTI55 completely blocked β PEA effect on extracellular levels of [^{3} H]DA whereas, in neuronal culture this effect was only partial. These results could simply reflect a different dose-related dependency of β PEA and/or RTI55 in transfected cells versus neuronal culture or suggest that β PEA involved proteins other than DAT-1, e.g. low affinity neurotransmitter transporter and/or DA D2 receptors (Horton et al. 2013, Duan and Wang 2010; Ishida et al. 2005, Mercuri et al. 1997, Rodriguez and Barroso 1995). Finally, our data demonstrate that *C. elegans* represents a powerful genetic model to perform *in vitro* studies in differentiated dopaminergic neurons.

Acknowledgments

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Abbreviations

DA	dopamine
DAT	DA transporter
β PEA	β -phenylethylamine

VMAT vesicular monoamine transporter

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HIGHLIGHTS

- β PEA increases the extracellular concentrations of DA in differentiated *C. elegans* neurons.
- The dopamine transporter (DAT) blocker RTI-55 partly blocks the β PEA-induced increase of extracellular DA.
- The vesicular monoamine transporter is not required to generate the β PEA-induced increase of extracellular DA.
- β PEA generates DA efflux in a subset of dopaminergic neurons
- β PEA does not generate DA efflux in DAT knockout neurons.

Figure 1.

βPEA increases the amount of extracellular DA in DAT-1 transfected cells. When DAT-1 expressing LLC-pk1 cells were treated for 1 minute with 100 μM βPEA, we measured a significant increase of extracellular [³H]DA with respect to control-treated samples (* $p = 0.0001$). Cells treated with βPEA/RTI-55 showed a significant reduction in extracellular [³H]DA with respect to βPEA-treated cells (° $p = 0.05$, one-way ANOVA). RTI55 applied alone did not have any effect.

Figure 2.

βPEA increases the amount of extracellular DA in *C. elegans* differentiated neuronal cultures. 100 μM βPEA applied for 1 minute in *C. elegans* cells caused a significant increase of extracellular [³H]DA with respect to control-treated cells (*p = 0.0001, one-way ANOVA). RTI-55 partly inhibited the βPEA-induced effect (*p=0.0001 vs. controls and ○p = 0.001 vs. βPEA-treated samples, one-way ANOVA), but caused no effect when applied alone.

Figure 3.

VMAT is not required by βPEA to increase the extracellular levels of DA. **A**, In cat-1 (VMAT) knockout neurons, 100 μM βPEA caused a significant increase of extracellular [³H]DA with respect to control-treated cells ($*p = 0.01$, t test). **B**, The increase of extracellular DA measured in cat-1 knockout neurons was comparable to that measured in wild-type neurons.

Figure 4.

β PEA generates amperometric currents in a subset of dopaminergic neurons. **A**, *C. elegans* dopaminergic neuron visualized with cytosolic GFP. **B**, Representative recording of fast-rising and self-inactivated amperometric currents generated by 50 μ M β PEA. **C**, Representative recording from a subset of dopaminergic neurons that did not respond to β PEA. **D**, Representative recording from a DAT-1 knockout neuron treated with 50 μ M β PEA. **E**, Average of amperometric currents measured in wild-type ($N=26$) and *dat-1* knockout ($N=20$) neurons (* $p = 0.0001$, t test). **F**, Average of time required to activate and inactivate the β PEA-induced amperometric currents.