ELSEVIER

European Journal of Pharmacology 451 (2002) 27–35

www.elsevier.com/locate/ejphar

Enhanced amphetamine-mediated dopamine release develops in PC12 cells after repeated amphetamine treatment

Lana Kantor [a], Yang Hae Park [a], Kevin K.W. Wang [a,b], Margaret E. Gnegy [a,*]

[a] *Department of Pharmacology, University of Michigan School of Medicine, 2220 MSRB III, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-0632, USA*

[b] *CNS Molecular Sciences, Pfizer Global R&D, Ann Arbor, MI 48105, USA*

Received 25 March 2002; received in revised form 25 July 2002; accepted 30 July 2002

Abstract

We previously demonstrated that rats treated with repeated, intermittent amphetamine displayed enhanced amphetamine-mediated dopamine release in the striatum. In this study, we examined whether amphetamine pretreatment would elicit enhanced amphetamine-mediated dopamine release in a cultured cell line in the absence of intact synaptic connections. PC12 cells pretreated with 1 μM amphetamine produced over twofold increase in amphetamine-mediated dopamine release upon challenge with 1 μM amphetamine as compared with vehicle-treated cells. No change in norepinephrine transporter density or [^{3}H]dopamine uptake was detected. A withdrawal time of 6 days was required to observe the enhanced amphetamine-mediated dopamine release. Differentiation of the cells with nerve growth factor did not alter the amphetamine-mediated dopamine release in control cells or the development of enhanced release in amphetamine-treated cells. Our results demonstrate that repeated, intermittent amphetamine leads to a neuroadaptation resulting in enhanced amphetamine-induced dopamine release in catecholaminergic cells without the need of an intact neuroanatomy.

Keywords: Nisoxetine; Norepinephrine transporter; Withdrawal time; Intermittent; Nerve growth factor; Dopamine uptake

1. Introduction

The psychomotor activating and reinforcing properties of amphetamine are due to its ability to increase extracellular concentrations of dopamine, norepinephrine or serotonin in the nucleus accumbens region of the forebrain (Di Chiara, 1995) via uptake through presynaptic monoamine transporters (Fischer and Cho, 1979; Seiden et al., 1993). Repeated and intermittent, but not continuous, administration of amphetamine leads to behavioral sensitization, identified as progressive and enduring modification in behavioral effects in response to amphetamine re-exposure in laboratory animals (Robinson and Becker, 1986) and in humans (Strakowski et al., 1996). A number of neurobiological adaptations develop in laboratory animals following repeated, intermittent amphetamine, such as subsensitivity of dopamine autoreceptors (Wolf et al., 1993), synapse remodeling (Robinson and Kolb, 1999), enhanced phosphorylation of synaptic proteins (Iwata et al., 1997) and enhanced amphetamine-induced dopamine release (Pierce and Kalivas, 1997; Robinson and Becker, 1986; Kantor et al., 1999).

Some of these neuroadaptations, such as the protein phosphorylation, synaptic remodeling and enhanced stimulus-induced dopamine release develop and strengthen with time following cessation of repeated amphetamine and are extremely persistent (Pierce and Kalivas, 1997; Robinson, 1991). It is unclear whether some of the neuroadaptations can be developed outside the laboratory animal without the complex circuitry provided by the brain. For instance, glutamatergic NMDA receptors and glial production of growth factors are important for the development of behavioral sensitization in the rat (Pierce and Kalivas, 1997; Wolf, 1998; Flores et al., 1998). We investigated whether repeated, intermittent amphetamine would elicit one specific neuroadaptation, enhanced amphetamine-induced dopamine release, in cultured cells. We chose to study the rat pheochromocytoma PC12 and human neuroblastoma SH-SY5Y cell lines because they synthesize and store dopamine and

* Corresponding author. Tel.: +1-734-763-5358; fax: +1-734-763-4450.
E-mail address: pgnegy@umich.edu (M.E. Gnegy).

0014-2999/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0014-2999(02)02190-8

norepinephrine and express the norepinephrine transporter (Friedrich and Bonisch, 1986; Apparsundaram et al., 1998; Cowell et al., 2000). Both amphetamine and dopamine are excellent substrates for the norepinephrine transporter (Zhu and Hexum, 1992; Gu et al., 1994; Wall et al., 1995) and the similarities between the norepinephrine transporter and the dopamine transporter result in similar regulation of the two transporters (Greene and Rein, 1977; Kantor et al., 2001). PC12 cells contain more dopamine than norepinephrine (Greene and Tischler, 1976); dopamine is readily released from PC12 cells in response to depolarization (Kittner et al., 1987) and amphetamine (Sulzer et al., 1995; Kantor et al., 2001). Therefore, we examined whether repeated, intermittent amphetamine treatment would result in enhanced amphetamine-mediated dopamine release in these cell lines. We also examined whether characteristics that were important for development of enhanced amphetamine-induced dopamine release in laboratory animals, such as the requirement for intermittent versus continuous treatments and dependence on a withdrawal period from amphetamine, were similarly important for the response in PC12 cells.

2. Materials and methods

2.1. Repeated, intermittent amphetamine treatment

PC12 and SH-SY5Y cells were plated in a 75-cm^2 tissue culture flask at 10^4 cells/plate. Cells were grown in monolayers in Dulbecco's Minimal Essential Media (Sigma, St. Louis, MO) supplemented with 5% (vol/vol) fetal bovine serum, 10% (vol/vol) horse serum, 100 μg/ml of streptomycin and 100 units/ml of penicillin at 7.5% CO_2. Cells were grown for 2 days before they were treated with drugs. For the repetitive, intermittent amphetamine treatment, media was removed from the cells and fresh media with or without 1 μM amphetamine was added to the plates for 5 min. The media was then removed from the plate and the cells were washed once before a subsequent addition of fresh media. This procedure was carried out once a day for 5 days. After the fifth day of amphetamine treatment, the cells were left drug-free for 10 days. The media was changed every other day, until cells were harvested for the experiments.

2.2. Continuous amphetamine treatment

For continuous amphetamine treatment, media with or without 1 μM amphetamine was added to the plates. Twenty-four hours later, the media was removed from the plate and cells were washed once before addition of fresh media with or without 1 μM amphetamine. This procedure was carried out once a day, for 5 days. After the fifth day of continuous amphetamine treatment, the cells were left drug-free for 10 days.

2.3. Nerve growth factor treatment

Two days following plating, cells were treated with media with or without 30 ng/ml nerve growth factor for 2 days. Neurite extension was clearly visible at that time. The 30 ng/ml nerve growth factor was present in the media throughout the entire course of the experiment. Following two full days of nerve growth factor treatment, the cells were treated for 5 days with 1 μM amphetamine or media alone for 5 min using the repeated, intermittent regimen described above. Cells were harvested 10 days following the last amphetamine treatment.

2.4. Superfusion assay

Cells were harvested by washing the flasks with Krebs–Ringer buffer containing 125 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 1.2 $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 24.9 mM $NaHCO_3$ and 0.25 mM ascorbic acid, oxygenated by 95% O_2 and 5% CO_2 for 1 h. The cell suspension was centrifuged at $500 \times g$ for 5 min; the supernatant was removed and cells were resuspended in Krebs–Ringer buffer to achieve a protein concentration of 1.2 mg/ml. Protein concentration in all experiments was determined by a modified Lowry method (Bio-Rad D-C protein assay kit). The suspension of PC12 cells was transferred to Whatman GF/B glass filters (Maidstone, England) in the appropriate chambers of a Brandel superfusion apparatus (Brandel SF-12, Gaithersburg, MD). Superfusion was performed at 37 °C at a rate of 100 μl/min. Samples were collected at 5-min intervals. After the cells were perfused for 1 h to establish a stable base line, a 2.5-min bolus of 1 μM amphetamine was delivered. This challenge dose of amphetamine was administered at fraction 7. Due to the flow rate and length of tubing, the amphetamine-mediated dopamine release began at fraction 9 and was complete by fraction 11. Replacing amphetamine with Krebs–Ringer buffer terminated the stimulation. Collection continued for another 40 min. Samples were collected into vials containing 25 μl of internal standard solution (0.05 $HClO_4$, 4.55 mM dihydrobenzylamine, 1 M metabisulfate and 0.1 M ethylenediaminetetraacetic acid). Samples were stored at −70 °C. The dopamine levels were measured by high pressure liquid chromatography with electrochemical detection using dihydrobenzylamine as an internal standard.

2.5. [^{3}H]dopamine uptake

PC12 cells were grown and harvested as discussed above. Cells (200 μl at 1.2 mg/ml protein) were placed in tubes and equilibrated to 37 °C. Uptake of [^{3}H]dopamine into PC12 cells was measured as described previously (Kantor et al., 2001). The cells were incubated with or without 100 nM nisoxetine, a specific norepinephrine transporter blocker used to determine specific binding (Buck and Amara, 1995), for 10 min followed by incubation with 70

nM [^{3}H]dopamine (Life Sciences, Boston, MA) for 1 or 5 min as indicated (specific activity, 60 Ci/mol). The reaction was stopped by filtering cells through GF/B filters on a Hoeffer filtering apparatus (San Francisco, CA) and washing several times with ice cold saline. Samples were counted in ScintiVerse BD in a Beckman LS 5800 Scintillation Counter.

2.6. [^{3}H]nisoxetine binding

PC12 cells were grown and harvested as described above. Cells (200 μl at 1.2 mg/ml protein) were placed in tubes and incubated with or without 10 μM cocaine at 37 °C for 10 min (Bryan-Lluka et al., 2001). [^{3}H]nisoxetine at 30 nM (Life Sciences) was added to all cells for 30 min (specific activity 80 Ci/mmol). The reaction was stopped by filtering the cells through GF/B filters on a Hoeffer filtering apparatus (San Francisco, CA) and washing several times with ice cold saline. All the samples were counted in ScintiVerse BD in a Beckman LS 5800 Scintillation Counter.

2.7. Immunoblot

Undifferentiated PC12 cells were removed from the plates, centrifuged, resuspended and homogenized in Krebs–Ringer buffer to achieve a protein concentration of 2 mg/ml. The PC12 lysate was subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel was transferred, blocked with 5% milk and incubated overnight in rabbit anti-rat norepinephrine transporter (1:1000; Alpha Diagnostic, San Antonio, TX) followed by 1 h of incubation with rat anti-rabbit 2° antibody (Chemicon, Temecula, CA) diluted 1:3000. The blot was developed with an alkaline phosphatase kit from Gibco (Gaithersburg, MD).

3. Results

3.1. Amphetamine-mediated dopamine release in PC12 cells following repeated, intermittent amphetamine treatment

We previously reported that repeated, intermittent treatment of rats with amphetamine elicited an enhanced dopamine release upon 1 μM amphetamine challenge in striatal brain slices. The rats were treated with amphetamine once a day for 5 days followed by 10 drug-free days (Kantor et al., 1999). The protocol for PC12 cell treatment was designed with a similar time frame such that cells were treated with amphetamine for a limited amount of time. PC12 cells were treated with 1 μM amphetamine once per day for 5 days and given 10 drug-free days. The dose of 1 μM amphetamine was chosen because it was in the range of the IC_{50} of amphetamine for inhibition of substrate transport into norepinephrine transporter (between 0.1 and 0.5 μM) (Gu et al., 1994; Wall et al., 1995) yet low enough to avoid cell toxicity. Following repeated, intermittent treatment of PC12 cells with 1 μM amphetamine, there was a significantly greater amount of dopamine released upon a challenge with 1 μM amphetamine as compared with vehicle-treated cells (Fig. 1; compare the peak, fraction 10, with baseline values, fractions 6–8). There was no significant change in the stable baseline between the two groups. The fold stimulation of dopamine released in response to 1 μM amphetamine over baseline increased from twofold in vehicle-treated cells to sevenfold in the amphetamine-pretreated cells. The repeated amphetamine treatment did not change the time of response to an amphetamine challenge as compared to acute treatment. The total dopamine measured in the cells did not change as a result of repeated amphetamine treatment. The concentration of dopamine in vehicle- and amphetamine-pretreated PC12 cells was 2123 ± 256 pmol/mg protein ($N=9$) and 1813 ± 212 pmol/mg protein ($N=10$), respectively.

We examined whether the enhanced dopamine release in response to a challenge of amphetamine following repeated amphetamine treatment of cells was due to an increase in V_{max} for the amphetamine-mediated reverse transport or an increase in affinity of amphetamine. As shown in Fig. 2, a complete dose–response curve revealed that the amount of dopamine released in response to amphetamine following repeated, intermittent amphetamine was increased at every concentration of amphetamine as compared to that in vehicle-treated cells. The V_{max} for this experiment was

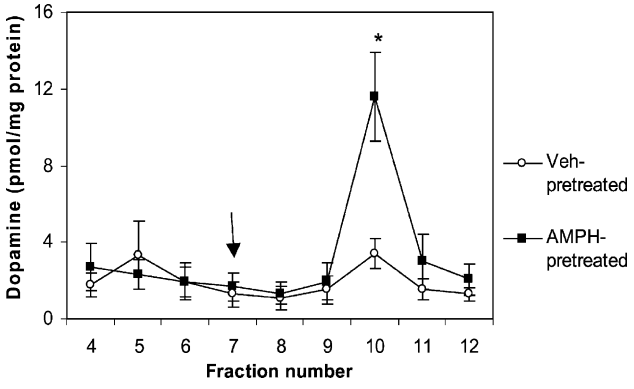

Fig. 1. Repeated, intermittent amphetamine elicits an increase in amphetamine-stimulated dopamine release. PC12 cells were treated for 5 min/day for 5 days with 1 μM amphetamine (AMPH-pretreated) or vehicle (vehicle-pretreated) followed by 10 drug-free days, harvested and perfused as described in Materials and methods. Cells were washed with Krebs–Ringer buffer for 1 h and fractions giving basal dopamine were collected. At fraction 7, shown by the arrow on the graph, a bolus of 1 μM amphetamine was administered for 2.5 min followed by Krebs–Ringer buffer. Due to the length of tubing and perfusion time, the amphetamine reaches the cells at fraction 9 and dopamine is eluted at fraction 10. The samples were analyzed on high performance liquid chromatography with electrochemical detection. Results are given in pmol dopamine/mg protein ± the standard error of the mean (S.E.M., $N=4$). Amphetamine-mediated dopamine release was enhanced following repeater, intermittent amphetamine. For fraction 10, * $P<0.02$ by two-tailed Student's t-test.

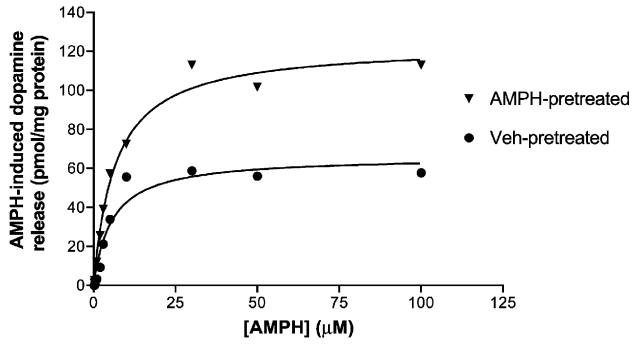

Fig. 2. Dose–response to amphetamine in PC12 cells pretreated with vehicle or 1 μM amphetamine. PC12 cells were treated with repeated, intermittent vehicle (veh-pretreated) or 1 μM amphetamine (AMPH-pretreated) and withdrawn from drug for 10 days as described in Materials and methods. Dopamine released in response to concentrations of amphetamine from 0.2 to 100 μM was measured as described in the legend of Fig. 1. The results demonstrate an increase in V_{max} for amphetamine-mediated dopamine release following repeated amphetamine treatment with no change in apparent K_a. The lines drawn represent the best-fit nonlinear regression analysis determined by Graph Pad Prism.

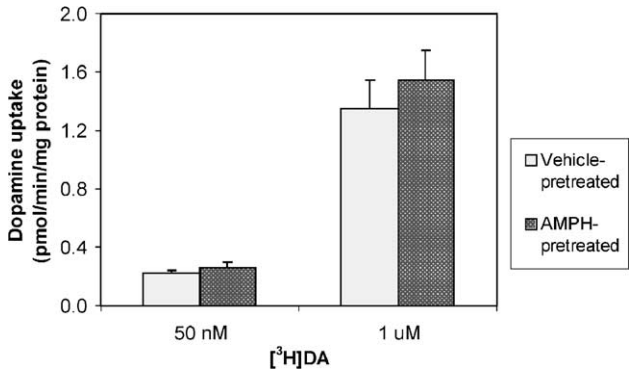

Fig. 3. Repeated, intermittent amphetamine treatment of PC12 cells did not alter [^{3}H]dopamine uptake. PC12 cells were repeatedly treated with 1 μM amphetamine or vehicle for 5 days and kept drug-free for 10 days. Dopamine uptake was measured with 70 nM [^{3}H]dopamine and 1 μM [^{3}H]dopamine as described in Materials and methods. Specific uptake was determined at 1 min using 100 nM nisoxetine for blank values. Values are given as pmol/min/mg ± S.E.M. Amphetamine pretreatment of PC12 cells did not alter the [^{3}H]dopamine uptake as compared to vehicle (N = 11).

nearly twofold greater in amphetamine-pretreated cells as compared to vehicle-pretreatment (123 and 66 pmol/mg protein, respectively). The apparent K_a for amphetamine did not change, being 5.5 μM for vehicle-pretreated cells and 6.6 μM in amphetamine-pretreated cells. Kinetic constants were calculated by nonlinear regression analysis in Graph Pad Prism. Baseline values for all doses of amphetamine remained constant and were not different between vehicle-pretreated cells (5.6 ± 0.5 pmol/mg protein) and amphetamine-pretreated cells (6.3 ± 0.7 pmol/mg protein), N = 10, for the 10 amphetamine doses.

3.2. Effect of repeated, intermittent amphetamine pretreatment in the PC12 cells on dopamine uptake

The repeated amphetamine treatment could have increased the turnover of the transporter such that both inward and outward transport was enhanced. To test this, we measured [^{3}H]dopamine uptake at two concentrations of [^{3}H]dopamine: at a lower concentration (70 nM) and 1 μM [^{3}H]dopamine, which is four times the K_m of dopamine for the norepinephrine transporter (Gu et al., 1994). The results in Fig. 3 show that there was no difference in nisoxetine-sensitive uptake of [^{3}H]dopamine in repeated amphetamine-treated cells as compared to the repeated vehicle-treated cells at either concentration of [^{3}H]dopamine.

3.3. Effect of repeated, intermittent amphetamine treatment on the concentration of the norepinephrine transporter in PC12 cells

To confirm that there was no change in the number of norepinephrine transporters, lysates from vehicle and amphetamine-pretreated cells were immunoblotted with anti-norepinephrine transporter. The data of Fig. 4A reveal that the concentration of norepinephrine transporter present in vehicle-treated PC12 cells was equivalent to the levels seen in amphetamine-pretreated PC12 cells. Densitometry of the bands in the immunoblot revealed the O.D. for vehicle- and for amphetamine-pretreated cells was

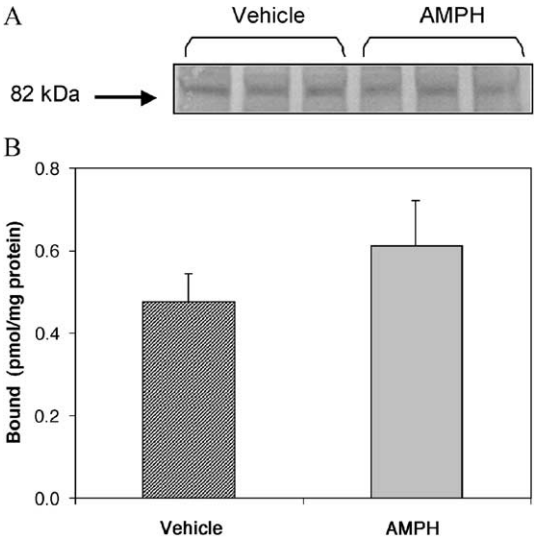

Fig. 4. Immunoblot and nisoxetine binding comparing norepinephrine transporter in amphetamine- and vehicle-treated PC12 cells. PC12 cells were treated with repeated, intermittent 1 μM amphetamine (AMPH-pretreated) or vehicle (vehicle-pretreated) and harvested as described in Materials and methods. (A) Whole cell lysates were used to analyze norepinephrine transporter density. Lanes 1–3 (left to right) are vehicle-pretreated cells (vehicle); lanes 4–6 are amphetamine-pretreated cells (AMPH). An arrow shows the position of 82 kDa as calculated from the molecular weight markers on the gel. Each lane contained 30 μg protein. Norepinephrine transporter density in PC12 cells did not change due to amphetamine pretreatment. (B) Cocaine-dependent [^{3}H]nisoxetine binding was measured on whole cells as described in Materials and methods. Results are given in pmol/mg protein ± S.E.M. Amphetamine pretreatment of PC12 cells did not significantly affect the [^{3}H]nisoxetine binding as compared to vehicle (N = 3).

15.5 ± 0.6 and 13.6 ± 1.2, respectively, $N=3$. To further test the levels of norepinephrine transporter present in amphetamine-pretreated cells, we measured the binding of the specific norepinephrine transporter ligand, [^{3}H]nisoxetine (Buck and Amara, 1995). The results in Fig. 4B reveal that there was no difference in the [^{3}H]nisoxetine binding between vehicle- and amphetamine-pretreated cells. Therefore, amphetamine treatment does not affect the expression of norepinephrine transporter or the available norepinephrine transporter on the outer membrane of the cells. In addition, lysates from vehicle- and amphetamine-pretreated PC12 cells were immunoblotted with antibody to the dopamine transporter. No band was visible in lysates from either control or amphetamine-pretreated cells (data not shown).

3.4. Effect of drug-free (withdrawal) time on the development of enhanced amphetamine-mediated dopamine release

In the rat, the development of enhanced amphetamine-mediated dopamine release requires days to develop following cessation of drug administration (Kolta et al., 1985; Paulson and Robinson, 1995). To investigate the requirement for drug withdrawal in amphetamine-pretreated PC12 cells, dopamine release in response to a challenge of 1 μM amphetamine was measured at various days following the last dose of amphetamine or vehicle. As shown in Fig. 5, the enhancement in amphetamine-mediated dopamine release in amphetamine-pretreated cells was not apparent until 6 days following the last dose of amphetamine. The response

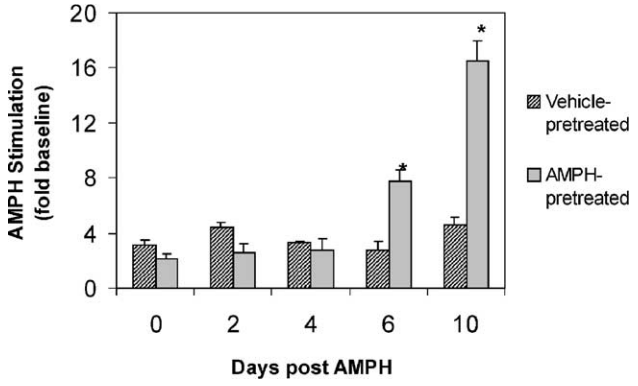

Fig. 5. Withdrawal time-dependence of the development of enhanced amphetamine-induced dopamine release following repeated, intermittent amphetamine of PC12 cells. PC12 cells were treated with repeated, intermittent 1 μM amphetamine (AMPH-pretreated) or vehicle (vehicle-pretreated) for 5 days. The cells were then left in a drug-free media for 0–10 days. The cells were harvested at the designated withdrawal time and perfused with a bolus of 1 μM amphetamine as described in Materials and methods and the legend of Fig. 1. Results are given as the fold-stimulation ± S.E.M. of amphetamine-mediated dopamine release over baseline values for fraction 10. The enhanced amphetamine-mediated dopamine release was observed after 6 days of withdrawal and significantly increased after 10 days of withdrawal. * $P<0.005$ as compared to vehicle-treated cells using a two-tailed Student's t-test ($N=4$).

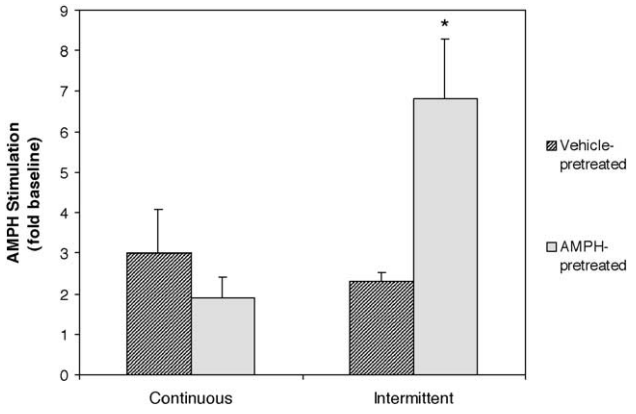

Fig. 6. Enhanced amphetamine-induced dopamine release develops following intermittent but not continuous amphetamine. One set of PC12 cells was treated with repeated, intermittent 1 μM amphetamine (AMPH-pretreated) or vehicle (vehicle-pretreated) for 5 days (intermittent). Another set of PC12 cells was treated with 1 μM amphetamine or vehicle continuously present in the media for 5 days (continuous). Both sets were withdrawn for 10 days, harvested and perfused with a 1 μM bolus of amphetamine as described in Materials and methods and the legend of Fig. 1. Results are given as the fold-stimulation of amphetamine (AMPH)-mediated dopamine release over baseline values for fraction 10. Repeated, intermittent treatment produced enhanced-amphetamine mediated dopamine release while continuous amphetamine treatment produced no change from control ($N=3$). * $P<0.05$, two-tailed Student's t-test.

became more robust with time. The highest and most robust increase in amphetamine-mediated dopamine release was found on withdrawal day 10. It was not possible to test longer withdrawal times due to confluence of the cells on the plate.

3.5. Effect of continuous treatment with amphetamine on the development of amphetamine-mediated dopamine release

We examined whether leaving the amphetamine in the cultures continuously would alter amphetamine-mediated outward transport in the same manner as did repeated, intermittent treatment. For the continuous treatment, the cells were incubated for 5 days continuously in the presence of 1 μM amphetamine in the media followed by 10 drug-free days. Fig. 6 displays the results obtained with continuous treatment of the cells with 1 μM amphetamine versus the intermittent treatment. Continuous amphetamine pretreatment did not produce an enhanced amphetamine-mediated dopamine release upon a challenge with 1 μM amphetamine.

3.6. The effect of repeated, intermittent amphetamine treatment on amphetamine-mediated dopamine release in nerve growth factor-differentiated PC12 cells

We wished to determine whether repeated, intermittent amphetamine would result in enhanced amphetamine-mediated dopamine release in nerve growth factor-differentiated PC12 cells for two reasons. First, we wished to determine if

the effect would occur in PC12 cells that possessed the 'neuronal' characteristics attained as a result of nerve growth factor treatment. Second, we wanted to determine whether the effect of repeated, intermittent amphetamine would occur in a culture in which cells were not rapidly dividing. Normally, cells continue to divide during the total 15 days of the experiment. Treatment with nerve growth factor would inhibit propagation of the cells. Prior nerve growth factor treatment did not alter the development of enhanced amphetamine-mediated dopamine release in response to repeated amphetamine (Fig. 7) nor the responsiveness to amphetamine in vehicle-pretreated cells. As reported previously (Greene and Tischler, 1976), we found that nerve growth factor lowered the dopamine content of the cells from 2133 ± 256 pmol/mg protein ($N=9$) in non-treated cells to 966 ± 83 pmol/mg protein ($N=4$) in nerve growth factor-treated cells. Despite the lowered total dopamine, the baselines and total amount of dopamine released remained fairly constant. Therefore, it appears that repeated, intermittent amphetamine regulates the transporter activity similarly in the endocrine-like undifferentiated PC12 cells and those that are differentiated to a neuronal state.

3.7. Effect of repeated, intermittent amphetamine on amphetamine-mediated dopamine release in human neuroblastoma SH-SY5Y cells

It was important to determine whether the effect of repeated, intermittent amphetamine on amphetamine-mediated dopamine release was specific to PC12 cells and to further establish whether it would occur in human neuronal cells. Human neuroblastoma, SH-SY5Y cells were selected since they synthesize dopamine and contain the norepinephrine catecholamine transporter. Fig. 8A illustrates that a challenge dose of amphetamine gave a 32-fold increase in dopamine release over baseline in amphetamine-pretreated cells as compared to the 17-fold increase in vehicle-pretreated cells. These results mirrored those seen in the PC12 cells and demonstrate a generalized response to the repeated, intermittent amphetamine. The data of Fig. 8B demonstrate that the repeated amphetamine treatment did not alter [^{3}H]dopamine uptake at either 70 nM or 1 μM [^{3}H]dopamine in SH-SY5Y cells.

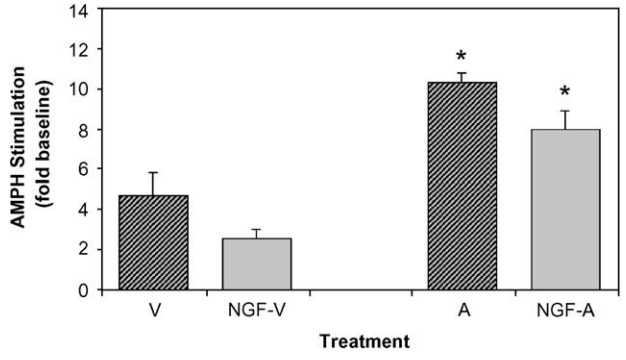

Fig. 7. The effect of nerve growth factor treatment on amphetamine-mediated dopamine release in PC12 cells given repeated, intermittent amphetamine. PC12 cells were incubated with 30 ng/ml of nerve growth factor (NGF) or vehicle (V) for 2 days prior to repeated, intermittent amphetamine treatment. Nerve growth factor remained in the media for the duration of the experiment. The cells were treated with 1 μM amphetamine (A, NGF-A) or vehicle (V, NGF-V) for 5 days, kept drug-free for 10 days, then were harvested and perfused with a bolus of 1 μM amphetamine. Results are given as the fold-stimulation of amphetamine (AMPH)-mediated dopamine release over baseline values ± S.E.M. for fraction 10. Nerve growth factor treatment did not affect dopamine basal release nor the development of enhanced amphetamine-mediated dopamine release ($N=3$). * In analysis by one-way analysis of variance, $P<0.001$. In post-hoc Tukey–Kramer analysis, both vehicle values differed from both amphetamine values at $P<0.01$. There was no significant difference between the two amphetamine values.

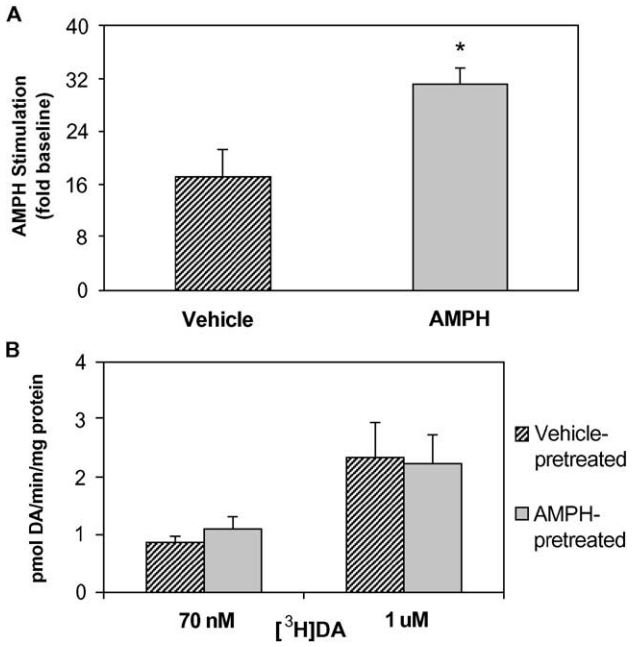

Fig. 8. Repeated, intermittent treatment of SH-SY5Y cells with amphetamine. SH-SY5Y cells were treated with repeated, intermittent 1 μM amphetamine (AMPH) or vehicle (vehicle) as described for PC12 cells in Materials and methods. After cells were harvested they were analyzed for (A) amphetamine-mediated dopamine release using a bolus dose of 1 μM amphetamine. Results are given as the fold stimulation of amphetamine (AMPH)-mediated dopamine release over baseline values ± S.E.M. for fraction 10. SH-SY5Y cells demonstrated an enhanced amphetamine-mediated dopamine release following repeated, intermittent dopamine as observed in PC12 cells. * $P<0.05$ as compared to vehicle-treated cells ($N=3$). (B) [^{3}H]dopamine uptake was measured in vehicle- and amphetamine-pretreated SH-SY5Y cells at both 70 nM and 1 μM [^{3}H]dopamine. Specific uptake was determined at 5 min using 100 nM nisoxetine for blank values. Values are given as pmol/min/mg ± S.E.M. Amphetamine pretreatment of SH-SY5Y cells did not significantly affect the [^{3}H]dopamine uptake as compared to vehicle ($N=3$).

4. Discussion

We report the original observation that repeated, intermittent treatment of cultured cells that contain a catecholamine transporter with relatively low doses of amphetamine

leads to an enhanced amphetamine-mediated outward transport of dopamine. The development of the enhanced release displays characteristics similar to those resulting in enhanced amphetamine-induced dopamine release in the rats: emergence with time following drug discontinuation, a progressive enhancement of the effect and a requirement for intermittent treatment (Robinson and Becker, 1986). Interestingly, these characteristics are also generally true of the development of behavioral sensitization, although enhanced amphetamine-induced dopamine release appears later than the enhanced behavior (Robinson, 1992). The fact that this phenomenon, which appears to be a function of outward transport through the catecholamine transporter, can occur in both laboratory animals and cultured cells suggests that it may be due to a direct effect of amphetamine on the transporter-containing cells. Intact neuroanatomical pathways and connections between specific cells are not required. It would appear that this particular neuroadaptation requires only the catecholaminergic cell and specific treatment requirements with amphetamine.

PC12 cells subjected to repeated, intermittent amphetamine treatment generated an increase in dopamine release in response to amphetamine challenge in contrast to cells receiving continuous amphetamine treatment. An intermittent regimen has proven important to the development of enhanced responsiveness of various neurochemical and behavioral paradigms while continuous treatments generally lead to a down-regulated response. Only the intermittent treatment with amphetamine resulted in the enhanced amphetamine-induced release in our experiments. Continuous treatment with 1 μM amphetamine had no effect on amphetamine-mediated dopamine release in our study. Zhu and Ordway (1997) found that three continuous days of treatment of PC12 and HEK-293 hNET (human embryonic kidney cells transfected with human norepinephrine transporter) cells with 10 to 100 μM amphetamine decreased the measurable norepinephrine transporter and reduced the uptake of [^{3}H]norepinephrine. Although we treated cells continuously for 5 days, we used only 1 μM amphetamine, which may not have the same down-regulating effect as 10 or 100 μM amphetamine. Intermittent treatment with amphetamine results in the development of sensitized locomotor and stereotyped behaviors while continuous treatment diminishes these behaviors (Post, 1980; Ellison and Morris, 1981; Robinson and Becker, 1986; Robinson, 1992). Similarly, intermittent but not continuous treatment with dopamine agonists results in an enhanced behavioral and electrophysiological response to dopamine agonists (Vaughn et al., 1990; Engber et al., 1989; White et al., 1990). On the contrary, continuous treatment with dopamine agonists led to a reduction in rotational behavior (Engber et al., 1989) as well as a down-regulation in tyrosine hydroxylase protein and mRNA levels (Iwata et al., 2000).

Thus, it appears that an intermittent or phasic stimulation of dopaminergic cells leads to a strengthening of certain responses that is not attained through continuous stimulation of the cell. The enhanced response to intermittent amphetamine is thus reminiscent of exaggerated responsiveness of systems to short, phasic challenges in long-term potentiation (Malenka and Nicoll, 1999), long-term depression, kindling (Glenthoj, 1995) and dopaminergic 'priming' (Morelli et al., 1989), all of which can occur in dopaminergic areas (Calabresi et al., 1992, 1997). Our study demonstrates that this type of synaptic plasticity can be elicited by repeated amphetamine in a cell culture.

Our results demonstrate that the neuroadaptation resulting in enhanced amphetamine-induced dopamine release does not depend on interactions with non-catecholaminergic neurons nor on an intact neuroanatomy. The fact that at least 6 days were required for the expression of the enhanced amphetamine-induced release suggests that RNA and/or protein synthesis is required for the phenomenon. We are in the process of examining this requirement. Repeated amphetamine has been demonstrated to lead to enhanced RNA and protein synthesis (Norman et al., 1993; Jaber et al., 1995; Simpson et al., 1995; Turgeon et al., 1997).

The repeated amphetamine pretreatment increased amphetamine-mediated dopamine release but did not modify dopamine uptake in undifferentiated PC12 cells or SH-SY5Y cells. Similarly, the repeated amphetamine treatment did not alter the concentration of norepinephrine transporter on the outer membrane. Therefore, the neuroadaptation elicited by repeated amphetamine primarily affects outward transport, suggesting the modification is not simply due to increase in turnover of the transporter, nor to an increased number of the transporters on the cell surface. Although one might expect that uptake and release would be similarly modulated, we and others have demonstrated that dopamine uptake and release can be independently modulated (Kantor and Gnegy, 1998; Danek Burgess and Justice, 1999; Sitte et al., 2001).

Differentiation of the PC12 cells with nerve growth factor did not alter the development of the enhanced amphetamine-induced dopamine release. Nerve growth factor treatment causes PC12 to differentiate into neuronal-like cells, while maintaining the biochemical characteristics of a PC12 cell line (Greene and Tischler, 1976). Although it is possible that amphetamine could be inducing the synthesis and release of a growth factor from PC12 cells, the development of the enhanced amphetamine-induced dopamine release was not induced by nerve growth factor treatment alone. Therefore, neither the growth-reducing nor neurite-extending properties of nerve growth factor altered the ability of amphetamine to elicit enhanced amphetamine-induced dopamine release. We further demonstrated that this phenomenon could be reproduced in neuronal cells by the treatment of norepinephrine transporter-containing SH-SY5Y cells.

In conclusion, the present data provide for the first time information on norepinephrine transporter-dependent amphetamine-mediated dopamine release and uptake after repeated, intermittent amphetamine treatment. Here we

propose that PC12 and SH-SY5Y cells produce similar neuroadaptations to repeated, intermittent amphetamine treatment as observed in the animal model. Based on our prior studies in the rat (Kantor et al., 1999) and in PC12 cells (Kantor et al., 2001), we suggest that amphetamine causes a change in the action of catecholamine plasmalemmal transporters that favors dopamine efflux. Therefore, we propose that PC12 and SH-SY5Y cells can be used as cellular models to study select actions of amphetamine after acute and chronic treatment regimens. Further experiments are in progress to elucidate the mechanisms of enhanced amphetamine-mediated dopamine release in these cells after repeated, intermittent amphetamine treatment.

Acknowledgements

Support for these studies came from the National Institutes of Drug Abuse, DA13417 and an NRSA Grant DA05912 (L.K.).

References

Apparsundaram, S., Schroeter, S., Giovanetti, E., Blakely, R.D., 1998. Acute regulation of norepinephrine transport: II. PKC-modulated surface expression of human norepinephrine transporter proteins. J. Pharmacol. Exp. Ther. 287, 744–751.

Bryan-Lluka, L.J., Paczkowski, F.A., Bonisch, H., 2001. Effects of short- and long-term exposure to c-AMP and c-GMP on the noradrenaline transporter. Neuropharmacology 40, 607–617.

Buck, K.J., Amara, S.G., 1995. Structural domains of catecholamine transporter chimeras involved in selective inhibition by antidepressants and psychomotor stimulants. Mol. Pharmacol. 48, 1030–1037.

Calabresi, P., Maj, R., Pisani, A., Mercuri, N.B., Bernardi, G., 1992. Long-term synaptic depression in the striatum: physiological and pharmacological characterization. J. Neurosci. 12, 4224–4233.

Calabresi, P., Pisani, A., Centonze, D., Bernardi, G., 1997. Synaptic plasticity and physiological interactions between dopamine and glutamate in the striatum. Neurosci. Biobehav. Rev. 21, 519–523.

Cowell, R.M., Kantor, L., Hewlett, G.H., Frey, K.A., Gnegy, M.E., 2000. Dopamine transporter antagonists block phorbol ester-induced dopamine release and dopamine transporter phosphorylation in striatal synaptosomes. Eur. J. Pharmacol. 389, 59–65.

Danek Burgess, K.S., Justice Jr., J.B., 1999. Effects of serine mutations in transmembrane domain 7 of the human norepinephrine transporter on substrate binding and transport. J. Neurochem. 73, 656–664.

Di Chiara, G., 1995. The role of dopamine in drug abuse viewed from the perspective of its role in motivation. Drug Alcohol Depend. 38, 95–137.

Ellison, G., Morris, W., 1981. Opposed stages of continuous amphetamine administration: parallel alterations in motor stereotypies and in vivo spiroperidol accumulation. Eur. J. Pharmacol. 74, 207–214.

Engber, T.M., Susel, Z., Juncos, J.L., Chase, T.N., 1989. Continuous and intermittent levodopa differentially affect rotation induced by D-1 and D-2 dopamine agonists. Eur. J. Pharmacol. 168, 291–298.

Fischer, J.F., Cho, A.K., 1979. Chemical release of dopamine from striatal homogenates: evidence for an exchange diffusion model. J. Pharmacol. Exp. Ther. 208, 203–209.

Flores, C., Rodaros, D., Stewart, J., 1998. Long-lasting induction of astrocytic basic fibroblast growth factor by repeated injections of amphetamine: blockade by concurrent treatment with a glutamate antagonist. J. Neurosci. 18, 9547–9555.

Friedrich, U., Bonisch, H., 1986. The neuronal noradrenaline transport system of PC-12 cells: kinetic analysis of the interaction between noradrenaline, Na^+ and Cl^- in transport. Naunyn-Schmiedeberg's Arch. Pharmacol. 333, 246–252.

Glenthoj, B.Y., 1995. The brain dopaminergic system. Pharmacological, behavioural and electrophysiological studies. Dan. Med. Bull. 42, 1–21.

Greene, L.A., Rein, G., 1977. Release, storage and uptake of catecholamines by a clonal cell line of nerve growth factor (NGF) responsive pheochromocytoma cells. Brain Res. 129, 247–263.

Greene, L.A., Tischler, A.S., 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. U. S. A. 73, 2424–2428.

Gu, H., Wall, S.C., Rudnick, G., 1994. Stable expression of biogenic amine transporters reveals differences in inhibitor sensitivity, kinetics, and ion dependence. J. Biol. Chem. 269, 7124–7130.

Iwata, S., Hewlett, G.H.K., Gnegy, M.E., 1997. Amphetamine increases the phosphorylation of neuromodulin and synapsin I in rat striatal synaptosomes. Synapse 26, 281–291.

Iwata, S., Nomoto, M., Kaseda, S., Tanoue, S., Shimosaka, M., Fukuda, T., 2000. TH protein and mRNA in nigrostriatal dopaminergic neurons are down-regulated by continuous but not intermittent apomorphine. Brain Res., Mol. Brain Res. 82, 133–136.

Jaber, M., Cador, M., Dumartin, B., Normand, E., Stinus, L., Bloch, B., 1995. Acute and chronic amphetamine treatments differently regulate neuropeptide messenger RNA levels and Fos immunoreactivity in rat striatal neurons. Neuroscience 65, 1041–1050.

Kantor, L., Gnegy, M.E., 1998. Protein kinase C inhibitors block amphetamine-mediated dopamine release in rat striatal slices. J. Pharmacol. Exp. Ther. 284, 594–598.

Kantor, L., Hewlett, G.H., Gnegy, M.E., 1999. Enhanced amphetamine- and K^+-mediated dopamine release in rat striatum after repeated amphetamine: differential requirements for Ca^{2+}- and calmodulin-dependent phosphorylation and synaptic vesicles. J. Neurosci. 19, 3801–3808.

Kantor, L., Hewlett, G.H., Park, Y.H., Richardson-Burns, S.M., Mellon, M.J., Gnegy, M.E., 2001. Protein kinase C and intracellular calcium are required for amphetamine-mediated dopamine release via the norepinephrine transporter in undifferentiated PC12 cells. J. Pharmacol. Exp. Ther. 297, 1016–1024.

Kittner, B., Brautigam, M., Herken, H., 1987. PC12 cells: a model system for studying drug effects on dopamine synthesis and release. Arch. Int. Pharmacodyn. Ther. 286, 181–194.

Kolta, M.G., Shreve, P., De Souza, V., Uretsky, N.J., 1985. Time course of the development of the enhanced behavioral and biochemical responses to amphetamine after pretreatment with amphetamine. Neuropharmacology 24, 823–829.

Malenka, R.C., Nicoll, R.A., 1999. Long-term potentiation—a decade of progress? Science 285, 1870–1874.

Morelli, M., Fenu, S., Garau, L., Di Chiara, G., 1989. Time and dose dependence of the 'priming' of the expression of dopamine receptor supersensitivity. Eur. J. Pharmacol. 162, 329–335.

Norman, A.B., Lu, S.Y., Klug, J.M., Norgren, R.B., 1993. Sensitization of c-*fos* expression in rat striatum following multiple challenges with D-amphetamine. Brain Res. 603, 125–128.

Paulson, P.E., Robinson, T.E., 1995. Amphetamine-induced time-dependent sensitization of dopamine neurotransmission in the dorsal and ventral striatum: a microdialysis study in behaving rats. Synapse 19, 56–65.

Pierce, R.C., Kalivas, P.W., 1997. A circuitry model of the expression of behavioral sensitization to amphetamine-like psychostimulants. Brain Res., Brains Res. Rev. 25, 192–216.

Post, R.M., 1980. Intermittent versus continuous stimulation: effect of time interval on the development of sensitization or tolerance. Life Sci. 26, 1275–1282.

Robinson, T.E., 1991. The neurobiology of amphetamine psychosis: evidence from studies with an animal model. In: Nakazawa, T. (Ed.), Taniguchi Symposia on Brain Sciences. Biological Basis of Schizophrenic Disorders, vol. 14. Japan Scientific Societies Press, Tokyo, pp. 185–201.

Robinson, T.E., 1992. Persistent sensitizing effects of drugs on brain dopamine systems and behavior: implications for addiction and relapse. In: Barchas, J., Korenman, S. (Eds.), The Biological Basis of Substance Abuse. Oxford Univ. Press, New York, pp. 373–403.

Robinson, T.E., Becker, J.B., 1986. Enduring changes in brain and behavior produced by chronic amphetamine administration: a review and evaluation of animal models of amphetamine psychosis. Brains Res. Rev. 11, 157–198.

Robinson, T.E., Kolb, B., 1999. Alterations in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine or cocaine. Eur. J. Neurosci. 11, 1598–1604.

Seiden, L.S., Sabol, K.E., Ricaurte, G.A., 1993. Amphetamine: effects on catecholamine systems and behavior. Annu. Rev. Pharmacol. Toxicol. 33, 639–677.

Simpson, J.N., Wang, J.Q., McGinty, J.F., 1995. Repeated amphetamine administration induces a prolonged augmentation of phosphorylated cyclase response element-binding protein and Fos-related antigen immunoreactivity in rat striatum. Neuroscience 69, 441–457.

Sitte, H.H., Hiptmair, B., Zwach, J., Pifl, C., Singer, E.A., Scholze, P., 2001. Quantitative analysis of inward and outward transport rates in cells stably expressing the cloned human serotonin transporter: inconsistencies with the hypothesis of facilitated exchange diffusion. Mol. Pharmacol. 59, 1129–1137.

Strakowski, S.M., Sax, K.W., Setters, M.J. , Keck Jr., P.E., 1996. Enhanced response to repeated D-amphetamine challenge: evidence for behavioral sensitization in humans. Biol. Psychiatry 40, 872–880.

Sulzer, D., Chen, T.-K., Lau, Y.Y., Kristensen, H., Rayport, S., Ewing, A., 1995. Amphetamine redistributes dopamine from synaptic vesicles to the cytosol and promotes reverse transport. J. Neurosci. 15, 4102–4108.

Turgeon, S.M., Pollack, A.E., Fink, J.S., 1997. Enhanced CREB phosphorylation and changes in c-Fos and FRA expression in striatum accompany amphetamine sensitization. Brain Res. 749, 120–126.

Vaughn, D.M., Severson, J.A., Woodward, J.J., Randall, P.K., Riffee, W.H., Leslie, S.W., Wilcox, R.E., 1990. Behavioral sensitization following subchronic apomorphine treatment—Possible neurochemical basis. Brain Res. 526, 37–44.

Wall, S.C., Gu, H., Rudnick, G., 1995. Biogenic amine flux mediated by cloned transporters stably expressed in cultured cell lines: amphetamine specificity for inhibition and efflux. Mol. Pharmacol. 47, 544–550.

White, F.J., Hu, X.-T., Brooderson, R.J., 1990. Repeated stimulation of dopamine D1 receptors enhances the effects of dopamine receptor agonists. Eur. J. Pharmacol. 191, 497–499.

Wolf, M.E., 1998. The role of excitatory amino acids in behavioral sensitization to psychomotor stimulants. Prog. Neurobiol. 54, 679–720.

Wolf, M.E., White, F.J., Nassar, R., Brooderson, R.J., Khansa, M.R., 1993. Differential development of autoreceptor subsensitivity and enhanced dopamine release during amphetamine sensitization. J. Pharmacol. Exp. Ther. 264, 249–255.

Zhu, J., Hexum, T.D., 1992. Characterization of cocaine-sensitive dopamine uptake in PC12 cells. Neurochem. Int. 21, 521–526.

Zhu, M.Y., Ordway, G.A., 1997. Down-regulation of norepinephrine transporters on PC12 cells by transporter inhibitors. J. Neurochem. 68, 134–141.