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Evoked Dopamine Overflow is Augmented in the Striatum of Calcitriol Treated Rats

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Abstract

Calcitriol, the active metabolite of vitamin D, has been shown to have significant effects on the brain. These actions include reducing the severity of some central nervous system lesions, possibly by upregulating trophic factors such as glial cell line-derived neurotrophic factor (GDNF). GDNF has substantial effects on the nigrostriatal dopamine (DA) system of young adult, aged and lesioned animals. Thus, the administration of calcitriol may lead to significant effects on nigrostriatal DA neuron functioning. The present experiments were designed to examine the ability of calcitriol to alter striatal DA release, and striatal and nigral tissue levels of DA. Male Fischer-344 rats were administered vehicle or calcitriol (0.3, 1.0, or 3.0 μg/kg, s.c.) once daily for 8 consecutive days. Three weeks later in vivo microdialysis experiments were conducted to measure basal and stimulus evoked overflow of DA from the striatum. Basal levels of extracellular DA were not significantly affected by the calcitriol treatments. However, the 1.0 and 3.0 μg/kg doses of calcitriol led to increases in both potassium and amphetamine evoked overflow of striatal DA. Although post-mortem tissue levels of striatal DA were not altered by the calcitriol injections, nigral tissue levels of DA and its main metabolites were increased by both the 1.0 and 3.0 μg/kg doses of calcitriol. In a separate group of animals GDNF levels were augmented in the striatum and substantia nigra after eight consecutive daily injections of calcitriol. These results suggest that systemically administered calcitriol can upregulate dopaminergic release processes in the striatum and DA levels in the substantia nigra. Increases in levels of endogenous GDNF following calcitriol treatment may in part be responsible for these changes. The ability of calcitriol to lead to augmented DA release in the striatum suggests that calcitriol may be beneficial in disease processes involving dopaminergic dysfunction.

Keywords

Calcitriol; Dopamine; Striatum; Substantia Nigra; GDNF; Microdialysis

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1. Introduction

Calcitriol (1,25-dihydroxyvitamin D_3), the active metabolite of vitamin D_3, is currently used clinically to treat several conditions such as hypocalcemia and hypoparathyroidism. While its classical role in bone metabolism and calcium homeostasis are well known, calcitriol has also been shown to modulate several other physiological processes including cell growth, apoptosis and immune responses (Dusso et al., 2005). Calcitriol has also been shown to have numerous effects in the nervous system (Fernandes de Abreu et al., 2009; Garcion et al., 2002). Although its ability to cross the blood brain barrier is limited, calcitriol is taken up by the brain in proportion to free circulating levels perfusing the blood brain barrier (Gascon-Barre and Huet, 1983); and vitamin D receptors are extensively distributed throughout the brain (Eyles et al., 2005; Prufer et al., 1999; Stumpf and O'Brien, 1987). Furthermore, the enzyme responsible for the final step in calcitriol synthesis has been localized to neuronal and glial cells (Eyles et al., 2005), and it has been demonstrated that activated microglial cells can produce calcitriol (Neveu et al., 1994b). Taken together, these results suggest that calcitriol may have important physiological functions in the central nervous system.

One of the apparent functions of calcitriol in the nervous system is modulation of trophic factors. Several studies have indicated that calcitriol regulates the expression of trophic factors in glial and neuroblastoma cell lines (Neveu et al., 1994a; Veenstra et al., 1997a and 1997b). One of the trophic factors that is regulated by calcitriol is glial cell line-derived neurotrophic factor (GDNF). Calcitriol has been shown to increase expression of GDNF in vitro in C6 glioma cells (Naveilhan et al., 1996), and increase release of GDNF from human glioblastoma cells (Verity et al., 1999). In vivo, calcitriol administration has been shown to increase expression and protein levels of GDNF in the brain (Sanchez et al., 2002 and 2009; Wang et al., 2000).

In young adult animals a single intracerebral administration of GDNF has significant and long-lasting effects on brain dopamine (DA) systems. These effects include changes in tissue levels of DA in the nigrostriatal system (Cass and Peters, 2010; Hudson et al., 1995; Martin et al., 1996;), augmentation of stimulus-evoked overflow of DA from the striatum (Hebert et al., 1996; Xu and Dluzen, 2000), and behavioral changes that suggest activation of dopaminergic systems (Hudson et al., 1995; Kobayashi et al., 1998; Martin et al., 1996). Similar changes have been reported in aged animals following acute or chronic administration of GDNF (Emerich et al., 1996; Grondin et al., 2003; Hebert and Gerhardt, 1997; Lapchak et al., 1997). Because of its striking effects on nigrostriatal DA neurons in normal and aged animals, GDNF has also been extensively evaluated as a potential therapeutic agent in several animal models of Parkinson's disease (Bjorklund et al., 2000; Cass et al., 1998; Gash et al., 1998; Grondin et al., 2002; Kordower et al., 2000).

Previous studies have documented that calcitriol can increase brain GDNF levels, and that GDNF leads to increases in dopaminergic activity in the brain. Thus, it is possible that calcitriol could lead to alterations in dopaminergic processes in the brains of normal animals. Understanding how calcitriol affects DA dynamics in intact animals may provide insight into its potential as a possible therapeutic agent for conditions associated with dopaminergic dysfunction. The purpose of the present study was to determine if calcitriol treatment can alter striatal DA release, and striatal and nigral tissue levels of DA, in normal animals. In vivo microdialysis was used to evaluate potassium- and amphetamine-evoked overflow of DA, and to monitor basal extracellular levels of DA and its primary metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) from the striatum of rats treated with various doses of calcitriol. Postmortem tissue levels of DA and metabolites in the striatum and substantia nigra were determined at the conclusion of each

experiment, and, in a separate group of animals the ability of calcitriol to upregulate GDNF levels was evaluated.

2. Materials and methods

2.1. Animals

Male Fischer-344 rats 14-15 weeks old were obtained from Harlan Laboratories (Indianapolis, IN). The animals weighed 277-303 g at the start of the experiments and 8 animals were included in each treatment group. The animals were housed in pairs under a 12-hr light-dark cycle with food and water freely available. All animal use procedures were approved by the Animal Care and Use Committee at the University of Kentucky and were in strict accordance with National Institutes of Health guidelines. All efforts were made to minimize the number of animals used and to minimize their pain and discomfort.

2.2. Calcitriol injections

Animals were injected once daily for eight consecutive days with calcitriol (0.3, 1.0 or 3.0 μg/kg/day) or vehicle. All injections were administered subcutaneously. The calcitriol (Sigma Chemical Co., St. Louis, MO) was first dissolved in propylene glycol at a concentration of 100 μg/ml. For injections the calcitriol in propylene glycol was diluted into 0.9% saline so that the final volume given was 1 ml/kg of body weight. Vehicle treated animals were injected with propylene glycol diluted in 0.9% saline.

2.3. In Vivo Microdialysis

Microdialysis studies were conducted three weeks after the last vehicle or calcitriol injection. The animals were anesthetized with urethane (1.25–1.50 g/kg, i.p.) and placed into a Kopf stereotaxic frame. Body temperature was maintained at 37°C with a heating pad coupled to a rectal thermometer. Microdialysis probes (CMA/11 probes, 3.0 mm length of dialysis membrane; CMA/Microdialysis, Acton, MA) were slowly lowered into both the left and right striata (0.0 mm anterior to bregma, 3.0 mm lateral from midline, tip of probe 6.3 mm below the surface of the brain). The probes were perfused at a rate of 1.2 μl/min with artificial cerebrospinal fluid containing 145 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.2 mM ascorbic acid, and 2.0 mM NaH_2PO_4 (pH adjusted to 7.4 using NaOH). Dialysate fractions were collected at 20-min intervals. Following a two-hour equilibration period and the collection of 3 baseline fractions, DA overflow was stimulated by increasing the KCl concentration in the perfusate to 100 mM (NaCl reduced to 47.7 mM) for a single 20-min fraction, and then two hours later by adding 100 μM d-amphetamine to the perfusate for a single 20-min fraction. Five final fractions with normal artificial cerebrospinal fluid were collected following the d-amphetamine stimulation. Dialysate samples were immediately frozen on dry ice and stored at −80°C until assayed for DA, DOPAC and HVA.

2.4. Tissue collection and HPLC analysis

After collecting the dialysate fractions the urethane-anesthetized animals were killed by decapitation and their brains rapidly harvested and chilled in ice-cold saline. A coronal slice of brain 2 mm thick at the level of the dialysis probes was made with the aid of an ice-chilled brain mold (Rodent Brain Matrix, ASI Instruments, Warren, MI). The location of all dialysis probes was confirmed to be centered in the dorsal striatum at the level of the crossing of the anterior commissure. The striatum was then dissected from each half of the slice. The substantia nigra was dissected from both sides of a 2 mm thick coronal slice through the midbrain. The tissue pieces were placed in preweighed vials, weighed, frozen on dry ice, and then stored at −80°C until assayed for DA and metabolites by high performance liquid chromatography (HPLC). For tissue analysis the samples were prepared as previously

described (Cass et al., 2003). For dialysis samples, 20 μl of the dialysate was injected directly onto the HPLC column.

The HPLC system consisted of a Beckman model 118 pump and model 507 autoinjector, and an ESA model 5200A Coulochem II electrochemical detector with a model 5011 dual-detector analytical cell (detector 1 set at +350 mV and detector 2 set at −300 mV). Separations were carried out using an ESA Hypersil ODS 3 μm particle C18 column (4.6 mm × 80 mm). Flow rate was 1.4 ml/min and the mobile phase was a 0.17 M citrate-acetate buffer containing 5 mg/l EDTA, 90 mg/l octanesulfonic acid, and 7–8 % methanol (pH 4.1). Chromatograms were recorded from both detectors using dual-channel strip chart recorders. Retention times of standards were used to identify peaks, and peak heights were used to calculate recovery of internal standard and amount of DA and metabolites.

2.5. Striatal and nigral GDNF levels

Rats were treated with calcitriol or vehicle once daily for eight consecutive days and striatal and nigral tissue was harvested 2–3 hours after the last injection as described above. GDNF levels were measured using Promega E_{max}^{TM} ImmunoAssay Kits as described previously (Smith et al., 2006; Yurek and Fletcher-Turner, 2001).

2.6. Data analysis

All dialysis probes were calibrated in vitro prior to use to determine acceptable probes (recovery of DA at least 12%). However, values were not corrected for in vitro recoveries as uncorrected values may be better correlated to true values (Glick et al., 1994). Basal levels of DA and metabolites were defined as the average value in the three fractions preceding stimulation by excess potassium. Dialysis data were expressed as nM concentration of DA or metabolite in the dialysate and, for evoked overflow, as the total amount of DA in the dialysate above baseline following stimulation with potassium or amphetamine. Tissue levels of DA and GDNF were expressed as ng/g wet weight of tissue. For animals used in dialysis experiments data from the left and right sides of the brain were averaged to get a single value for each time point and neurochemical per animal. Results were analyzed statistically using analysis of variance (ANOVA) as indicated in the results section. When the ANOVA results indicated significant effects, Newman-Keuls tests were used for post hoc comparisons.

3. Results

3.1. Dialysate levels of DA and metabolites

Basal extracellular dialysate levels of DA and metabolites in urethane anesthetized rats are shown in Table 1. The results were analyzed using one-way ANOVA, and, while there was a trend for basal DA levels to increase with increasing doses of calcitriol, the increase did not reach statistical significance. However, with both DOPAC and HVA there was a significant effect. In the animals treated with the highest dose of calcitriol basal DOPAC levels were increased by 31%, and basal HVA levels were increased by 23%, compared to the vehicle treated control animals.

The complete time course for the dialysis experiments for DA levels is shown in Fig. 1. Overall extracellular DA levels from the animals treated with either 1.0 or 3.0 μg/kg/day of calcitriol were greater than those from the vehicle group ($p < 0.05$ for both; main effect of treatment group). In order to facilitate comparisons of the potassium and amphetamine evoked overflow of DA between the groups the data were expressed as total amount of DA in the dialysate fractions above basal levels following stimulation by excess potassium or d-amphetamine (Fig. 2). Compared to the vehicle treated control animals, potassium-evoked

overflow of DA was increased by 57% in the animals treated with 1.0 μg/kg/day of calcitriol, and by 42% in the animals treated with 3.0 μg/kg/day of calcitriol ($p < 0.05$ for both). Similarly, amphetamine-evoked DA overflow was increased by 36% and 32%, respectively, in the animals treated with 1.0 or 3.0 μg/kg/day of calcitriol, compared to the vehicle treated group ($p < 0.05$ for both).

3.2. Tissue levels of DA

Striatal and nigral tissue levels of DA, DOPAC and HVA are shown in Table 2. The calcitriol treatments had no significant effect on striatal levels of DA or DOPAC. However, the highest dose of calcitriol, 3.0 μg/kg/day, did increase striatal levels of HVA by 26% compared to vehicle treated control animals ($p < 0.05$). The effects of the calcitriol treatments were more substantial in the substantia nigra, where both the 1.0 and 3.0 μg/kg/day doses of calcitriol led to significant increases in DA and metabolites. For the 1.0 and 3.0 μg/kg/day doses of calcitriol, the respective increases in nigral DA levels were 17% and 20%, the increases in DOPAC levels were 21% and 23%, and the increases in HVA levels were 46% and 55% ($p < 0.05$ for all). Metabolite to DA ratios were also calculated as an indicator of potential changes in DA turnover. However, there were no significant differences between any of the doses of calcitriol in either the striatum or substantia nigra for DOPAC/DA ratios, HVA/DA ratios, or (DOPAC+HVA)/DA ratios (data not shown), suggesting that the calcitriol treatments did not have a significant effect on DA turnover.

3.3 Tissue levels of GDNF

In a separate group of animals, tissue levels of GDNF were measured on the eighth day of vehicle or calcitriol treatment. In the striatum, eight days of calcitriol treatment led to 80% and 82% increases in GDNF levels in the animals treated with the 1.0 and 3.0 μg/kg/day doses of calcitriol, respectively ($p < 0.05$), compared to vehicle injected control animals (Fig. 3). In the substantia nigra, eight days of calcitriol treatment led to increases in GDNF levels of 131% and 107% in the animals treated with the 1.0 and 3.0 μg/kg/day doses of calcitriol, respectively ($p < 0.05$), compared to the control animals (Fig. 3).

4. Discussion

The present study was undertaken to determine if administration of calcitriol would lead to changes in DA release in the striatum or in DA content in the striatum or substantia nigra of normal young adult rats. The results demonstrated that calcitriol, at doses of 1.0 or 3.0 μg/kg/day for eight days, led to significant changes in DA release and content in the nigrostriatal system three weeks after the end of the calcitriol injections.

Previous in vivo studies that examined the protective effects of calcitriol for DA neurons (Sanchez et al., 2009; Smith et al., 2006; Wang et al., 2001), or the ability of calcitriol to alter GDNF levels (Sanchez et al., 2002 and 2009; Wang et al., 2000) examined a single dose of calcitriol (1.0 μg/kg/day). In the current study we examined the effects of three doses of calcitriol on DA neurons and GDNF levels. The lowest dose used, 0.3 μg/kg/day, did not have statistically significant effects on any of the parameters examined. The middle and higher doses (1.0 and 3.0 μg/kg/day) both had significant, and similar, effects. On a few of the parameters examined only the 3.0 μg/kg/day dose, and not the 1.0 μg/kg/day dose, was significantly different from controls (basal dialysate levels of DOPAC and HVA, and striatal tissue levels of HVA). However, there were no differences between the 1.0 and 3.0 μg/kg/day groups when compared to each other. Thus, the differences between the effects of the middle and high doses were relatively minor. In addition, during the 8 days of calcitriol injections, the animals treated with the 1.0 μg/kg/day dose did not lose body weight, while the animals treated with the 3.0 μg/kg/day dose did lose weight during the treatment (%

change in body weight from day 1 to day 8 of treatment: 1.0 μg/kg/day group, +0.24 ± 0.49%; 3.0 μg/kg/day group, −13.43 ± 1.56%; $p < 0.001$, t-test). Thus, the highest dose, while effective, may have been high enough to produce adverse side effects, possibly due to calcitriol-induced hypercalcemia (Chavhan et al., 2011; Wang et al., 2000). Therefore, for the parameters examined in the present study, the 1.0 μg/kg/day was the optimal dose for producing changes in DA neuron functioning and GDNF levels.

Other in vivo studies, all using the 1.0 μg/kg/day dose, have also examined the ability of calcitriol to alter GDNF levels. Wang et al. (2000) found that eight consecutive daily injections of calcitriol led to a 92% increase in cortical levels of GDNF. Sanchez et al. (2002) report a 40% increase in striatal GDNF protein levels after seven consecutive days of treatment, and Smith et al. (2006) report a 37% increase in nigral GDNF levels after eight days of calcitriol. In the current study we found an 80% increase in striatal GDNF levels, and a 131% increase in nigral GDNF levels, with the 1.0 μg/kg/day dose after eight days of treatment. Thus, the present results support previous studies and provide further evidence that calcitriol can act as a modulator of endogenous GDNF levels.

The dose response of calcitriol on GDNF levels was similar to the dose response for changes in DA overflow and nigral tissue DA levels. In addition, the effects observed on evoked DA overflow and tissue levels of DA are similar to what has been reported in rats treated with GDNF. For example, following the injection of a single dose of GDNF into the brain of unlesioned rats, previous studies have reported increases, or a trend for an increase, in potassium-evoked overflow of striatal DA (Cass and Manning, 1999; Cass and Peters, 2010; Hebert et al., 1996; Hebert and Gerhardt, 1997; Xu and Dluzen, 2000), and increases in amphetamine-evoked overflow of striatal DA (Cass and Peters, 2010; Hebert et al., 1996; Hebert and Gerhardt, 1997). In addition, tissue levels of DA in the substantia nigra are also significantly increased following a single intracerebral administration of GDNF (Cass and Manning 1999; Cass and Peters, 2010; Hebert et al., 1996; Hudson et al., 1995; Martin et al., 1996). Thus, the parallel changes in dopaminergic parameters and levels of GDNF, along with the previously documented effects of GDNF, suggest that the calcitriol-induced changes in DA release and tissue DA levels may be due in part to upregulation of endogenous GDNF levels. However, we cannot rule out the possibility that other effects of calcitriol may be responsible for the present results. For instance, calcitriol has been shown to regulate expression of other trophic factors, such as transforming growth factor- ß, nerve growth factor and neurotrophin-3 (Neveu et al., 1994a; Saporito et al., 1994; Veenstra et al., 1997a and 1997b), which could affect neuronal function; and calcitriol has been shown to increase levels of tyrosine hydroxylase expression (Puchacz et al., 1996; Sanchez et al., 2009), which could modulate dopaminergic processes. Thus, further studies will be necessary to more completely define the mechanism by which calcitriol affects DA systems.

The microdialysis experiments were carried out three weeks after ending the calcitriol injections. This interval was chosen as it is a time point when significant changes in DA release and content have been demonstrated in rats following GDNF injection (Cass and Peters, 2010; Hebert et al., 1996; Hebert and Gerhardt, 1997). In addition, several studies have demonstrated significant behavioral or neurochemical effects 3 to 4 weeks after GDNF treatment (for example: Aoi et al., 2000; Cass et al., 2000; Date et al., 1998; Hoffer et al., 1994; Salvatore et al., 2004). Thus, if GDNF is involved with the effects of calcitriol, the three week interval should, and did, represent a time point likely to demonstrate potential effects of calcitriol on DA neurons.

The timing for the duration of the calcitriol treatment and for the measurement of GDNF levels (8 days) was based on previous calcitriol studies. Wang et al. (2001) found that eight days of calcitriol partially protected against the neurotoxic effects of 6-hydroxydopamine (6-

OHDA), and Sanchez et al. (2009) found that seven days of calcitriol, given before or after a 6-OHDA lesion, led to a reduction in 6-OHDA-induced loss of tyrosine hydroxylase positive neurons in the substantia nigra. In addition, seven or eight consecutive days of calcitriol administration (1.0 μg/kg/day) has been shown to upregulate GDNF expression and protein levels in the brain (Sanchez et al., 2002 and 2009; Smith et al., 2006; Wang et al., 2000). Thus, we choose eight days of treatment for examining the potential ability of calcitriol to augment dopaminergic release processes and tissue DA and GDNF levels.

In conclusion, the systemic administration of calcitriol led to increases in evoked overflow of DA from the striatum, to increases in DA levels in the substantia nigra, and to increases in endogenous GDNF levels in the striatum and substantia nigra. Taken together, the results of this study indicate that calcitriol can augment dopaminergic processes in the nigrostriatal system of normal animals and provide further evidence that calcitriol may be beneficial in treating disease processes involving dopaminergic dysfunction.

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Highlights

- Effects of calcitriol (active metabolite of vitamin D) were studied in normal rats.
- Calcitriol increases stimulus- evoked overflow of dopamine from the striatum.
- Calcitriol increases tissue levels of dopamine in the substantia nigra.
- Calcitriol upregulates GDNF levels in the striatum and substantia nigra.
- Calcitriol may have therapeutic potential for disorders such as Parkinson's disease.

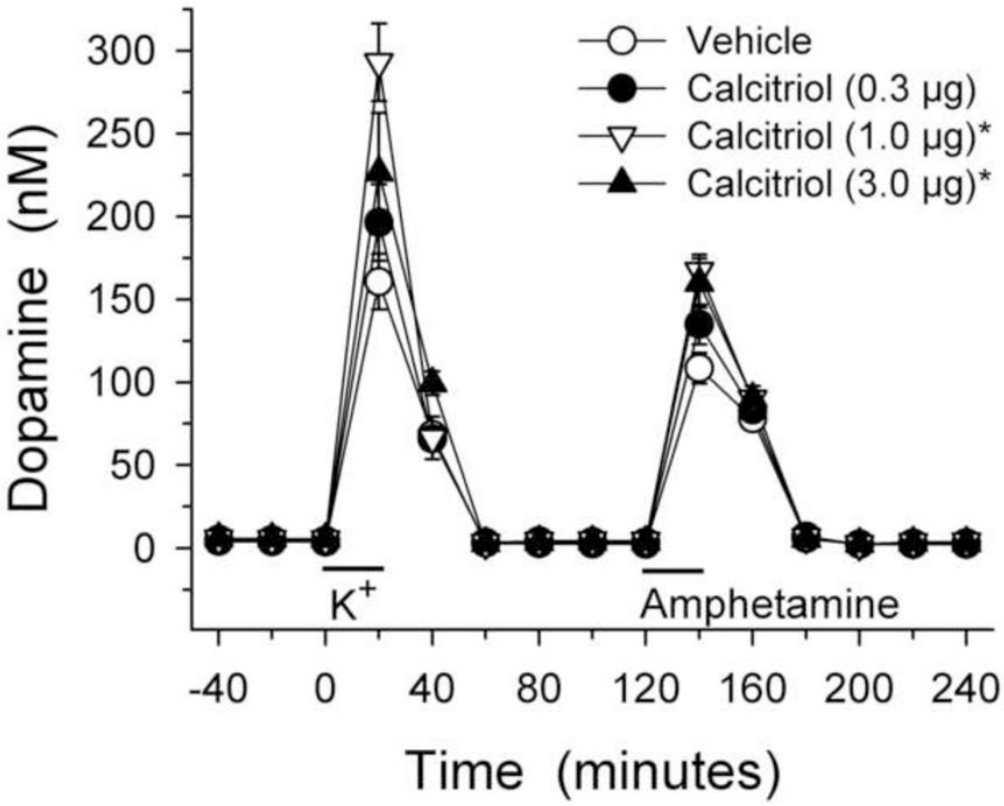

Figure 1.

Dialysate levels of DA from the striata of animals treated with vehicle or calcitriol for eight consecutive days. Microdialysis experiments were performed three weeks after the last day of treatment. Excess potassium (100 mM) was included in the perfusate for 20-min starting at 0 min (horizontal bar above K^+), and 100 µM amphetamine was included in the perfusate for 20-min starting at 120 min (horizontal bar above Amphetamine). Values shown are mean ± SEM from 8 animals per group. * $p < 0.05$ vs. vehicle group (mixed ANOVA with time of dialysis sample collection as a within factor, and treatment group as a between factor; significant main effect of treatment group ($p < 0.01$) followed by Newman-Keuls post-hoc comparisons).

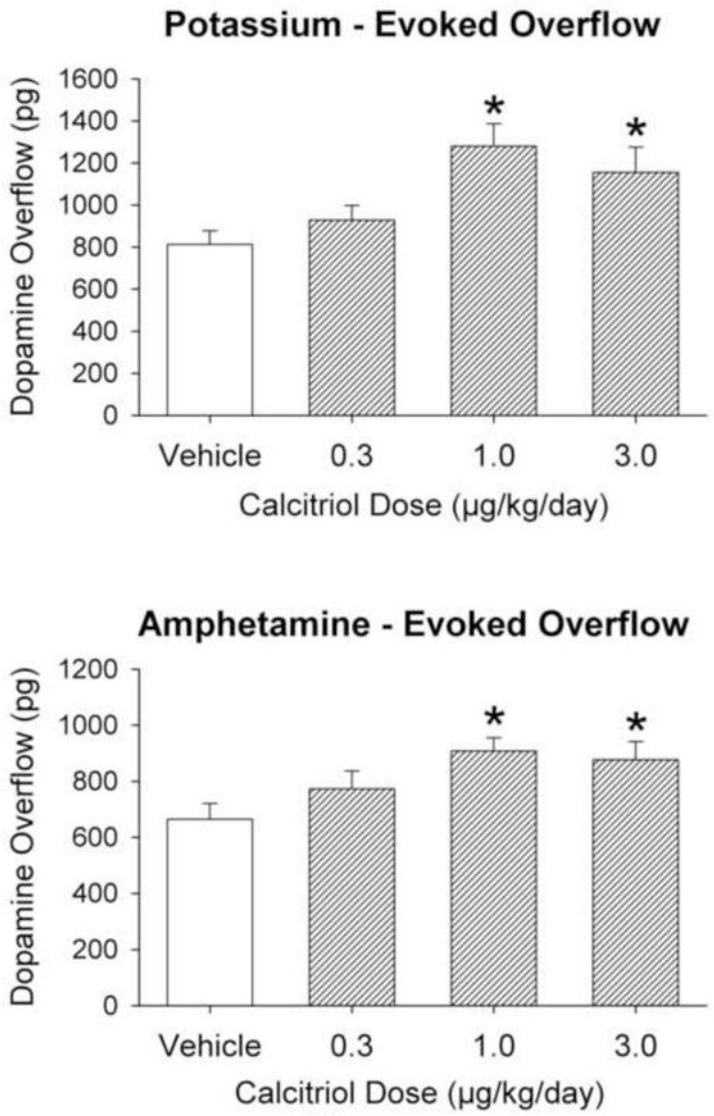

Figure 2.

Potassium-evoked (top panel) and amphetamine-evoked (bottom panel) overflow of DA from the striatum of animals treated with vehicle or calcitriol for eight consecutive days. Microdialysis experiments were performed three weeks after the last day of treatment. Values shown are mean ± SEM from 8 animals per group. * $p < 0.05$ vs. vehicle group (one way ANOVA followed by Newman-Keuls post-hoc comparisons).

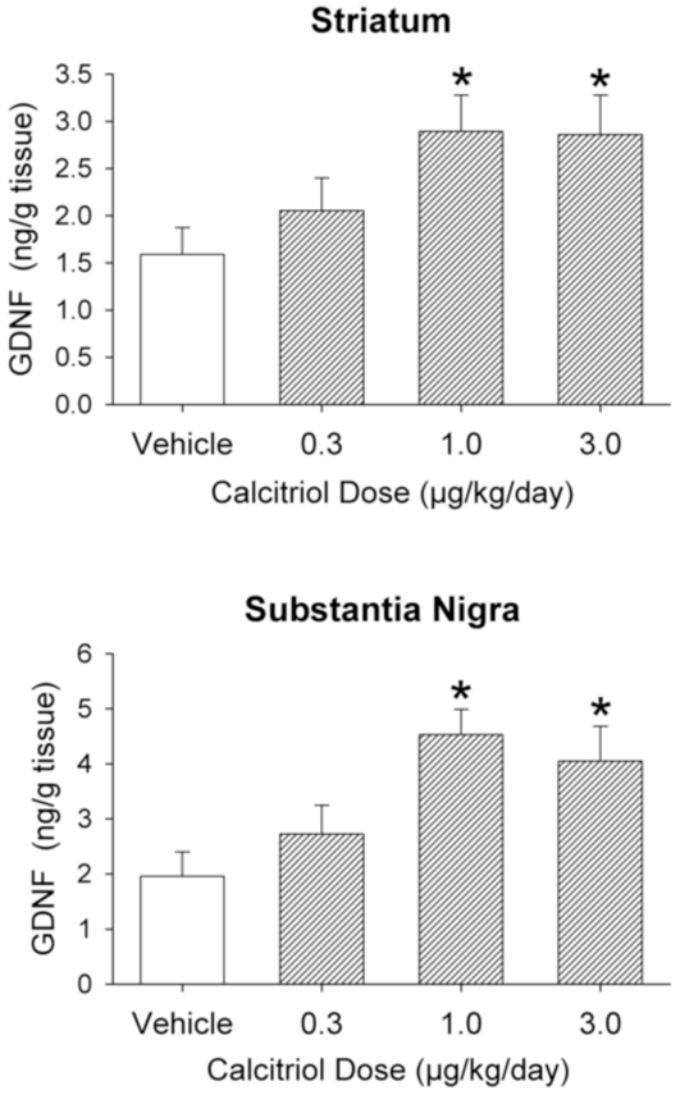

Figure 3.

Striatal (top panel) and substantia nigra (bottom panel) GDNF levels from animals treated with vehicle or calcitriol for eight consecutive days. Tissue was harvested on the eighth day of treatment. Values shown are mean ± SEM from 8-10 animals per group. * $p < 0.05$ vs. vehicle group (one way ANOVA followed by Newman-Keuls post-hoc comparisons).

Table 1

Basal dialysate levels of DA, DOPAC and HVA from the striatum of animals treated for eight days with vehicle or calcitriol. Dialysis experiments were performed three weeks after the final day of treatment.

	Striatal dialysate level (nM)		
Calcitriol Dose	**DA**	**DOPAC**	**HVA**
Vehicle	4.03 ± 0.44	1575 ± 82	911 ± 39
0.3 μg/kg/day	4.96 ± 0.41	1699 ± 84	982 ± 38
1.0 μg/kg/day	5.32 ± 0.44	1779 ± 86	1012 ± 47
3.0 μg/kg/day	5.77 ± 0.60	2056 ± 43[a]	1116 ± 26[a]

Values are mean ± SEM from 8 animals per group.

[a] $p < 0.05$ vs. vehicle group (one-way ANOVA followed by Newman-Keuls post hoc comparisons).

Table 2

Tissue levels of DA, DOPAC and HVA from the striatum and substantia nigra of animals treated for eight days with vehicle or calcitriol. Tissue was harvested three weeks after the final day of treatment.

Region and calcitriol dose	Tissue content (ng/g wet weight of tissue)		
	DA	DOPAC	HVA
Striatum			
Vehicle	15,474 ± 552	2,428 ± 108	1,373 ± 41
0.3 μg/kg/day	16,183 ± 460	2,496 ± 73	1,409 ± 98
1.0 μg/kg/day	15,900 ± 980	2,657 ± 164	1,591 ± 92
3.0 μg/kg/day	16,477 ± 654	2,664 ± 125	1,743 ± 60[a]
Substantia Nigra			
Vehicle	764 ± 16	143 ± 4.0	65 ± 2.0
0.3 μg/kg/day	788 ± 31	152 ± 9.3	70 ± 5.1
1.0 μg/kg/day	891 ± 41 [a]	173 ± 9.1 [a]	95 ± 7.8 [a]
3.0 μg/kg/day	914 ± 38 [a]	176 ± 8.7 [a]	101 ± 5.2 [a]

Values are mean ± SEM from 8 animals per group.

[a] $p < 0.05$ vs. vehicle group (one-way ANOVA followed by Newman-Keuls post hoc comparisons).