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## Reduced ability of calcitriol to promote augmented dopamine release in the lesioned striatum of aged rats

Wayne A. Cass\* and Laura E. Peters

Department of Anatomy and Neurobiology, University of Kentucky College of Medicine, Lexington, KY, USA

### Abstract

Parkinson's disease (PD) is a progressive and debilitating neurodegenerative disorder that affects over one million people in the United States. Previous studies, carried out in young adult rats, have shown that calcitriol, the active metabolite of vitamin D, can be neuroprotective in 6-hydroxydopamine (6-OHDA) models of PD. However, as PD usually affects older individuals, the ability of calcitriol to promote dopaminergic recovery was examined in lesioned young adult (4 month old), middle-aged (14 month old) and aged (22 month old) rats. Animals were given a single injection of 12 µg 6-OHDA into the right striatum. Four weeks later they were administered vehicle or calcitriol (1.0 µg/kg, s.c.) once a day for eight consecutive days. In vivo microdialysis experiments were carried out three weeks after the calcitriol or vehicle treatments to measure potassium and amphetamine evoked overflow of DA from both the left and right striata. In control animals treated with 6-OHDA and vehicle there were significant reductions in evoked overflow of DA on the lesioned side of the brain compared to the contralateral side. The calcitriol treatments significantly increased evoked overflow of DA from the lesioned striatum in both the young adult and middle-aged rats. However, the calcitriol treatments did not significantly augment DA overflow in the aged rats. Postmortem tissue levels of striatal DA were also increased in the young and middle-aged animals, but not in the aged animals. In the substantia nigra, the calcitriol treatments led to increased levels of DA in all three age groups. Thus, the effects of calcitriol were similar in the young adult and middle-aged animals, but in the aged animals the effects of calcitriol were diminished. These results suggest that calcitriol may help promote recovery of dopaminergic functioning in injured nigrostriatal neurons; however, the effectiveness of calcitriol may be reduced in aging.

### Keywords

Aging; Striatum; Substantia Nigra; Dopamine; Calcitriol; 6-OHDA

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\*Corresponding Author: Wayne A. Cass, Ph.D., Department of Anatomy and Neurobiology, MN-225 Chandler Medical Center, University of Kentucky, Lexington, Kentucky 40536-0298, Telephone (859) 323-1142, FAX (859) 323-5946, wacass1@uky.edu.

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## 1. Introduction

Increasing evidence indicates that vitamin D has significant actions in the brain including regulation of calcium homeostasis (Gezen-Ak et al., 2011; Groves et al., 2014; Zanatta et al., 2012), modulation of neurotrophic factors (Neveu et al., 1994a; Sanchez et al., 2002; Saporito et al., 1994; Veenstra et al., 1997a) and neurotransmitter systems (Cass et al., 2012; Jiang et al., 2014; Sonnenberg et al., 1986), immunomodulation (Fernandes de Abreu et al., 2009; Kesby et al., 2011), and neuroprotection (Garcion et al., 2002; Groves et al., 2014; Kesby et al., 2011). The active metabolite of vitamin D<sub>3</sub> is calcitriol (1,25-dihydroxyvitamin D<sub>3</sub>), which can cross the blood brain barrier to a limited extent (Gascon-Barre and Huet, 1983; Partridge et al., 1985). In addition, the brain itself may be able to synthesize calcitriol (Eyles et al., 2005; Neveu et al., 1994b). The effects of calcitriol are mediated through genomic pathways, via the vitamin D receptor (VDR), and nongenomic pathways, via membrane bound VDR's or membrane-associated rapid response steroid binding receptors (Fernandes de Abreu et al., 2009; Garcion et al., 2002; Groves et al., 2014). Both types of receptors are found in the adult brain (Cui et al., 2013; Eyles et al., 2005, 2014; Pendyala et al., 2012; Prufer et al., 1999; Stumpf and O'Brien, 1987). Together these studies suggest that calcitriol has significant effects in the brain.

Parkinson's disease (PD) is a neurodegenerative disorder that is likely due in part to the progressive degeneration of nigrostriatal dopamine (DA) neurons. Several studies have linked deficiencies in vitamin D, or changes in the vitamin D receptor, with an increased risk of developing PD (Butler et al., 2011; Evatt et al., 2008; Newmark and Newmark, 2007). In animal models of PD, calcitriol has been shown to have beneficial effects. Using 6-hydroxydopamine (6-OHDA) models of PD, several studies have demonstrated that calcitriol can partially protect against the behavioral, neurochemical and histological effects of the toxin (Kim et al., 2006; Sanchez et al., 2009; Smith et al., 2006; Wang et al., 2001). Similarly, neuroprotective effects of calcitriol have been reported in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD (Kim et al., 2006). Two studies have also reported that calcitriol administration, starting 3 to 4 weeks after lesioning with 6-OHDA, was able to help restore tyrosine hydroxylase (TH) positive cells in the lesioned substantia nigra (Sanchez et al., 2009) and DA release and tissue levels in the lesioned nigrostriatal system (Cass et al., 2014). In addition, calcitriol has also been reported to be neuroprotective in several in vitro studies that have examined dopaminergic toxins or cells (for example see: Ibi et al., 2001; Jang et al., 2014, 2015b; Orme et al., 2013; Shinpo, 2000). Together, these studies demonstrate that calcitriol has neuroprotective and dopaminergic promoting properties in vitro, and in animal models of PD.

Although calcitriol has been shown to have beneficial effects in animal models of PD, previous studies have used young adult animals in their studies. PD is primarily a disease of the elderly, and there can be differences in how young and aged animals respond to pharmacological treatments. Thus, the present experiments were designed to examine and compare the ability of calcitriol to promote restoration of DA overflow and tissue content of DA in young adult (4 month old), middle-aged (14 month old) and aged (22 month old) rats previously lesioned with 6-OHDA. In vivo microdialysis was used to evaluate basal extracellular levels of DA and its primary metabolites 3,4-dihydroxyphenylacetic acid

(DOPAC) and homovanillic acid (HVA), and stimulus-evoked overflow of DA, from the striatum of rats treated with 6-OHDA and calcitriol. Postmortem tissue levels of DA in the striatum and substantia nigra were also evaluated to further assess the effects of calcitriol in the three age groups.

## 2. Materials and methods

### 2.1. Animals

Young adult (4 months old, 303 – 366 g), middle-aged (14 months old, 461 – 536 g) and aged (22 months old, 405 – 499 g) male Fischer-344 rats were obtained from Harlan Laboratories (Indianapolis, IN). 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. Every effort was made to reduce the number of animals used and to minimize their pain and discomfort.

### 2.2. 6-OHDA Injections

Animals were anesthetized with isoflurane (2.0–2.5% as needed) and positioned in a stereotaxic apparatus. Using sterile procedures, the skull was exposed, and a small hole drilled in the skull over the right striatum (1.0 mm anterior to bregma, 2.8 mm lateral from midline). A microliter syringe with a 26 gauge blunt tapered needle was used to inject 12 µg 6-OHDA (Sigma-Aldrich, St. Louis, MO) into the striatum 5.0 mm below the surface of the cortex. The 6-OHDA was injected in a total of 4 µl 0.9 % saline with 0.1% ascorbic acid (pH 5.5) at a rate of 0.5 µl/min. The needle was left in place for an additional 5 minutes following the injection before being withdrawn. Gelfoam was placed in the burr hole, the incision closed with wound clips, and the animals allowed to recover.

### 2.3. Calcitriol Treatment

Four weeks after the 6-OHDA injections the animals were injected once daily for eight consecutive days with calcitriol (1.0 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.

### 2.4. In Vivo Microdialysis

Microdialysis studies were conducted three weeks after the final calcitriol or vehicle treatment. The rats were anesthetized with urethane (1.25–1.50 g/kg, i.p.) and positioned in a 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<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 0.2 mM ascorbic acid, and 2.0 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4). 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 potassium 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  $\mu$ M 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  $-80^{\circ}\text{C}$  until assayed for DA, DOPAC and HVA.

## 2.5. Tissue Collection and HPLC Analysis

After collecting the dialysate fractions the urethane-anesthetized animals were killed by decapitation and their brains rapidly removed 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, or just rostral to, the crossing of the anterior commissure. The site of the intrastriatal injection was also visible and was confirmed to be located in the dorsal striatum. 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, and frozen on dry ice. Samples were stored at  $-80^{\circ}\text{C}$  until assayed for DA by high performance liquid chromatography (HPLC) as previously described (Cass et al., 2003). For dialysis samples, 20  $\mu$ l of the dialysate was injected directly onto the column.

## 2.6. Data Analysis

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 were expressed as ng/g wet weight of tissue. Results were analyzed statistically using analyses of variance (ANOVA) followed by Newman-Keuls tests for post hoc comparisons.

## 3. Results

### 3.1. Baseline Values for Parameters Measured

Table 1 shows the baseline values of all parameters on the non-lesioned side of the control animals. Basal microdialysate DA levels did not change over age. However, basal DOPAC levels decreased with age (one-way ANOVA,  $F_{(2, 20)} = 6.83$ ,  $p < 0.01$ ). Newman-Keuls post hoc comparisons indicated that DOPAC levels in middle-aged and aged animals decreased by 15% ( $p < 0.05$ ) and 28% ( $p < 0.01$ ), respectively, compared to the young animals. While all of the other parameters tended to decrease with age, the decreases did not reach statistical significance.

### 3.2. Dialysate Levels of DA and Metabolites

Basal extracellular dialysate levels of DA and metabolites on the lesioned side of the brain, expressed as percentage of the unlesioned side, are shown in Fig. 1. Basal levels of DA on the lesioned side ranged from 65–87% of the unlesioned side. A two-factor ANOVA revealed no statistically significant differences in basal DA levels between groups or treatments. For basal extracellular DOPAC the levels on the lesioned side ranged from 24–41% of the unlesioned side, and for basal extracellular HVA the levels on the lesioned side ranged from 37–53% of the unlesioned side (Fig. 1). Similar to the results for basal DA levels, two-factor ANOVAs indicated no statistically significant differences in basal DOPAC or HVA levels between groups or treatments.

Figure 2 shows the time course data for DA levels from the in vivo microdialysis experiments for the three age groups. The data in each age group was analyzed using a mixed ANOVA with side of brain and time of dialysis sample collection as within factors, and treatment group as a between factor. Significant side of brain by treatment group interactions were found for the young adult ( $F_{(1, 14)} = 4.84, p < 0.05$ ) and middle-aged ( $F_{(1, 14)} = 6.35, p < 0.05$ ) animals. Newman-Keuls post-hoc comparisons indicated that within each age and treatment group, overall DA levels on the lesioned side were lower than those from the contralateral side ( $p < 0.01$  for all groups). In addition, on the lesioned side, DA levels from the groups treated with calcitriol were greater than those from the vehicle treated group for both the young adult and middle-aged animals ( $p < 0.05$  for both). However, there was not a significant difference in DA levels on the lesioned side of the aged animals between the vehicle and calcitriol treated groups. In order to facilitate comparison of the evoked overflow of DA between the groups and ages the data were converted to total amount of DA in the dialysate fractions above basal levels following stimulation by excess potassium or d-amphetamine, and then expressed as percentage of DA on the lesioned side compared to the unlesioned side (Fig. 3). In the vehicle treated rats, potassium-evoked DA overflow was reduced on the lesioned side of the brain compared to the contralateral side by 80%, 76% and 79% in the young, middle-aged and aged animals, respectively. In the calcitriol treated rats the decreases in potassium-evoked DA overflow on the lesioned side were 53%, 47% and 70% in the young, middle-aged and aged animals, respectively. The data were analyzed using two-way ANOVA with age and treatment group as between factors. A significant interaction was found ( $F_{(2, 40)} = 4.02, p < 0.05$ ), and Newman-Keuls post-hoc comparisons indicated that the potassium-evoked overflow of DA on the lesioned side of the brain was significantly greater in both the young adult and middle-aged animals treated with calcitriol compared to the groups treated with vehicle ( $p < 0.05$  for both ages). Amphetamine-evoked DA overflow was reduced on the lesioned side of the brain in the vehicle treated rats by 69%, 66% and 69% in the young, middle-aged and aged animals, respectively. In the calcitriol treated rats the decreases in amphetamine-evoked DA overflow on the lesioned side were 46%, 34% and 66% in the young, middle-aged and aged animals, respectively. Similar to above, a two-way ANOVA indicated a significant age by treatment group interaction ( $F_{(2, 40)} = 3.24, p < 0.05$ ), and Newman-Keuls post-hoc comparisons indicated that the amphetamine-evoked overflow of DA on the lesioned side of the brain was significantly greater in the young adult and middle-aged animals treated with calcitriol compared to the groups treated with vehicle ( $p < 0.05$  for both ages). In the aged animals

there was not a significant difference between the vehicle and calcitriol treated groups for either potassium- or amphetamine-evoked overflow of DA.

### 3.3. Tissue Levels of DA

The 6-OHDA treatments and the systemic injections of calcitriol affected postmortem levels of DA in all age groups in both the striatum and substantia nigra (Fig. 4). In the young adult, middle-aged and aged rats treated with vehicle, striatal DA levels were decreased in the respective age groups by 66%, 68% and 64% on the lesioned side compared to the contralateral side. In the calcitriol treated rats striatal DA levels were decreased by 45%, 42% and 59% in the young, middle-aged and aged groups on the lesioned side. The data were analyzed using two-way ANOVA with age and treatment group as between factors. A significant interaction was found ( $F_{(2, 40)} = 3.49$ ,  $p < 0.05$ ), and Newman-Keuls post-hoc comparisons indicated that in the young adult and middle-aged groups the decrease in DA levels in the calcitriol treated animals was significantly less than the decrease in the vehicle treated animals ( $p < 0.05$  for both groups). The calcitriol treatments had no significant effect on striatal DA levels in the aged animals. In the substantia nigra, decreases in DA on the lesioned side of the vehicle treated rats were 38% in the young adult, 43% in the middle-aged and 43% in the aged groups. In the calcitriol treated rats nigral DA levels on the lesioned side were decreased by 13%, 10% and 22% in the young, middle-aged and aged groups. A two-way ANOVA indicated a significant age by treatment group interaction ( $F_{(2, 40)} = 3.25$ ,  $p < 0.05$ ), and Newman-Keuls post-hoc comparisons indicated that in all three age groups the decrease in DA levels in the calcitriol treated animals was significantly less than the decrease in the vehicle treated animals ( $p < 0.05$  for all three groups).

## 4. Discussion

The present experiments were designed to examine the ability of calcitriol to promote restoration of DA overflow and tissue content of DA in young adult, middle-aged and aged rats that had previously been lesioned with 6-OHDA. The calcitriol treatments led to increases in potassium and amphetamine evoked overflow of striatal DA, and to increases in striatal and nigral tissue levels of DA, on the lesioned side in both the young adult and middle-aged rats. However, in the aged rats the calcitriol treatments had no statistically significant effect on evoked overflow or tissue content of striatal DA. However, nigral levels of DA did increase on the lesioned side of the aged rats treated with calcitriol. These results suggest that calcitriol may be able to help promote recovery of dopaminergic functioning in injured nigrostriatal neurons; however, the effectiveness of calcitriol may be reduced in aging. It should also be pointed out that 6-OHDA produces an acute injury of dopaminergic neurons, not a slow progressive loss as is seen in PD, and thus our results may not translate directly to the clinical situation of patients with PD

In both humans and monkeys, striatal DA levels are often reported to decrease during normal aging (Haycock et al., 2003; Irwin et al., 1994; Kish et al., 1992; Wenk et al., 1989). However, this is not always the case with rodents, as some studies report significant decreases in aged Fischer-344 rats (Ling et al., 2000; Marshall and Altar, 1986; Marshall and Rosenstein, 1990), while other reports indicate little to no significant differences (Cass et al.,

2005; Hebert et al., 1998; Stanford et al., 2003). In the current study we report a significant decrease in basal microdialysate levels of DOPAC in the aged rats, and a trend for a decrease in the aged rats for basal HVA levels, evoked overflow of DA, and tissue content of DA. There was no effect of aging on basal extracellular DA levels, suggesting that compensatory responses (Robinson et al., 1994; Schwarting and Huston, 1996, Zigmond et al., 1990) are able to maintain normal basal release. Similarly, on the lesioned side of the brain, compensatory mechanisms are likely maintaining relatively normal basal DA levels while basal metabolite levels are reduced to a greater extent. It should also be kept in mind that our control values for aging in this study were obtained from the non-lesioned side of 6-OHDA treated animals. Nonetheless, our current results suggest that any age-related decreases in the nigrostriatal dopaminergic system in Fischer-344 rats are minimal.

In the young adult and middle-aged animals the calcitriol treatments led to increases in both potassium- and amphetamine-evoked overflow of DA. Potassium-evoked stimulation reflects calcium-dependent release of DA (vesicular, exocytotic release of DA), while amphetamine-evoked stimulation reflects calcium-independent release of DA (cytosolic release of DA). The fact that both potassium and amphetamine-evoked overflow of DA were increased following calcitriol treatment suggests that the calcitriol was promoting recovery of both calcium-dependent and calcium-independent release of DA. Baseline release of DA was not significantly affected by the 6-OHDA lesions, or the calcitriol treatments, likely due to compensatory responses aimed at maintaining normal extracellular levels of DA (Robinson et al., 1994; Schwarting and Huston, 1996, Zigmond et al., 1990). However, under the conditions of stimulation that examine extent of release from vesicular and cytosolic stores of DA, the evoked DA was increased suggesting that both vesicular and cytosolic pools of DA were increased by the calcitriol treatments.

The present study examined the ability of calcitriol to restore dopaminergic parameters in previously lesioned animals. However, several studies have demonstrated the ability of calcitriol, administered prior to lesioning, to protect against the effects of dopaminergic toxins in young adult animals (Kim et al., 2006; Sanchez et al., 2009; Smith et al., 2006; Wang et al., 2001). It is thus possible that the protective effects of calcitriol (i.e. given before the neurotoxin lesion) may be more pronounced than the restorative effects in aged animals.

The mechanisms by which calcitriol promotes recovery of dopaminergic processes after lesioning have not been defined. Several reports have demonstrated that calcitriol can lead to increases in expression or release of GDNF in various cell lines (Naveihan et al., 1996a; Orme et al., 2013; Verity et al., 1999) and to upregulation of endogenous GDNF levels in the brain (Cass et al., 2012; Sanchez et al., 2002, 2009). GDNF can promote dopaminergic recovery by several possible mechanisms including its effects on DA synthesis, storage, release, and number of functional terminals (Bourque and Trudeau, 2000; Cass and Peters, 2010; Hebert et al., 1996; Pothos et al., 1998; Salvatore et al., 2004). Thus, calcitriol-induced upregulation of GDNF could be one possible mechanism for calcitriol's effects on dopaminergic systems. Other neurotrophic factors could also be involved as calcitriol has been shown to regulate several trophic factors and receptors (Naveilhan et al., 1996b; Neveu et al., 1994a; Saporito et al., 1994; Veenstra et al., 1997a, 1997b). Interestingly, several studies have reported that upregulation of brain trophic factors or trophic activity may be

reduced in aged animals (Kaseloo et al., 1996; Ling et al., 2000; Yurek and Fletcher-Turner, 2000, 2001), and that aged animals can have a reduced response to exogenous factors (Date et al., 1993; Fox et al., 2001; Schneider, 1992). This could help explain the reduced effectiveness of the calcitriol treatments in the striatum of the aged animals. Another possible mechanism could be calcitriol-induced increases in tyrosine hydroxylase expression and/or activation (Cui et al., 2015; Puchacz et al., 1996), which could lead to increased stores and release of DA. Calcitriol and vitamin D also have numerous other effects in the brain which could alter the functional status of neurons, such as reducing the production of free radicals or upregulating free radical scavenging systems (Garcion et al., 1998, 1999; Ibi et al., 2001; Shinpo et al., 2000), modulating neuro-immunological function (Fernandes de Abreu et al., 2009; Harms et al., 2011; Kesby et al., 2011) and regulating neuronal calcium signaling (Brewer et al., 2006; de Viragh et al., 1989). Thus, there are numerous mechanisms that could be involved with calcitriol's effects on dopaminergic neurons. Further studies will be needed to determine the mechanisms at work, and what differences may account for the reduced effects observed in the aged animals.

The dose of calcitriol and the time course followed in this study were based on previously published reports. The 1 µg/kg/day dose of calcitriol was found to be optimal in studies with young adult rats, with higher doses possibly leading to adverse effects (Cass et al., 2012). In addition, a seven to eight day treatment schedule has been found to be effective in numerous studies examining calcitriol's ability to modulate brain chemistry and physiology (Cass et al., 2012, 2014; Sanchez et al., 2002, 2009; Wang et al., 2000, 2001). The microdialysis studies were performed three weeks after the final calcitriol treatment for a couple of reasons: 1) it is a time point in which significant changes in evoked overflow and content of DA have been found in young adult normal, and lesioned, rats following calcitriol administration (Cass et al., 2012, 2014); and 2) since it is possible that the effects of calcitriol may be partially mediated by increased GDNF levels, the three week time period has been reported to lead to changes in stimulus evoked release and content of DA in animals following treatment with exogenous GDNF (Cass and Peters, 2010; Hebert and Gerhardt, 1997; Hebert et al., 1996). It should be kept in mind that the previous calcitriol studies examined young adult animals, and different treatment doses and schedules may be able to produce an improved outcome in the aged animals.

In the present study the 6-OHDA was administered directly into the striatum, and there was greater depletion of DA in the striatum than in the substantia nigra. This suggests that damage to striatal DA terminals is greater than damage to cell bodies in the substantia nigra. Thus, the extent of terminal damage in the aged animals, compared to the younger animals, may be more difficult to overcome, leading to a partial recovery in the younger animals but not in the aged animals. The less extensive damage in the nigra may allow for improved recovery in the aged as well as younger rats.

Although in the aged rats the calcitriol had no significant effect on dopaminergic parameters in the striatum, it did increase tissue DA levels in the substantia nigra. Several studies have pointed out the important role of nigral DA in regulating motor performance (Andersson et al., 2006; Gerhardt et al., 2002; Pruet and Salvatore, 2013; Robertson and Robertson, 1989; Salvatore et al., 2009; Trevitt et al., 2001). Thus, the positive effects of calcitriol in the



substantia nigra of the aged animals, while not as pronounced as the overall effects in the two younger age groups, suggests that calcitriol may be beneficial even in older individuals.

While this study found a less robust effect of calcitriol in aged animals compared to young adult and middle-aged animals, there are studies that support positive effects of calcitriol in the brains of aged animals, particularly concerning cognitive function. For example, in aged rats, vitamin D and calcitriol supplementation have led to improved hippocampal function and reduced cognitive decline (Brewer et al., 2006; Briones and Darwish, 2012; Latimer et al., 2014), while in vitamin D deficient rats, the brains of aged animals show increased levels of nitrosative stress, and changes in glucose metabolism and mitochondrial proteins that may increase the risk for cognitive decline (Keeney et al., 2013). Similarly, studies in elderly humans indicate lower cognitive performance in individuals with vitamin D deficiencies (Kueider et al., 2016; Llewellyn et al., 2010; Przybelski and Binkley, 2007). These studies support that vitamin D and calcitriol have beneficial effects in the aged brain.

The current results suggest that calcitriol may help promote recovery of neurotoxin-injured dopaminergic systems in animals; although the effects may be less pronounced in aged compared to younger adult animals. Several studies with PD patients have associated vitamin D insufficiency, or variations in the vitamin D receptor, with an increased risk of developing PD (Butler et al., 2011; Evatt et al., 2008; Newmark and Newmark, 2007; Peterson, 2014); and lower levels of vitamin D have also been associated with non-motor symptoms of PD (Jang et al., 2015a; Kwon et al., 2016; Peterson et al., 2013), suggesting a possible link between vitamin D levels and PD. Additionally, a published vitamin D intervention study has demonstrated positive effects of vitamin D supplementation on clinical rating scales in PD patients (Suzuki et al., 2013), providing clinical evidence for a potential therapeutic role for vitamin D or calcitriol. However, not all studies have found an association between vitamin D levels and PD (Petersen et al., 2014; Shrestha et al., 2016). Thus, the link between low levels of vitamin D and PD is controversial, and may only apply to some individuals with PD. However, taken together, the animal studies provide evidence that calcitriol may have protective and restorative effects on damaged dopaminergic neurons, and the clinical studies indicate that in some cases low levels of vitamin D may be associated with PD. These results suggest that additional studies on the effects of calcitriol on the brain are warranted.

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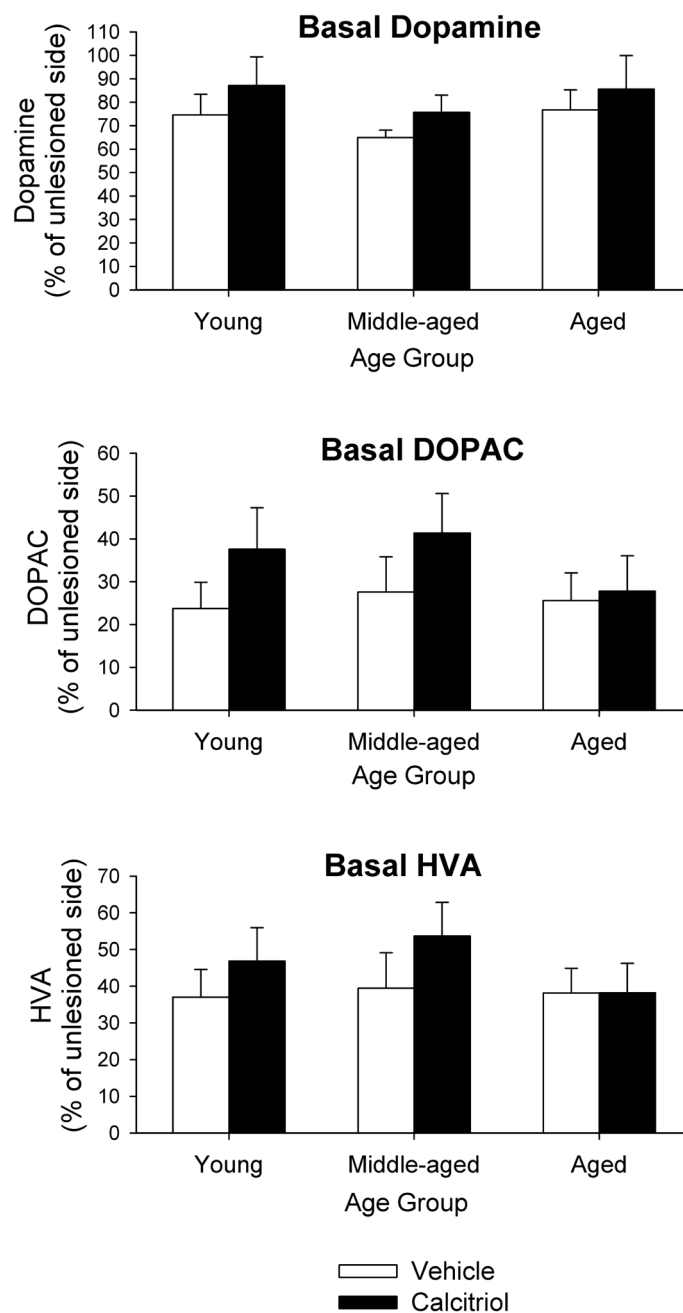
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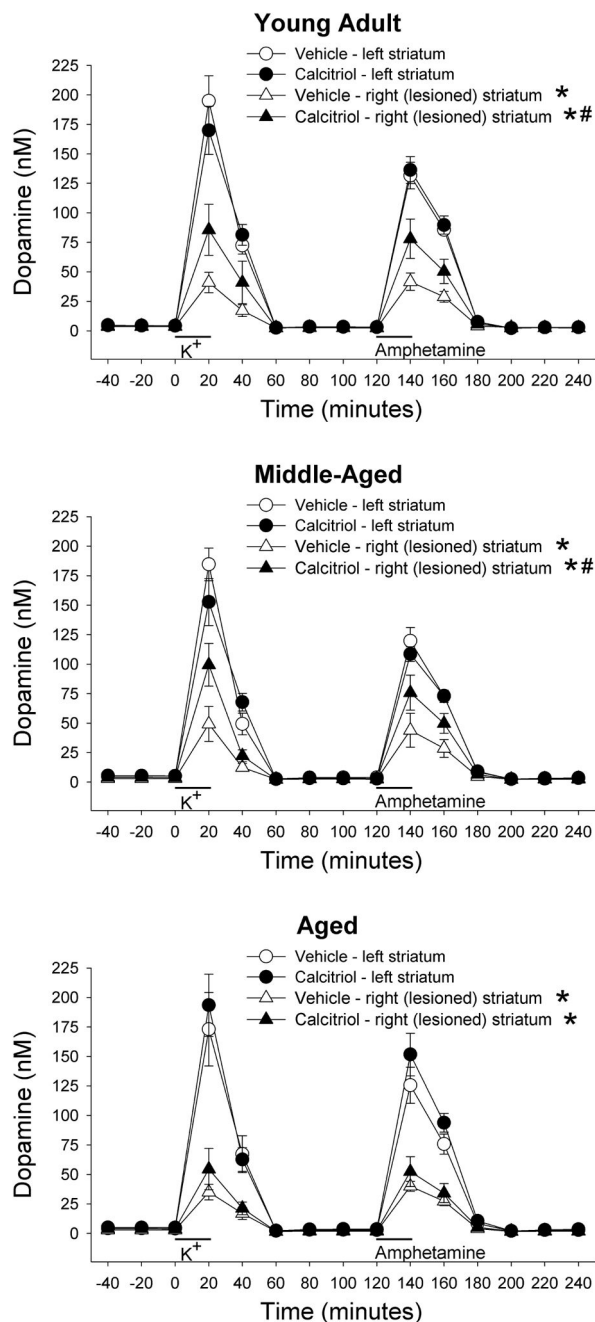
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- The effects of calcitriol were studied in rats previously lesioned with 6-OHDA
- Calcitriol increased dopamine in the striatum of young and middle-aged rats
- Calcitriol did not increase dopamine in the striatum of aged rats
- Calcitriol increased nigral tissue dopamine levels in all three age groups
- The dopaminergic effects of calcitriol are diminished in lesioned, aged rats



**Fig. 1.** Basal dialysate levels of DA, DOPAC and HVA from the striatum of young adult, middle-aged and aged animals injected in the right striatum with 6-OHDA followed four weeks later with eight days of calcitriol treatment. Microdialysis experiments were performed three weeks after the end of the calcitriol injections. The results are expressed as percentage of DA, DOPAC or HVA on the lesioned side compared to the contralateral, unlesioned side. Values shown are mean  $\pm$  SEM from 7–8 animals per group.





**Fig. 2.**

Time course of dialysate levels of DA from the left and right striata of animals injected in the right striatum with 6-OHDA four weeks prior to treatment with vehicle or calcitriol. Microdialysis experiments were performed three weeks after the last vehicle or calcitriol injection. Excess potassium (100 mM) was included in the perfusate for 20-min starting at 0 min (horizontal bar above  $K^+$ ), and 100  $\mu$ M amphetamine was included in the perfusate for 20-min starting at 120 min (horizontal bar above Amphetamine). Values shown are mean  $\pm$  SEM from 7–8 animals per group. \*  $p < 0.01$  vs. left striatum of same group, #  $p < 0.05$  vs. right striatum of vehicle treated group (mixed ANOVA with side of brain and time of

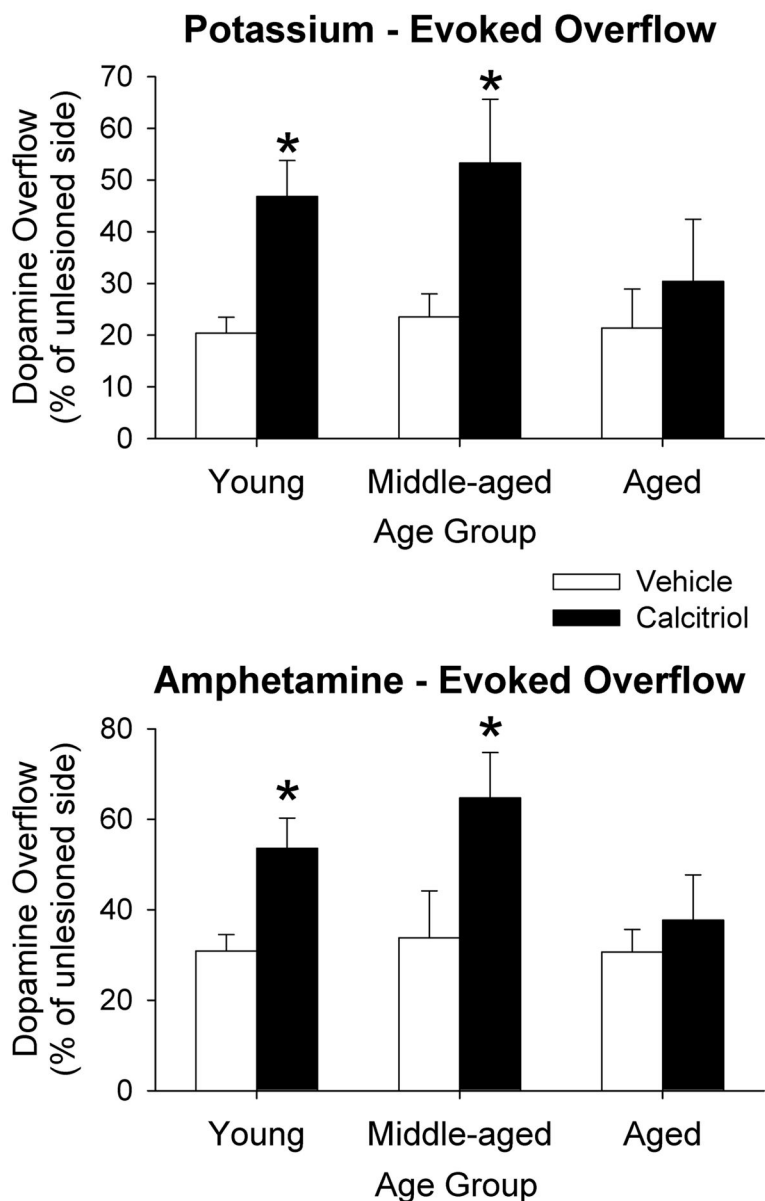
dialysis sample collection as within factors, and treatment group as a between factor; followed by Newman-Keuls post-hoc comparisons).

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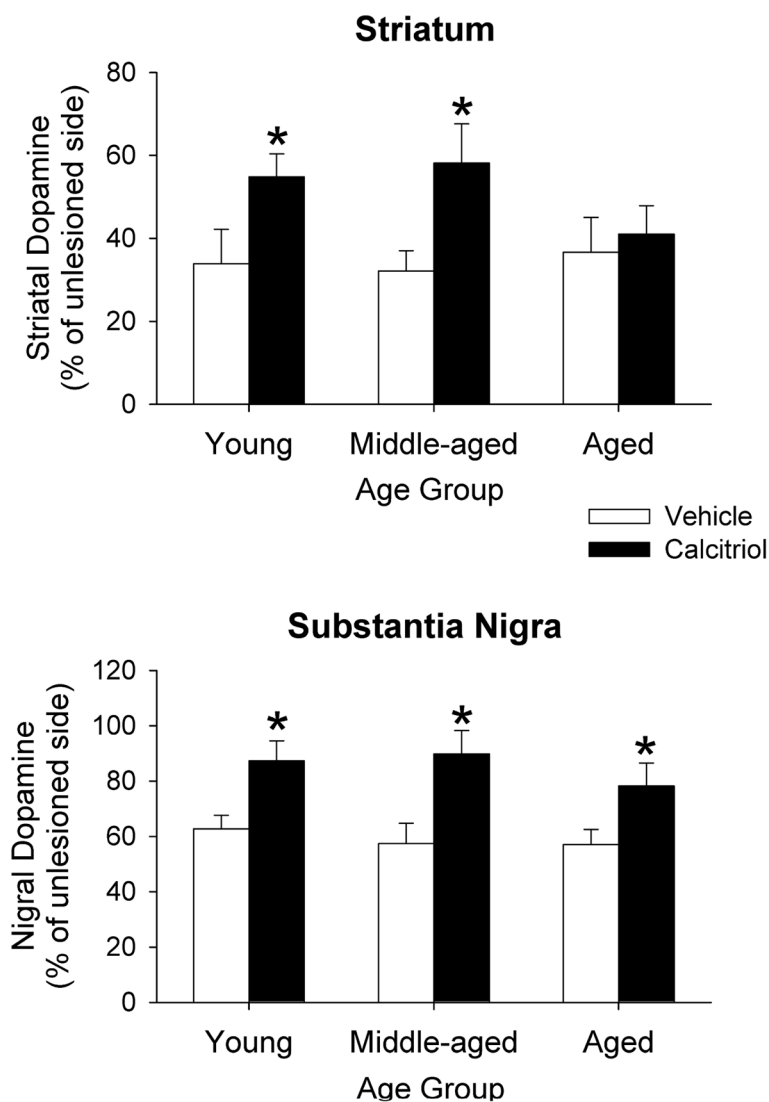
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**Fig. 3.** Potassium-evoked and amphetamine-evoked overflow of DA from the striatum of young adult, middle-aged and aged animals injected in the right striatum with 6-OHDA four weeks prior to treatment with vehicle or calcitriol. Microdialysis experiments were performed three weeks after the last vehicle or calcitriol injection. The results are expressed as percentage of DA overflow on the lesioned side compared to the contralateral, unlesioned side. Values shown are mean  $\pm$  SEM from 7–8 animals per group. \*  $p < 0.05$  vs. vehicle treated group of the same age (two-way ANOVA with age and treatment group as between factors; followed by Newman-Keuls post-hoc comparisons).



**Fig. 4.** Postmortem tissue levels of DA from the striatum and substantia nigra of young adult, middle-aged and aged animals injected in the right striatum with 6-OHDA four weeks prior to treatment with vehicle or calcitriol. Tissue was harvested three weeks after the last vehicle or calcitriol injection. The results are expressed as percentage of striatal or nigral DA on the lesioned side compared to the contralateral, unlesioned side. Values shown are mean  $\pm$  SEM from 7–8 animals per group. \*  $p < 0.05$  vs. vehicle treated group of the same age (two-way ANOVA with age and treatment group as between factors; followed by Newman-Keuls post-hoc comparisons).

**Table 1**

Baseline control values (left side of vehicle treated animals) for the neurochemical parameters examined in the rats used in this study.

Parameter	Age Group		
	Young	Middle-Aged	Aged
Basal DA (nM)	4.77 ± 0.45	5.12 ± 0.44	4.95 ± 0.66
Basal DOPAC (nM)	1631 ± 110	1379 ± 58 *	1175 ± 83 *
Basal HVA (nM)	929 ± 57	844 ± 35	778 ± 39
Potassium-evoked overflow of DA (pg)	950 ± 57	914 ± 117	853 ± 142
Amphetamine-evoked overflow of DA (pg)	776 ± 54	745 ± 73	725 ± 82
Striatal DA content (ng/g tissue wt.)	13,966 ± 846	13,574 ± 757	11,656 ± 677
Nigral DA content (ng/g tissue wt.)	877 ± 75	815 ± 60	776 ± 54

Rats were injected daily with vehicle for 8 consecutive days starting 4 weeks after a right, unilateral 6-OHDA lesion. Microdialysis and tissue harvesting were performed 3 weeks after the vehicle injections. Values are mean ± SEM for 7–8 rats per group.

\*  $p < 0.05$  vs. young animals (one-way ANOVA followed by Newman-Keuls post hoc comparisons).