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# Paraquat exposure reduces nicotinic receptor-evoked dopamine release in monkey striatum

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## Abstract

Paraquat, an herbicide widely used in the agricultural industry, has been associated with lung, liver, and kidney toxicity in humans. In addition, it is linked to an increased risk of Parkinson's disease. For this reason, we had previously investigated the effects of paraquat in mice and showed that it influenced striatal nicotinic receptor (nAChR) expression but not nAChR-mediated dopaminergic function. Since non-human primates are evolutionarily closer to humans and may better model the effects of pesticide exposure in man, we examined the effects of paraquat on striatal nAChR function and expression in monkeys. Monkeys were administered saline or paraquat once weekly for six weeks, after which nAChR levels and receptor-evoked $^{3}$H-dopamine ($^{3}$H-DA) release were measured in striatum. The functional studies showed that paraquat exposure attenuated dopamine (DA) release evoked by α3/α6β2* nAChRs, a subtype present only on striatal dopaminergic terminals, with no decline in release mediated by α4β2* nAChRs, present on both DA terminals and striatal neurons. Paraquat treatment decreased α4β2* but not α3/α6β2* nAChR expression. The differential effects of paraquat on nAChR expression and receptor-evoked $^{3}$H-DA release emphasize the importance of evaluating changes in functional measures. The finding that paraquat treatment has a negative impact on striatal nAChR-mediated dopaminergic activity in monkeys but not mice indicates the need for determining the effects of pesticides in higher species.

## Introduction

Paraquat is an herbicide that is widely used in the agricultural industry but has been linked to lung, liver, and kidney toxicity in humans (Van Vleet and Schnellmann, 2003; Dudka, 2006; Dinis-Oliveira et al., 2008). Epidemiological studies have also implicated paraquat in the etiology of Parkinson's disease (Di Monte et al., 2002; Di Monte, 2003; Brown et al., 2006). In fact, a dose-dependent relationship has been reported between the two, such that the risk for Parkinson's disease increases in parallel with cumulative lifetime exposure to paraquat (Liou et al., 1997).

Although the exact molecular and cellular changes that result in these negative consequences are not entirely clear, previously we demonstrated that chronic paraquat treatment in mice results in alterations in striatal nicotinic acetylcholine receptors (nAChRs) (Khwaja et al., 2007). NAChRs are pentameric ligand-gated ion channels, with two main nAChR subtypes present in the striatum. One is composed of the α3 or α6, and β2 subunits (α3/α6β2*; the asterisk indicates the possible presence of additional subunits). Within the striatum, these nAChRs are located exclusively on the dopaminergic terminals projecting from the substantia nigra

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(Champtiaux et al., 2002; Zoli et al., 2002). The second subtype, α4β2* (containing α4 and β2 subunits, but not α3 or α6), is expressed on both the dopamine (DA) terminals as well as on intra-striatal neurons (Zoli et al., 2002; Luetje, 2004; Salminen et al., 2004). The cholinergic and dopaminergic systems are closely associated in the striatum as demonstrated by co-localization of numerous molecular markers, including cholinergic measures such as choline acetyltransferase, acetylcholinesterase and nAChRs, and dopaminergic markers such as dopamine, the dopamine transporter, and tyrosine hydroxylase (Fuxe et al., 1964; Fonnum, 1973; Zhou et al., 2001; Zhou et al., 2002). A dense network of tonically active cholinergic neurites surrounds dopaminergic neurons and terminals, thereby regulating DA function (Bennett and Wilson, 1999; Zhou et al., 2001; Zhou et al., 2002). Activation of both nAChR subtypes stimulates DA release in the striatum, indicating that the cholinergic system plays a key role in mediating dopaminergic neurotransmission in this region (Wonnacott et al., 2000; Grady et al., 2002; Salminen et al., 2004; McCallum et al., 2005).

Our previous studies in mice demonstrated that chronic paraquat treatment selectively influences α3/α6β2* nAChR expression, whereas there was no effect on α4β2* nAChR levels. Paraquat exposure also did not induce alterations in the functional interaction between either of the nAChR subtypes and the dopaminergic system, as measured by synaptosomal nicotine-evoked [$^3$H]DA release assays (Khwaja et al., 2007). In rodents, α3/α6β2* nAChRs account for ~15% of all striatal nAChRs and mediate ~40% of nAChR-evoked DA release (Kulak et al., 1997; Whiteaker et al., 2000; Quik et al., 2003b; Salminen et al., 2004). However, in non-human primates these numbers are closer to 40 and 70%, respectively (Quik et al., 2001; Quik et al., 2002; McCallum et al., 2005). Thus paraquat may differentially affect the striatal nicotinic receptor system in mice and monkeys. In fact, the existence of numerous other species differences suggests that non-human primates may ultimately be a better model of human pesticide exposure because they more closely resemble humans. Therefore, we have extended the mouse studies to determine the effects of paraquat exposure on striatal nAChR expression and mediation of DA release in a non-human primate model.

## Methods

### Animals

Adult female squirrel monkeys (*Saimiri sciureus*) weighing between 0.5 and 0.7 kg were purchased from Worldwide Primates (Miami, FL) and quarantined for one month according to California State regulations. All care and treatments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at the Parkinson's Institute. Monkeys were housed in a room with a 13:11-h light/dark cycle with food given once daily, and water *ad libidum*. Animals were divided into two treatment groups: control (n=6) and paraquat-treated (n=8). The paraquat group received one subcutaneous injection of 2.5 mg/kg paraquat dichloride hydrate (Sigma, St. Louis, MO) per week for 6 weeks and were euthanized 2 or 4 weeks following the final injection. This treatment paradigm was chosen for several reasons. We used a dose of 2.5 mg/kg paraquat as higher doses induced lung toxicity, as previously reported in other species including humans and mice (Tomita et al., 2007; Dinis-Oliveira et al., 2008). Injections were administered once weekly as in mouse studies (McCormack et al., 2002; Khwaja et al., 2007), and to protect against acute toxicity. General observations of the animals failed to detect any paraquat-induced behavioral abnormalities and thus are not discussed here. Animals were humanely killed two and four weeks after the last injection to allow for sufficient time for paraquat to induce its effects. No statistical differences were observed in results from animals euthanized at the two time points; data were therefore pooled. All animals were euthanized according to the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. The animals were first given an

ip injection of 1.5ml of euthanasia solution (390 mg sodium pentobarbital and 50 mg phenytoin sodium/ml), followed by 2.2 ml/kg iv of the same solution.

### Tissue preparation

Tissue was prepared as previously described (McCallum et al., 2005). Brains were removed and sectioned along the midline. Half was sliced into 6 mm thick blocks in a plexiglass mold, and blocks were quick frozen on glass slides in isopentane on dry ice, and stored at −80°C for future use. These blocks were sectioned (20 μm) on a cryostat (Leica Microsystems Inc., Deerfield, IL, USA), mounted and air-dried on Superfrost Plus™ slides (Fisher Scientific, Pittsburgh, PA, USA), and stored at −80°C for autoradiography experiments. The second half of the brain was sliced into 2 mm blocks. The medial and lateral caudate were dissected out from the region between 15 and 13.5 mm anterior to Bregma (Emmers and Akert, 1963) and used fresh for $^{3}$H-dopamine ($^{3}$H-DA) release assays.

### $^{3}$H-dopamine release

Striatal synaptosome preparation and $^{3}$H-DA release assays were performed as previously described (McCallum et al., 2005). Fresh striatal tissue (~15 mg per region) was homogenized in 2 ml of cold homogenization buffer (0.32 M sucrose, 5 mM HEPES, pH 7.5) and centrifuged at 12,000 g for 20 min. P1 pellets were resuspended in 0.8 ml uptake buffer (128 mM NaCl, 2.4 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 25 mM HEPES, pH 7.5, 10 mM glucose, 1 mM ascorbic acid, 0.01 mM pargyline), and incubated for 10 min. at 37°C. 4 μCi of $^{3}$H-DA (final concentration of 100 nM dopamine; Perkin Elmer Life Sciences, Waltham, MA, USA) was then added and tissue was incubated for another 5 min. An 80 μl aliquot of synaptosomes (approximately 0.5–2 mg tissue) was placed on 5 mm diameter A/E glass-fiber filters (Gelman Instruments Co., Ann Arbor, MI, USA) and perfused with uptake buffer also containing 0.1% bovine serum albumin and 10 μM nomifensine for 10 min at a rate of 1 ml/min before fraction collection was initiated. Following 54 sec of sample collection, tissue was stimulated with either nicotine (0.03–30 μM) or 20 mM $K^+$ buffer for 18 sec. When the antagonist α-conotoxinMII (50 nM) was used, tissue was pre-exposed for 3 min prior to agonist stimulation. For each tissue aliquot 15 fractions (18 sec each) were collected, including basal release before and after stimulation. Econosafe scintillation cocktail (Research Products International Corp., Mount Prospect, IL, USA) was added to the fractions and radioactivity was counted on a Beckman Coulter LS6500 scintillation counter (Fullerton, CA, USA).

### $^{125}$I-epibatidine autoradiography

$^{125}$I-epibatidine (Amersham Biosciences, Piscataway, NJ, USA) binding was performed as described (Kulak et al., 2002). Briefly, thawed sections were preincubated for 30 min at RT in Tris buffer (50 mM pH 7.5, containing 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1 mM $MgCl_2$. α-ConotoxinMII (100 nM) was used to distinguish between $^{125}$I-epibatidine binding to α3/α6β2* (α-conotoxinMII-sensitive) and α4β2* (α-conotoxinMII-resistant) nAChRs. Nicotine (100 μM) was used to define non-specific binding. When used, these compounds were added to both preincubation and incubation buffers. Sections were incubated with 0.015 nM $^{125}$I-epibatidine buffer for 40 min at RT, washed 2 × 5 min in 4°C buffer, and dipped in ice-cold water. They were air-dried and exposed to Kodak Biomax MR film with $^{125}$I standards, for several days.

### $^{125}$I-α-ConotoxinMII autoradiography

$^{125}$I-α-ConotoxinMII was synthesized and radiolabeled as described (Whiteaker et al., 2000), and binding was performed as previously reported (Quik et al., 2001). HEPES buffer (20 mM) containing 144 mM NaCl, 1.5 mM KCl, 2mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.5, was used throughout the experiment. Thawed sections were preincubated at RT for 15 min in buffer plus

0.1% bovine serum albumin and 1 mM phenylmethylsulfonyl fluoride. Sections were then incubated in buffer that included 0.5nM $^{125}$I-α-conotoxinMII, 0.5% BSA, 5 mM EDTA, 5 mM EGTA, and 1 μl/ml each of aprotinin, leupeptin, and pepstatin A. Nicotine (100 μM) was used to define non-specific binding and when used, was added to both preincubation and incubation buffers. Sections were washed with 1X HEPES buffer plus 0.1% bovine serum albumin buffer for 10 min at RT and then 4°C, followed by 2 × 10 min in 4°C 0.1X buffer, and dipped in 4° C water twice. They were air-dried and exposed to Kodak Biomax MR film with $^{125}$I standards for several days.

### Data Analysis

$^{3}$H-DA release was quantified as previously described (McCallum et al., 2005). Release was plotted as counts per minute (cpm) versus fraction number using a curve-fitting algorithm in SigmaPlot 5.0 for MS-DOS (Jandel Scientific, San Rafael, CA, USA). Fractions before and after the peak were selected to calculate basal release by plotting values as a single exponential decay function. Baseline was subtracted out, and fractions above 10% of baseline were added to obtain evoked release. This value was then normalized to wet tissue weight per filter to obtain cpm/mg tissue. α4β2* and α3/α6β2* nAChR components of release were discriminated by the addition of α-conotoxinMII to perfusion buffer. Release remaining in the presence of α-conotoxinMII was mediated by α4β2* nAChRs. The α3/α6β2* nAChR-mediated component was determined by subtraction of the α4β2* component from total release. $R_{max}$ and $EC_{50}$ values for dose-response curves were calculated by non-linear regression equations in GraphPad Prism (GraphPad Software Co., San Diego, CA, USA).

The ImageQuant program (GE Healthcare, Sunnyvale, CA) was used to obtain optical density values from autoradiographic films. Specific binding was calculated by subtracting background tissue levels from total binding and these values were converted to fmol/mg tissue using standard curves generated from $^{125}$I radioactivity standards. The binding value for each brain region for each animal was obtained from at least two independent experiments with 1–2 sections per experiment. Data is reported as mean ± SEM of 6–8 animals. Sample optical density readings were within the linear range of the film.

### Statistical Analysis

All statistical analyses were performed in GraphPad Prism. Statistical comparisons were conducted using either one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test, or two-way ANOVA followed by Bonferroni post hoc test. A level of $p < 0.05$ was considered significant for all analyses. All data are expressed as mean ± SEM for indicated number of animals.

## Results

### Paraquat treatment selectively reduces α3/α6β2* nAChR-mediated $^{3}$H-DA release from medial and lateral caudate synaptosomes

We first examined the effects of paraquat treatment on nicotine-evoked $^{3}$H-DA release from medial and lateral caudate synaptosomes. Both of these regions were tested because previous work has shown that the medial and lateral caudate are differentially affected by toxic insults with the lateral caudate more affected (Kish et al., 1988; Alexander et al., 1992; Moratalla et al., 1992; McCallum et al., 2005; McCallum et al., 2006). Experiments were performed in the presence and absence of the α3/α6β2*-selective antagonist α-conotoxinMII in order to distinguish between DA release mediated by the two main nAChR subtypes (α3/α6β2* and α4β2*) located on presynaptic DA terminals in the striatum. α4β2* nAChR-mediated nicotine-evoked $^{3}$H-DA release was defined as that remaining in the presence of α-conotoxinMII. The

difference between total and α4β2*-mediated release represented α3/α6β2* receptor-stimulated release.

Paraquat treatment decreased nicotine-evoked α3/α6β2*-mediated $^{3}$H-DA release from medial and lateral caudate synaptosomes (Fig. 1). The synaptosomal nicotine dose response curve from paraquat-exposed animals was significantly lower than that from synaptosomes from control monkeys ($p < 0.05$ and $p < 0.001$ in medial and lateral caudate respectively). In contrast, paraquat did not affect nicotine-evoked α4β2* nAChR-mediated $^{3}$H-DA release in either region, although there was a trend towards an increase in release in lateral caudate (Fig. 2). Paraquat also did not alter $EC_{50}$ values for either nicotine-evoked α3/α6β2* or α4β2* nAChR-mediated release in either region (Table 1).

### α3/α6β2* and α4β2* nAChR expression is differentially affected by paraquat treatment

We utilized two autoradiographic approaches to determine the effects of paraquat treatment on α3/α6β2* nAChRs. First, $^{125}$I-α-conotoxinMII binding was used to provide a direct measure of α3/α6β2* nAChR binding sites in the caudate. Paraquat exposure did not change α3/α6β2* nAChR expression levels in either medial or lateral caudate (Fig. 3A, B). We also examined $^{125}$I-epibatidine binding in the presence and absence of 100 nM α-conotoxinMII to assess α3/α6β2* nAChR binding sites since previous results suggest that the α3/α6β2* nAChR sites measured using this approach are not identical to those measured using $^{125}$I-α-conotoxinMII (Quik et al., 2003a; Khwaja et al., 2007). Paraquat treatment did not alter $^{125}$I-epibatidine binding to α3/α6β2* nAChRs (α-conotoxinMII-sensitive) in either the medial or lateral caudate (Fig. 3C, D), consistent with results using $^{125}$I-α-conotoxinMII.

Paraquat produced a significant reduction in α-conotoxinMII-resistant $^{125}$I-epibatidine binding in both medial (13.0%, $p < 0.05$) and lateral caudate (12.5%, $p < 0.05$), indicating a decline in α4β2* nAChR expression (Fig. 4).

## Discussion

The present results are the first to show that paraquat exposure reduces nAChR-evoked dopaminergic activity in monkey striatum. This appears to be due to a decrease specifically in α3/α6β2* nAChR-mediated DA release with no change in function of α4β2* nAChRs located on nigrostriatal terminals. α3/α6β2* nAChRs are responsible for the majority of nAChR-evoked DA release (~70%), and thus play a key role in regulation of striatal dopaminergic activity (Kulak et al., 1997; Quik et al., 2001; Quik et al., 2002; McCallum et al., 2005). The observed changes in synaptosomal α3/α6β2* nAChR-mediated DA release may therefore be indicative of substantial paraquat-induced alterations in overall striatal dopaminergic function *in vivo*.

In our previous studies in mice, paraquat exposure influenced only the expression of striatal α3/α6β2* nAChRs, with no change in receptor-mediated function (Khwaja et al., 2007). This is in contrast to our current results in monkey caudate that demonstrate paraquat-induced changes in α3/α6β2* nAChR function but not expression. In rodents, α3/α6β2* nAChRs account for ~15% of all striatal nAChRs and mediate ~40% of nAChR-evoked DA release, compared to 40% and 70% respectively in monkeys (Kulak et al., 1997; Whiteaker et al., 2000; Quik et al., 2001; Quik et al., 2002; Quik et al., 2003b; Salminen et al., 2004; McCallum et al., 2005). These differences in relative proportions of the main striatal nAChR subtypes may play a role in the differential effects of paraquat exposure we observed in the two species. There are also numerous other species differences between monkeys and mice that may contribute to these differential results. Given that non-human primates more closely resemble humans than do rats, the paraquat-induced alterations in monkey striatum that we report here

may better model functional changes in the human striatum that arise as a consequence of paraquat exposure.

The present results show that changes in nAChR subtype-mediated DA release do not correlate with alterations in expression levels of the corresponding receptor subtype. The discrepancies between the observed changes in nAChR subtype function and expression following paraquat exposure may be a reflection of several factors. First, the decrease in α4β2* nAChR numbers in the absence of a corresponding reduction in α4β2* nAChR-mediated DA release from striatal synaptosomes may be due to a loss of receptors located on striatal cell bodies as opposed to nigrostriatal DA terminals. Such a change would not be detectable by our functional assays, which utilize only isolated nerve terminals. However, the observed decrease in α4β2* nAChRs may lead to alterations in DA release via an indirect pathway that would only be evident in experimental preparations that retain striatal circuitry. Additionally, it is not uncommon for receptor binding and functional assays to yield disparate results because of inherent experimental differences between these two techniques. For instance, radioligand-binding studies typically quantify both intracellular and extracellular receptors regardless of their functionality, and binding assay conditions favor desensitized receptors. In contrast, functional measures reflect only activatable membrane-bound receptors. The use of pharmacologically distinct ligands employed in the two techniques may also contribute to the apparent inconsistencies in paraquat-induced alterations in nAChR subtype function versus expression because specific ligands may differentially interact with different subpopulations of the α3/α6β2* and α4β2* nAChR subtypes. Our results emphasize the importance of performing functional studies which may be more reflective of the ultimate behavioral consequences of nigrostriatal damage.

In conclusion, paraquat treatment significantly reduced α3/α6β2* nAChR-mediated $^{3}$H-DA release in monkey medial and lateral caudate, as well as α4β2* nAChR expression. These data suggest that chronic paraquat may modulate nicotinic receptor-mediated function resulting in a consequent decline in dopaminergic neurotransmission. Such a mechanism may be linked to the increased risk of neurological disorders, such as Parkinson's disease, associated with environmental exposure to this herbicide.

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## ABBREVIATIONS

**ANOVA**
: analysis of variance

**DA**
: dopamine

**nAChR**
: nicotinic acetylcholine receptor

*****
: denotes nicotinic receptors containing the indicated α and β subunit and possible additional subunits

**α3/α6β2***

refers to a nAChR that is composed of the α3 or α6 subunits, and β2

**α4β2***

refers to a nAChR containing the α4 and β2 subunits, but not α3 or α6

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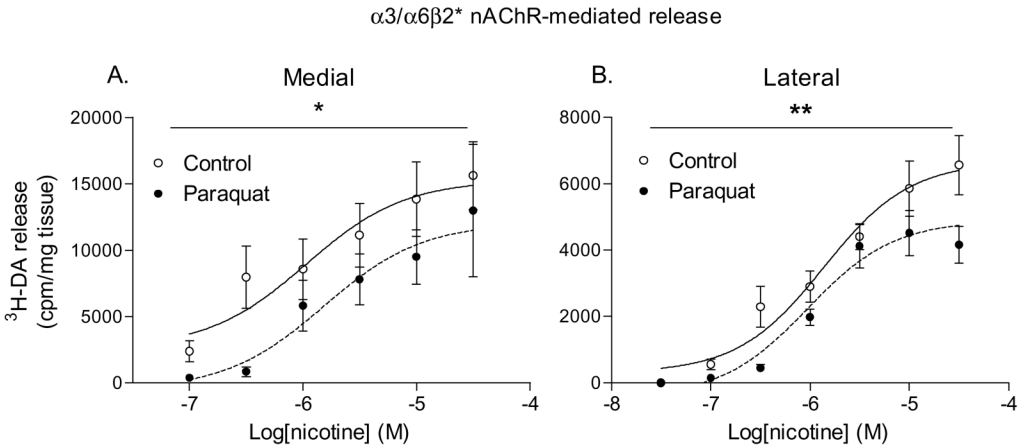


**Fig. 1.**

Paraquat exposure reduced nicotine-evoked α3/α6β2*-mediated $^3$H-DA release from medial (A) and lateral (B) caudate synaptosomes. Animals were administered 2.5 mg/kg paraquat once weekly for six weeks. The α3/α6β2* nAChR-selective antagonist, α-conotoxinMII, was used to define α3/α6β2* nAChR-evoked $^3$H-DA release as described in Results. Dose response curves from the paraquat group were significantly different from control, * $p < 0.05$, ** $p < 0.001$ (two-way ANOVA). Curves were generated by non-linear regression analysis in GraphPad Prism. All values represent mean ± SEM of 6–8 animals.

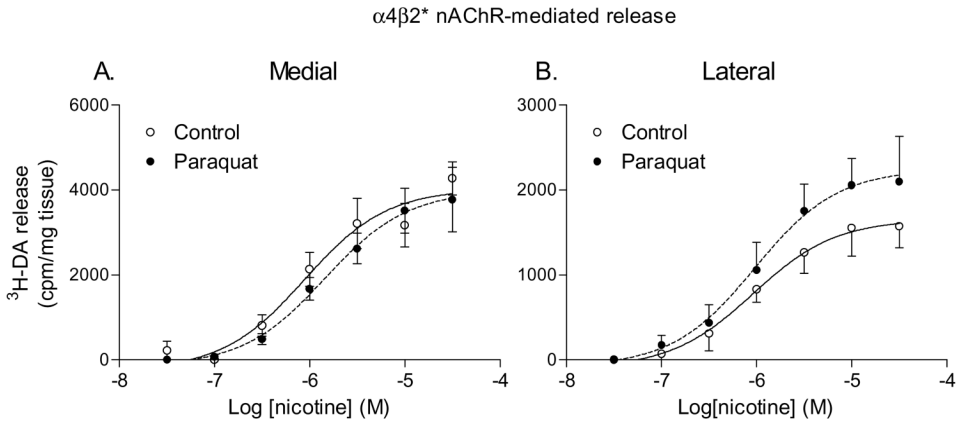


**Fig. 2.**

Paraquat exposure did not affect nicotine-evoked α4β2* nAChR-mediated $^3$H-DA release. Paraquat treatment did not alter α4β2* nAChR-mediated $^3$H-DA release from either medial (A) or lateral (B) caudate synaptosomes. Animals were administered 2.5 mg/kg paraquat once weekly for six weeks. α4β2* nAChR-mediated release was determined as described in Results. Dose response curves were generated by non-linear regression analysis in GraphPad Prism. All values represent mean ± SEM of 6–8 animals.

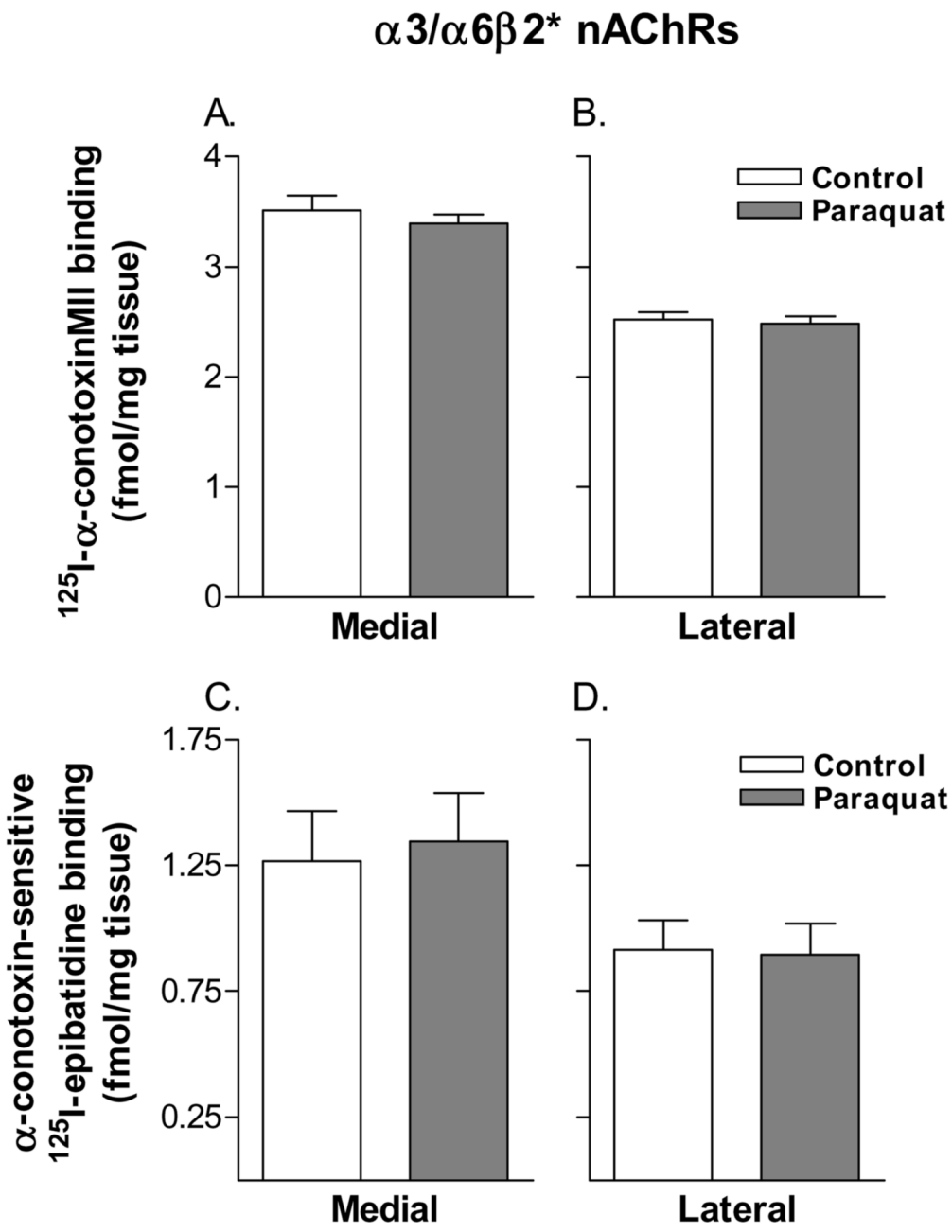


**Fig. 3.**

Paraquat exposure did not change α3/α6β2* nAChR expression in the caudate. Paraquat treatment did not significantly alter binding of the α3/α6β2* nAChR-selective ligand $^{125}$I-α-conotoxinMII in medial (A) or lateral (B) caudate. It also did not affect α-conotoxinMII-sensitive $^{125}$I-epibatidine binding, an additional measure of α3/α6β2* nAChRs, in medial (C) or lateral (D) caudate. All values represent mean ± SEM of 6–8 animals per group.

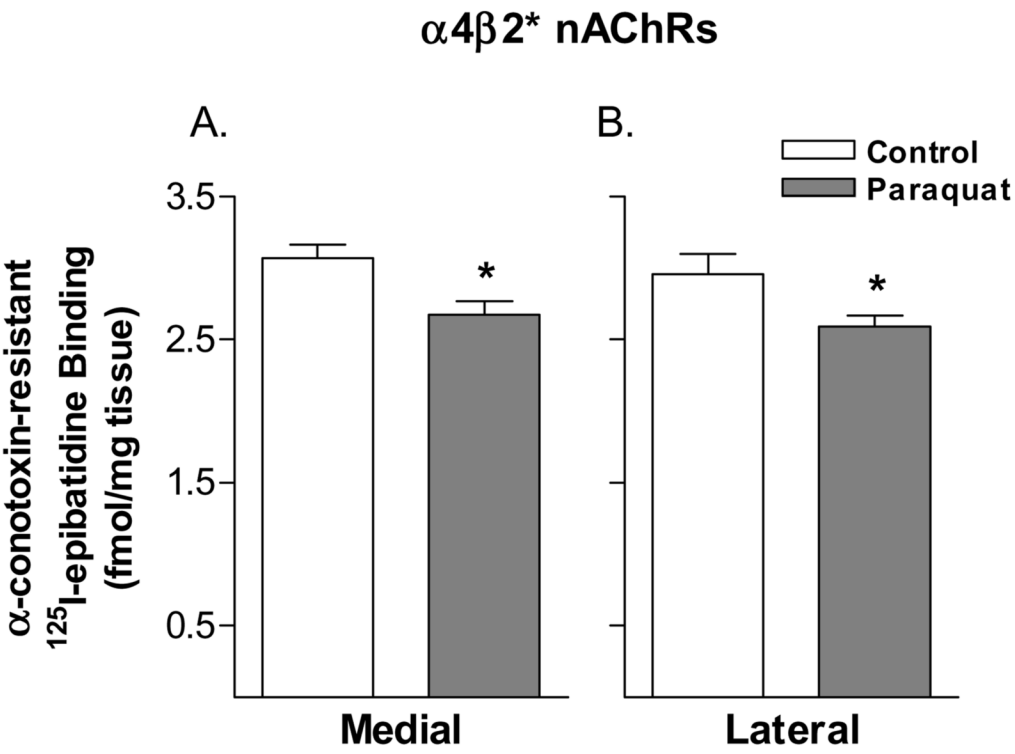


**Fig. 4.**

Paraquat treatment reduced α4β2* nAChR expression in the medial and lateral caudate (A. and B. respectively). α4β2* nAChR expression was measured as the level of $^{125}$I-epibatidine binding remaining in the presence of the α3/α6β2* nAChR-selective antagonist α-conotoxinMII. Significance of difference from control, * $p < 0.05$ (one-way ANOVA followed by Dunnett's post hoc test). All values represent mean ± SEM of 6–8 animals per group.

## TABLE 1

$EC_{50}$ values for α3/α6β2* and α4β2* nAChR-mediated $^{3}$H-DA release from medial and lateral caudate synaptosomes do not change with paraquat treatment

| | | $EC_{50}$ (CI) μM | |
|---|---|---|---|
| **Subtype** | **Region** | **Control** | **Paraquat** |
| α3/α6β2* | Medial | 0.94 (0.15–5.78) | 1.67 (0.26–10.8) |
| | Lateral | 1.38 (0.57–3.36) | 0.95 (0.30–3.01) |
| α4β2* | Medial | 0.89 (0.33–2.39) | 1.45 (0.50–4.19) |
| | Lateral | 0.92 (0.30–2.83) | 0.96 (0.24–3.90) |

$EC_{50}$ and confidence interval (CI) values were derived from non-linear regression analysis using GraphPad Prism. Data are from 6–8 animals.