Neuropsychopharmacology (2007) 32, 919–931
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www.neuropsychopharmacology.org

Amphetamine Sensitization Alters Dendritic Morphology in Prefrontal Cortical Pyramidal Neurons in the Non-Human Primate

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Amphetamine (AMPH) sensitization in the nonhuman primate induces persistent aberrant behaviors reminiscent of the hallmark symptoms of schizophrenia, including hallucinatory-like behaviors, psychomotor depression, and profound cognitive impairment. The present study examined whether AMPH sensitization induces similarly long-lasting morphologic alterations in prefrontal cortical pyramidal neurons. Three to $3\frac{1}{2}$ years postsensitization, sensitized, and AMPH-naïve control monkeys were killed. Blocks of prefrontal cortex were Golgi-impregnated for elucidation of pyramidal dendritic morphology in layers II/superficial III (II/IIIs), deep III, and V/VI. In AMPH-sensitized animals as compared to AMPH-naïve controls, pyramidal dendrites in layer II/IIIs exhibited reduced overall dendritic branching and reduced peak spine density (22%) on the apical trunk. Across all layers, the distance from soma to peak spine density along the apical trunk was decreased ($126.38 \pm 7.65\,\mu m$ in AMPH-sensitized compared to $162.98 \pm 7.26\,\mu m$ in AMPH-naïve controls), and basilar dendritic length was reduced (32%). These findings indicate that chronic dopamine dysregulation, consequent to AMPH sensitization, results in enduring, atrophic changes in prefrontal pyramidal dendrites that resemble the pathologic alterations described in patients with schizophrenia and may contribute to the persistence of schizophrenia-like behavioral changes and cognitive dysfunction associated with sensitization. These findings may also provide key insights into the etiologic origin of the pronounced behavioral disturbances and cognitive dysfunction associated with schizophrenia.
Neuropsychopharmacology (2007) **32,** 919–931. doi:10.1038/sj.npp.1301179; published online 16 August 2006

Keywords: dopamine; schizophrenia; amphetamine sensitization; Golgi impregnation; working memory

INTRODUCTION

Subchronic exposure to amphetamine (AMPH) in the nonhuman primate induces behavioral sensitization such that a subsequent acute AMPH challenge results in augmented expression of fine-motor stereotypies, static posturing, and hallucinatory-like behaviors as compared to the behavioral responses elicited by the same dose of AMPH prior to sensitization (Castner and Goldman-Rakic, 1999, 2003; Castner *et al*, 2000). We have documented that behavioral sensitization is still present more than 3 years following termination of the sensitizing AMPH regimen, suggesting that the altered behavioral sensitivity in these animals is seemingly 'permanent.' Moreover, the responses to acute AMPH challenge become more 'intense' over time in the postwithdrawal phase (Castner and Goldman-Rakic, 1999). Notably, AMPH sensitization profoundly impairs spatial working memory performance as measured by performance on the Delayed Response Task, and this spatial working memory deficit appears to be associated with a significant reduction in dopamine turnover in both the dorsolateral prefrontal cortex and striatum (Castner *et al*, 2005). Further evidence that the prefrontal cortex plays a critical role in behavioral sensitization is demonstrated by our finding that lesions of the dorsal and lateral prefrontal cortex performed prior to AMPH sensitization block augmentation of hallucinatory-like behaviors in response to an AMPH challenge (Castner and Goldman-Rakic, 2003). Thus, our findings suggest that neurochemical and/or structural alterations of primate prefrontal cortex may be instrumental in the development of psychotomimetic behaviors and cognitive deficits in response to prolonged dopamine dysregulation as produced by the AMPH sensitizing regimen.

Studies in rodents have explored in depth the mechanisms underlying behavioral sensitization to AMPH and other psychomotor stimulants and have highlighted the

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*Deceased 31 July 2003.
Received 29 September 2005; revised 6 June 2006; accepted 7 July 2006
Online publication: 12 July 2006 at http://www.acnp.org/citations/Npp071206050596/default.pdf

importance of intermittent *vs* continuous drug exposure regimens for producing behavioral sensitization (Robinson and Becker, 1986). Recently, morphometric analyses in rodents have shown that AMPH sensitization induces marked alterations in the dendritic structure of neurons receiving midbrain, dopaminergic inputs (Robinson and Kolb, 1997, 1999; Li *et al*, 2003). At intervals of up to 3.5 months postsensitization, morphologic changes, including increased dendritic length and spine density, have been observed in medium spiny neurons of the nucleus accumbens and caudate-putamen, as well as in the apical dendritic arbors of pyramidal neurons in medial prefrontal cortex. These seminal anatomic findings indicate that the long-lasting effects on behavior consequent to repeated, intermittent AMPH exposure may be mediated at least in part by structural reorganization of synaptic connectivity.

If chronic dysregulation of the dopaminergic system were to have deletorious morphologic consequences for neurons in the prefrontal cortex, this would have important implications for the pathophysiology of schizophrenia. With the discovery of antipsychotic drugs in the 1950s, the pharmacotherapy of schizophrenia has been directed primarily toward the dopamine system. Indeed, the therapeutic efficacy of antipsychotic drugs for the amelioration of positive symptoms has been directly linked to their potency at D2-type dopamine receptor sites (Seeman, 1992). However, evidence linking disturbances in dopamine neurotransmission to structural pathology in the brain has been lacking. For example, post-mortem studies of the prefrontal cortex from patients with schizophrenia patients have described reductions in spine density, dendritic length, and complexity in the basilar dendrites of pyramidal cells in layers III and V (Garey *et al*, 1998; Glantz and Lewis, 2000; Kalus *et al*, 2000; Broadbelt *et al*, 2002; Black *et al*, 2004). However, it is not known whether there is a causal effect between the purported neurochemical imbalance in the dopaminergic innervation of the prefrontal cortex and the observed abnormalities in dendritic architecture of pyramidal cells in this region.

In this study, we examined the morphology of prefrontal cortical pyramidal neurons in AMPH-sensitized nonhuman primates as compared to AMPH-naïve controls 3–$3\frac{1}{2}$ years following repeated AMPH exposure. We hypothesized that the persistent aberrant behaviors and profound cognitive dysfunction induced by AMPH sensitization, particularly in the realm of spatial working memory, might be mediated at least in part by alterations in the spinodendritic morphology of pyramidal cells in the prefrontal cortex.

MATERIALS AND METHODS

A total of 11 young adult (5–10 years of age) rhesus macaques (*Macaca mulatta*) were included in this study. With the exception of one individual, all monkeys experienced some form of pair or group housing during their lifetimes. The one exception was a control monkey who, although singly-housed, experienced the same sensory environment and interaction with animal caretaking staff, investigators, and veterinary staff as pair/group-housed members of the primate colony.

Five monkeys (3 females, 2 males; all 5–6 years of age during treatment) received intermittent, escalating doses of AMPH (0.1–1.0 mg/kg; i.m.; b.i.d. with weekends off) for either a 6-week ($N=2$) or 12-week ($N=3$) period, and six monkeys (4 females, 2 males) served as AMPH-naïve controls. For the duration of the sensitization and postsensitization periods, all animals in both groups were singly housed in the Yale primate colony and maintained on a 12 : 12 light–dark cycle. The monkeys' appetites during sensitization were closely monitored and supplemented as necessary. Data for behavioral sensitization and cognitive deficits induced by repeated AMPH exposure have been previously reported (Castner and Goldman-Rakic, 1999, 2003; Castner *et al*, 2000, 2005). It is noteworthy that animals treated with sensitizing regimens of AMPH for either 6 or 12 weeks showed significant behavioral sensitization to the same acute challenge dose (0.4 mg/kg, i.m.) of AMPH (Castner and Goldman-Rakic, 1999; Castner *et al*, 2000) and exhibited the same neurochemical alterations in the dorsolateral prefrontal cortex (Castner *et al*, 2005). Approximately, 3–$3\frac{1}{2}$ years postsensitization, animals were killed via intracardial perfusion. Housing, daily care, experimental treatment, enrichment, and killing of the animals were performed in accordance with Yale Animal Use and Care Committee and federal guidelines for nonhuman primates.

Perfusion and Brain Dissection

Monkeys were sedated with ketamine and atropine and then overdosed with sodium pentobarbital (100 mg/kg; i.v.). The chest cavity was opened, and a catheter was inserted into the left ventricle to infuse a solution of ice-cold Ringer's buffer. The brain was immediately removed from the skull, and small blocks of dorsolateral prefrontal area 9 were dissected for Golgi impregnation (Figure 1).

Histologic Processing

Cortical tissue blocks were trimmed to approximately 1 cm^2 on face but no thicker than 0.5 cm and processed with a modified Rapid Golgi method. according to the following procedure. Tissue blocks were postfixed for 2 h in 4% paraformaldehyde in phosphate-buffered saline. The blocks were then transferred to a freshly prepared aqueous solution of 2.5% potassium dichromate and 0.2% osmium tetroxide for 3–5 days in the dark. Subsequently, the blocks were washed several times with 0.75% silver nitrate and reacted in this same solution for 24–48 h in the dark. The blocks were then dehydrated through increasing concentrations of ethanol, embedded in celloidin, and sectioned at 120 μm. Sections were mounted on slides with Permount, coverslipped, and air-dried on a flat surface.

Microscopy and Neurolucida Drawing

Sections were examined on a Zeiss Akioskop microscope that had been custom fitted to a Neurolucida system for computer-aided, quantitative microscopy (MicroBrightfield, Williston, VT); drawing and analysis were performed using the Neurolucida software package (ver. 5.05.4). All analyses were performed by one observer (AB) who was blind to the

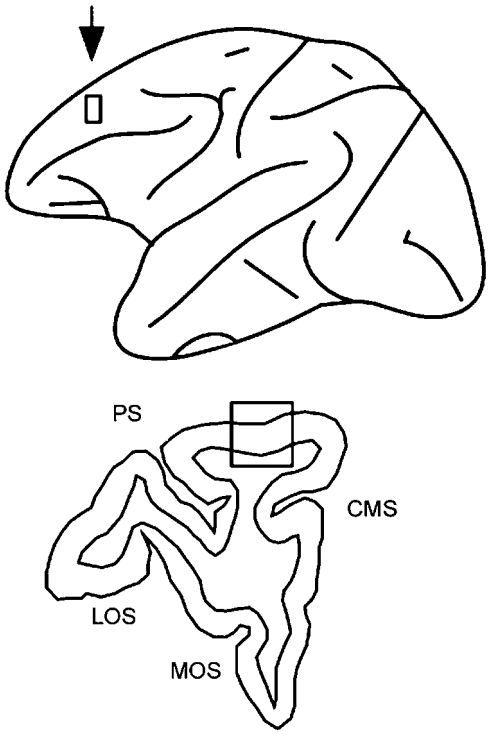

Figure 1 Schematic drawings of the macaque brain showing the dissection of area 9 for Golgi analysis. Arrow and boxes indicate the location of the dissected block on the lateral surface of the hemisphere (above) and on a coronal section (below). CMS, callosomarginal sulcus; LOS, lateral orbital sulcus; MOS, medial orbital sulcus; PS, principal sulcus.

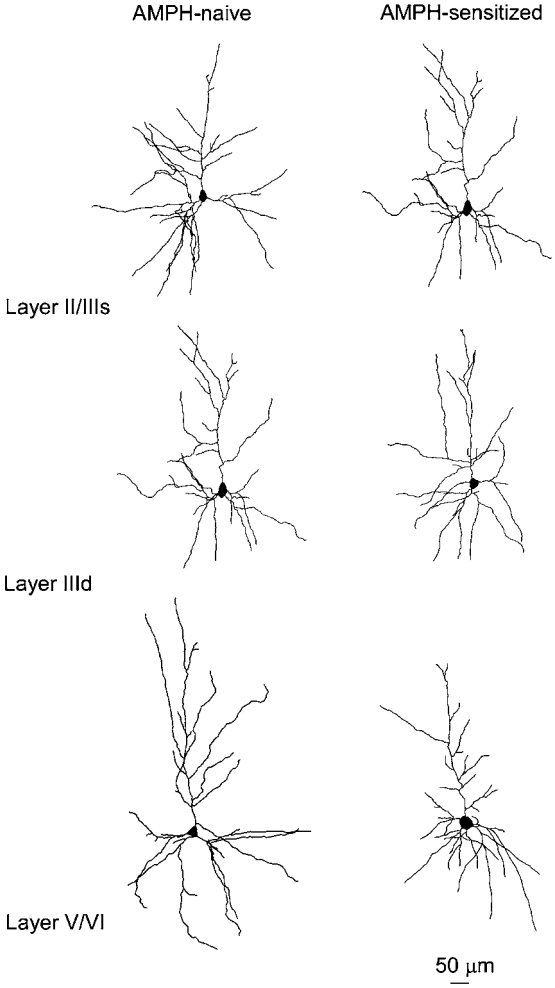

Figure 2 Drawings of representative pyramidal neurons in layers II/IIIs (top), IIId (middle) and V/VI (bottom) illustrate dendritic complexity and length of apical and basilar dendritic processes of the dendritic tree. Scale bar = 50 μm.

experimental treatment status of the animals. At low power (× 350), the sections were outlined, and the position of Golgi-impregnated neurons was indicated on the section drawing. Pyramidal neurons were identified by their characteristic pyramidal somal shape and by the pattern of their dendritic arborization, that is, one primary apical dendrite extending outward from the apex of the soma and multiple basilar dendrites emanating from the base of the soma. The depth of the cell soma from the pial surface was measured, and cells were categorized as residing in one of three layers based on previous cytoarchitectural analyses in monkey prefrontal cortex (Selemon *et al*, 1999): layer II/superficial layer III (IIIs) (250–650 μm), deep layer III (IIId) (700–1000 μm), or layers V/VI (>1100 μm). At higher magnification (× 1500), pyramidal cells were drawn for Sholl analysis and measurement of dendritic length (Figure 2).

Five pyramidal cells from each of the three layers in each monkey were selected on the basis of the following criteria. (1) The apical trunk could be traced for at least 150 μm from the soma. (2) At least two basilar dendrites were present. (3) Basilar and apical branch dendritic processes were well impregnated and extended to a natural tapered ending. (4) Dendrites were not obscured by overlying glial processes, other neuronal processes, or artifact. Another five cells per layer per case were selected for analysis of spine density. Apical trunk dendrites, apical branch dendrites, and basilar dendrites (Figure 2) were drawn at high magnification (× 1500). Five pyramidal cells in each layer that exhibited a well-impregnated apical trunk that extended for at least 100 μm distal to the peak density were selected, and the apical trunk dendrites were drawn; an additional five in each layer with well impregnated apical branch dendrite and another five in each layer with well impregnated basilar dendrites were selected, and these processes were drawn. Spines extending perpendicularly outward from the parent dendritic process were drawn. As has been described by others (Feldman and Peters, 1979), this represents an underestimation of the total number of spines as those that extend downward or upward in the plane of the section were obscured by the shaft of the dendrite and therefore were not counted. Cases were excluded from the analysis of spine density of specific processes if five well-impregnated and unobscured dendrites were not found (two control cases for apical trunk spine density in layer II/IIIs, one AMPH-sensitized monkey for apical trunk spine density in layer V/VI, and one AMPH-sensitized monkey for apical branch spine density in layer V/VI).

Sholl Analysis

The number of intersections with concentric circles placed 50, 100, and 150 μm from the cell soma was calculated as a

measure of dendritic complexity, an index that roughly corresponds to the number of dendritic branches.

Analysis of Dendritic Length

The sum of all apical dendritic segments within 150 μm of the cell soma was calculated. As very few apical dendritic trees were present in entirety within the 100-μm thick section, analysis of dendritic length was limited to a 150 μm radius from the soma in order to avoid skewing by apical trunk dendrites that remained within the section for longer distances. The sum of all basilar dendritic segments regardless of distance from the cell soma was calculated.

Analysis of Spine Density

Spine density is expressed as number of spines per micron of dendritic segment. For apical dendritic trunks, spine density was averaged over 25 μm lengths of the trunk starting at the somal origin and extending distally. As spine density varied considerably along the length of the dendritic trunk (Figure 3), the points of peak spine density for the five neurons in each layer were aligned. Spine densities 50 μm proximal to the peak, at peak, 50 μm distal to the peak, and 100 μm distal to the peak were then averaged for each case. For apical dendrite branches, spine densities were measured along secondary (2°), tertiary (3°), and 4th order (4°) branches. Likewise, spine densities along all primary (1°), (2°), and (3°) branches of basilar dendrites were measured. Spine density from five neurons in each layer was then averaged for each case.

Figure 3 Photomontage of the apical dendritic trunk of a layer II pyramidal cell. Note that spine density proximal to the soma is very low, increases to peak density approximately 150 μm from the soma, and decreases progressively distal to the peak. Box indicates region shown at high power in Figure 6a. Scale bar = 20 μm.

Statistical Analysis

Data used for statistical analysis represented mean values for each animal, that is, mean of measurements from five neurons per layer. Data were checked for normality prior to analysis using normal probablility plots and Kolmogorov–Smirnov tests. No transformation was necessary. Effects significant at the 0.05 level were considered statistically meaningful except where noted below. All measurements are expressed as mean ± standard error of the mean.

For data collected from the Sholl analysis (intersections with concentric circles), a mixed model with fixed effects of group (AMPH-sensitized animals *vs* AMPH-naïve controls), layer (II/IIIs, IIId, or V/VI), circle radius (50, 100, 150 μm), and all possible interactions was fitted. Cell depth and somal size were included as covariates. The correlation structure of the data was modeled by random effects for monkey and for layer within monkey. Least squares means for each radius (averaged over the other factors) were then computed, and all possible comparisons between least-square means were performed.

For measurements of dendritic length, a mixed model with fixed effects of group, layer, dendritic region (apical dendrite or basilar dendrite), and all possible interactions was fitted. Cell depth and somal size were included in the model as covariates. The correlation structure of the data was modeled by random effects for monkey and by structured variance–covariance matrix for observations on the two dendritic regions within each layer. The latter variance–covariance structure was the best fitting one according to the Akaike Information Criterion and to the Schwartz' Bayesian criterion. Least-square means for each group by dendritic region (averaged across layers) were then computed, and all possible comparisons between least-square means were performed. The significance level for group by layer interactions was adjusted to 0.02.

As a primary analysis of spine density, a mixed model with fixed effects of group, layer and dendritic region (apical dendritic trunk, apical branch, or basilar dendrite), and all possible interactions was fitted. The correlation structure of the data was modeled by random effects for monkey, layer within monkey, and dendritic region within layer within monkey. As a secondary analysis, a separate mixed model for each dendritic region with fixed effects of group, layer, position within each dendritic region (ie distance from peak along apical dendrite trunk or branch order for apical branches or basilar dendrites), and all possible interactions was fitted. The correlation structure was modeled by using random effects for monkey and unstructured variance–covariance matrix within dendritic region within monkey. The significance level was adjusted to 0.02 for this analysis. The analysis of spine density along the apical dendritic trunk was performed using cell depth and somal size as covariates; cell depth and somal size were not available for apical branch and basilar dendritic analyses. Finally, a mixed model with fixed effects of group, layer, and the interaction was fitted only to the peak spine density of the apical dendrite trunk. An unstructured matrix was used to model the variances and covariances across

layers within monkeys. Distance from the cell soma to peak spine density (DTP) was also tested for significance using a mixed model with fixed effects of group, layer, and the interaction of the two. The correlation structure of the data was modeled by random effects for monkey and layer within monkey.

RESULTS

Sholl Analysis

Analysis of Sholl data revealed a highly significant effect of circle radius ($F_{2,54} = 148.61$, $p < 0.001$) on the number of intersections. The number of intersections (least-square means averaged across all layers) with a circle of 50 μm radius (12.15 ± 0.76) was greater than the number of intersections at 100 μm radius (9.47 ± 0.76), which in turn was greater than the number of intersections at the 150 μm radius (5.29 ± 0.76) (Figure 4). A significant group by layer interaction was also observed ($F_{2,14.8} = 6.01$, $p = 0.012$). The number of intersections for pyramidal cells in layer II/IIIs was significantly reduced in the AMPH-sensitized monkeys in comparison to the AMPH-naïve controls ($F_{1,9.98} = 5.28$, $p = 0.04$), and the number of intersections for pyramidal cells in layer IIId showed a trend reduction for the AMPH-sensitized monkeys ($F_{1,9.98} = 4.44$, $p = 0.06$) (Figure 4). Neither somal size nor somal depth differed between groups for any of the layers (Table 1).

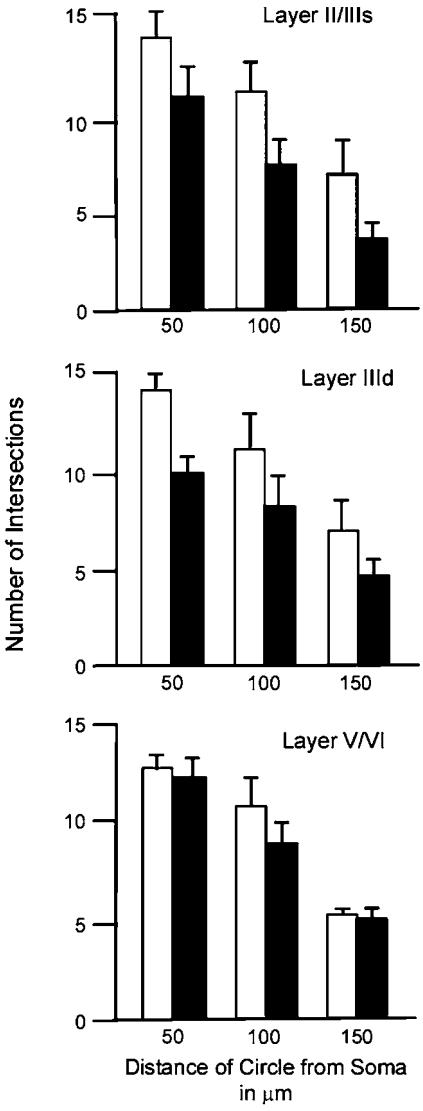

Figure 4 Bar graphs illustrating the results of a Sholl analysis of dendritic complexity. The number of intersections with concentrically placed circles with radii extending 50, 100, and 150 μm from the cell soma are shown for pyramidal cells in layers II/IIIs (top), IIId (middle), and V/VI (bottom) in AMPH-sensitized (solid bars) and AMPH-naïve monkeys (open bars). Dendritic complexity was significantly reduced in pyramidal cells in layer II/IIIs ($F_{1,9.98} = 5.28$, $p = 0.04$) and marginally reduced in layer IIId ($F_{1,9.98} = 4.44$, $p = 0.06$) following AMPH sensitization.

Dendritic Length

Mixed model analysis of dendritic length uncovered a significant interaction between group and dendritic region across layers ($F_{1,24} = 8.16$, $p = 0.009$), a significant effect of dendritic region ($F_{1,24} = 51.63$, $p < 0.001$), and a significant group-by-layer interaction ($F_{2,14.8} = 5.65$, $p = 0.015$). Comparison of all possible combinations of least-square means indicated that basilar dendritic length was longer than apical dendirtic length across layers and groups, and that basilar dendritic length was reduced in the AMPH-sensitized monkeys (820.15 ± 97.06 μm) in comparison to AMPH-naïve controls (1211.31 ± 88.60 μm; $t_{12.4} = 2.98$, $p = 0.012$) whereas the difference for apical dendritic length (AMPH-sensitized: 698.75 ± 88.60 μm; AMPH-naïve controls: 586.41 ± 97.06 μm) was not significant ($t_{12.4} = 0.85$, $p = 0.409$) (Figure 5). In layers II/IIIs and IIId, trend reductions in dendritic length (apical and basilar combined) were found when the significance level was adjusted for multiple comparisons (II/IIIs: $F_{1,10.1} = 5.25$, $p = 0.045$; IIId: $F_{1,9.99} = 7.26$, $p = 0.023$) whereas dendritic length did not differ between AMPH-sensitized and control animals in layer V/VI ($F_{1,9,91} = 0.65$, $p = 0.439$).

Spine Density

The primary mixed model analysis with fixed effects of group, layer, dendritic region and all interactions indicated significant effects for dendritic region ($F_{2,71.4} = 195.66$, $p < 0.001$) and group by dendritic region ($F_{2,71.4} = 3.32$, $p = 0.045$). Comparison of least-square means indicated that for both AMPH-sensitized monkeys and controls spine density was highest on the apical dendrite trunk, next highest on the apical branch and lowest on the basilar dendrite (Figure 6). Although the difference between groups was greatest for spine density on the apical dendritic trunk (AMPH-sensitized: 0.60 ± 0.03 spines/μm; AMPH-naïve: 0.67 ± 0.03 spines/μm), even this did not approach statistical significance ($t_{12.3} = 1.64$, $p = 0.127$). Secondary mixed model analysis of each dendritic region revealed no significant effects for the apical branch dendrites (Table 2; Figure 6c and d). A significant effect of branch order was found for the basilar dendrites ($F_{2,24.8} = 7.00$, $p = 0.0039$) with least-square means averaged across all layers and both groups indicating increasing spine density at higher branch orders ($1^\circ = 0.310 \pm 0.028$ spines/μm $< 2^\circ = 0.357 \pm 0.021$ spines/μm $< 3^\circ = 0.378 \pm 0.020$ spines/μm) (Table 2). There were no significant effects of group or group interactions

(Figure 6e and f). For the apical dendritic trunk, a significant effect of place (distance from peak) was found ($F_{3,22} = 152.08$, $p < 0.001$). Spine density on the apical dendritic trunks of pyramidal cells in both AMPH-sensitized and AMPH-naïve monkeys exhibited an inverted V pattern (Figures 3 and 7). Spine density proximal to the soma was very low but increased to peak density approximately 150–170 μm from the soma and decreased less sharply distal to the peak. Analysis of peak spine density on the apical dendritic trunk indicated that there was a significant group by layer interaction ($F_{2,5.31} = 5.88$, $p = 0.045$). *Post hoc* comparisons between groups indicated that peak spine density was reduced in the AMPH-sensitized monkeys (0.73 ± 0.06 spines/μm) relative to controls (0.93 ± 0.08 spines/μm) in layer II/IIIs ($F_{1,7.49} = 19.15$, $p = 0.0028$) and did not differ between groups in layer IIId ($F_{1,9} = 0.31$, $p = 0.594$) or layer V/VI ($F_{1,8.84} = 3.77$, $p = 0.085$) (Figures 6a, b and 7). Note that width of

Table 1 Somal Size and Depth

	AMPH-naïve		AMPH-sensitized		
	Mean	SE	Mean	SE	Statistic[a]
Size (μm^2)					
Layer					
II/IIIa	254.34	25.14	239.93	21.93	$t_{(1,9)} = -0.422$; $p = 0.683$
IIId	266.02	38.73	267.78	14.91	$t_{(1,9)} = -0.039$; $p = 0.970$
V/VI	261.87	42.05	284.44	19.88	$t_{(1,9)} = -0.453$; $p = 0.661$
Depth (μm)					
Layer					
II/IIIa	513.19	28.16	503.57	26.49	$t_{(1,9)} = 0.245$; $p = 0.812$
IIId	839.71	14.58	863.57	14.77	$t_{(1,9)} = -1.140$; $p = 0.284$
V/VI	1557.54	49.95	1414.56	75.96	$t_{(1,9)} = 1.624$; $p = 0.139$

[a]Statistic = independent *t*-test, two-tailed.

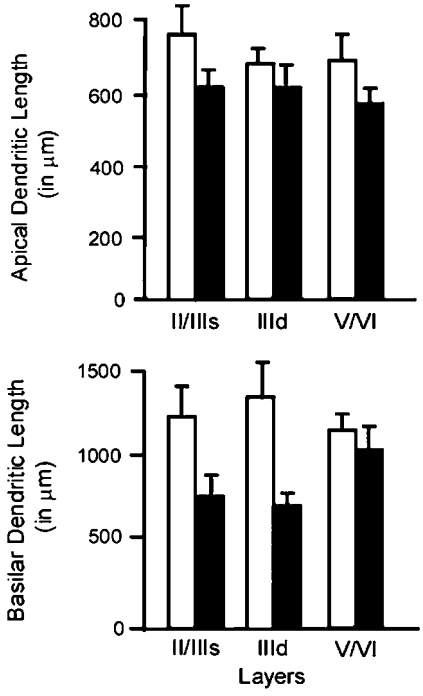

Figure 5 Graphs depicting apical dendritic length (above) and basilar dendritic length (below) of pyramidal dendrites in layers II/IIIs, IIId, and V/VI in AMPH-naive controls (open bars) and AMPH-sensitized monkeys (solid bars). Basilar dendritic length was reduced in the AMPH-sensitized monkeys compared to controls when averaged across all layers ($t_{12.4} = 2.98$, $p = 0.012$). In addition, there was a trend reduction in combined apical and dendritic length for pyramidal cells in layers II/IIIs and IIId in the AMPH-sensitized group.

Table 2 Dendritic Spine Density[a]

	Apical branch Mean (SE)			Basilar dendrite Mean (SE)	
Layer/branch order	AMPH-naïve	AMPH-sens	Branch order	AMPH-naïve	AMPH-sens
II/IIIs					
2°	0.44 (0.03)	0.45 (0.02)	1°	0.33 (0.03)	0.28 (0.05)
3°	0.42 (0.03)	0.44 (0.03)	2°	0.40 (0.02)	0.34 (0.03)
4°	0.40 (0.03)	0.42 (0.04)	3°	0.42 (0.02)	0.37 (0.03)
IIId					
2°	0.36 (0.05)	0.43 (0.04)	1°	0.31 (0.06)	0.33 (0.08)
3°	0.41 (0.03)	0.43 (0.04)	2°	0.35 (0.04)	0.33 (0.04)
4°	0.41 (0.03)	0.38 (0.06)	3°	0.37 (0.04)	0.38 (0.04)
V/VI					
2°	0.41 (0.04)	0.42 (0.05)	1°	0.34 (0.06)	0.28 (0.09)
3°	0.43 (0.02)	0.39 (0.01)	2°	0.37 (0.03)	0.37 (0.04)
4°	0.40 (0.03)	0.36 (0.02)	3°	0.39 (0.02)	0.37 (0.04)

[a]Number of spines/μm of dendrite.

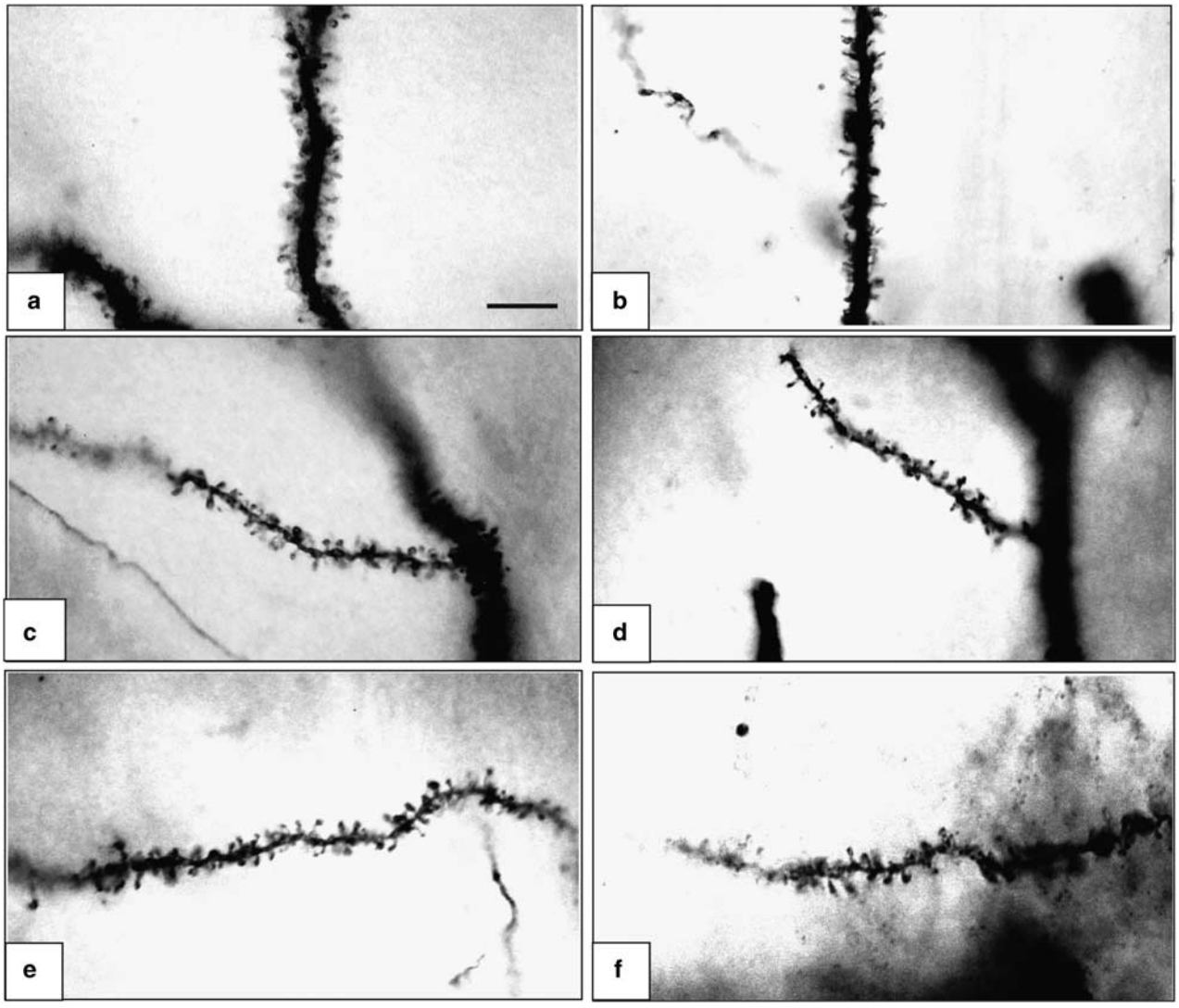

Figure 6 Photomicrographs illustrating the distribution of spine density in layer II/IIIs neurons from AMPH-naive controls (a, c, e) and AMPH-sensitized monkeys (b, d, f). (a, b) Peak density along the apical dendritic trunk was reduced in AMPH-sensitized monkeys compared to controls whereas (d, c) spine density on 2° apical branches and (e, f) spine density on 3° basilar dendrites did not differ between groups. Scale bar = 10 μm.

the apical trunk was not different in AMPH-sensitized and AMPH-naïve monkeys (Table 3) and therefore did not account for the differences in peak density in layer II/IIIs. Finally, analysis of DTP indicated that there was a significant effect of group ($F_{1,9.39} = 12.04$; $p = 0.007$). Comparison of all possible least-square means indicated that DTP was reduced in AMPH-sensitized animals (126.38 ± 7.65 μm) compared to controls (162.98 ± 7.26 μm) across all layers (Figure 8).

DISCUSSION

The present study found significant, albeit subtle, changes in the dendritic architecture of prefrontal cortical pyramidal neurons up to 3–$3\frac{1}{2}$ years after monkeys had undergone an AMPH sensitizing regimen. Specifically, AMPH sensitization in the nonhuman primate resulted in a significant reduction in overall dendritic complexity and in peak spine density along the apical trunk for pyramidal cells in layer II/IIIs. AMPH-sensitization also resulted in a reduction in basilar dendritic length and distance to peak spine density along the apical trunk for pyramidal cells across all layers. These findings indicate that a state of chronic dopamine dysregulation can lead to morphologic changes in cortical pyramidal dendrites that are enduring, if not permanent, in nature, suggesting that aspects of aberrant behavior and cognitive dysfunction induced by our AMPH sensitizing regimen (Castner and Goldman-Rakic, 1999, 2003; Castner *et al*, 2000) may be mediated at least in part by structural alterations in prefrontal cortical neurons. Dendritic morphology, including size of the dendritic arbor, branching pattern, and spine density, are shaped by both intrinsic (genetic) and extrinsic factors (Rakic, 1975; Purves, 1998). Our findings suggest that some extrinsic factors, such as exposure to stimulants, can induce long-term morphologic changes in spinodendritic architecture that may contribute to changes in cognition and behavior that persist for many years and possibly for life.

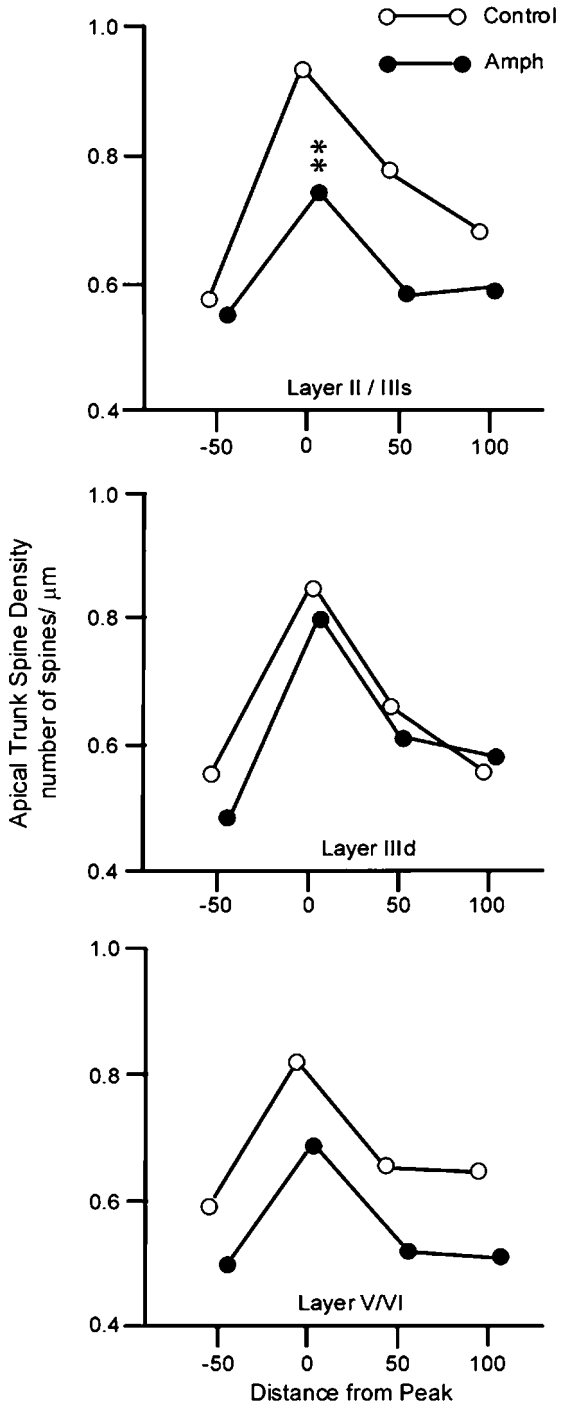

Figure 7 Graphs showing the distribution of spine density along the apical trunk for the AMPH- sensitized (filled circles) relative to AMPH-naive (open circles) monkeys in layers II/IIIs (top), IIId (middle), and V/VI ((bottom). Peak spine density in layer II/IIIs was significantly reduced in the AMPH-sensitized monkeys as compared to controls (($F_{1,7.49} = 19.15$, $p = 0.0028$).

Limitations and Possible Confounds of the Study

The primates in this study all experienced pair/group housing at some point during their lifetimes with the exception of one monkey that was singly-housed. While we have considered the possibility that rearing condition could have influenced our measurements of spine density and dendritic complexity, if anything, the inclusion of a singly housed animal, that is, a monkey deprived of physical but not sensory interaction with the colony, in the AMPH-naïve group would tend to mask the atrophic changes in dendritic architecture that were observed following sensitization. A careful review of the rodent literature suggests that alterations of dendritic morphology following differential rearing in these studies cannot be attributed to social *vs* isolation rearing but rather to the relative richness of sensorimotor stimulation of the environment (Volkmar and Greenough, 1972; Greenough *et al*, 1973; Wallace *et al*, 1992), and that even these effects were not observed in the frontolateral cortex (Greenough *et al*, 1973). Likewise, studies of singly-housed *vs* colony-reared monkeys indicates that physical isolation decreases spine density and dendritic complexity of neurons only in the primary sensory and motor cortices (Struble and Riesen, 1978; Bryan and Riesen, 1989) without effect in the prefrontal cortex (Struble and Riesen, 1978) and that sensorimotor enrichment can not only compensate for the effects of social isolation but also in some instances can result in enhanced dendritic branching relative to the socially housed condition (Stell and Riesen, 1987; Bryan and Riesen, 1989). Therefore, there is little evidence to suggest that the extent and or duration of social housing would significantly affect the dendritic morphology of pyramidal neurons in the prefrontal cortex of the monkeys in this study.

The aim of the present study was to examine the morphologic state that underlies the persistent altered behavioral and cognitive state observed many months following discontinuation of the amphetamine sensitization (Castner and Goldman-Rakic, 1999, 2003; Castner *et al*, 2000, 2005). Additional studies are needed to determine whether the morphologic states during sensitization and immediately following the sensitization period during withdrawal differ from the atrophic dendritic profile observed many months postsensitization. As discussed in detail below, previous studies of amphetamine sensitization in rodent models have examined the structural pathology of the prefrontal cortex at relatively short intervals following sensitization, perhaps accounting in part for the discrepancy in findings (Robinson and Kolb, 1997, 1999).

Only small blocks of tissue were available for Golgi impregnation from prefrontal cortical area 9. Because of the limited quantity of tissue for processing, the number of optimally impregnated cells for each analysis was small. This may have limited the power of the analysis as additional group by layer differences may have been detected with a larger sample of cells. It should be noted however that other studies examining the effects of subchronic AMPH on pyramidal dendritric morphology (Robinson and Kolb, 1997, 1999; described in detail below) also examined only five cells per animal. Therefore, differences in findings between studies cannot be attributed to sample size. Moreover, the statistical method that we employed enabled us to pool cells across all layers (15 neurons/animal) to detect significant group differences in dendritic length and complexity in this study, indicating that the analytic protocol was adequate for detection of alterations in dendritic architecture.

In this study, as in previous studies of dendritic morphology (Robinson and Kolb, 1997, 1999), linear spine

Table 3 Apical Trunk Width (in μm)

	AMPH-naïve		AMPH-sensitized		
Distance from soma	**Mean**	**SE**	**Mean**	**SE**	**Statistic[a]**
Layer II/IIIa					
75 μm	3.93	0.46	4.44	0.61	$t_{(1,7)} = -0.650$; $p = 0.537$
125 μm	3.33	0.37	3.47	0.83	$t_{(1,7)} = -0.260$; $p = 0.802$
175 μm	2.89	0.37	2.57	0.24	$t_{(1,7)} = 0.751$; $p = 0.477$
Peak	3.38	0.55	3.68	0.46	$t_{(1,7)} = -0.421$; $p = 0.687$
Layer IIId					
75 μm	4.14	0.36	4.35	0.41	$t_{(1,9)} = -0.329$; $p = 0.750$
125 μm	4.07	0.40	3.50	0.41	$t_{(1,9)} = 0.997$; $p = 0.345$
175 μm	3.08	0.29	3.31	0.23	$t_{(1,9)} = -0.584$; $p = 0.574$
Peak	3.57	0.23	3.37	0.27	$t_{(1,9)} = -0.585$; $p = 0.573$
Layer V/VI					
75 μm	3.94	0.49	4.27	0.39	$t_{(1,8)} = -0.485$; $p = 0.641$
125 μm	3.44	0.55	3.71	0.43	$t_{(1,8)} = -0.355$; $p = 0.732$
175 μm	2.93	0.52	3.49	0.63	$t_{(1,8)} = -0.678$; $p = 0.517$
Peak	3.29	0.64	3.8	0.41	$t_{(1,8)} = -0.588$; $p = 0.572$

[a]Statistic = independent *t*-test, two-tailed.

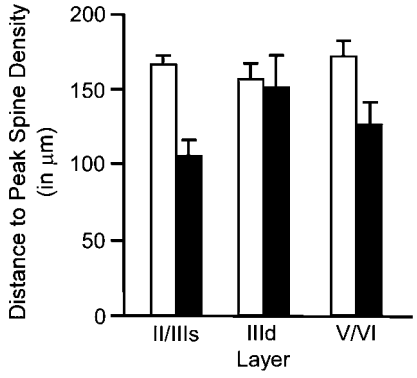

Figure 8 Graph depicting distances from soma to peak spine density (DTP) along the apical trunk in AMPH-naive (open bars) and AMPH-sensitized (solid bars) monkeys. Averaged across all layers, DTP was shorter in AMPH-sensitized monkeys relative to AMPH-naïve controls. Although differences between groups were not significant for individual layers, layers II/IIIs and V/VI exhibited greater group differences in DTP than layer IIId.

density measurements underestimated true spine density because spines hidden behind the shaft of the dendrite were not counted (Feldman and Peters, 1979). Although dendritic thickness is a potential confound for spine density, we have carefully matched different regions of the dendritic arbor for comparison and thereby minimized differences in dendritic thickness between groups. Moreover, measurements of dendritic width along the apical trunk, the only region of the dendritic tree that exhibited altered spine density in the AMPH-sensitized animals, did not differ between groups and therefore could not account for the observed reduction in spine density (Table 3).

Comparison with Golgi Findings in Rodents

AMPH sensitization in rodents has been shown to produce increases in dendritic length, spine density, and the number of branched spines on the apical dendrites of layer III pyramidal cells in the prelimbic cortex of the rat and to have similar effects on all portions of the dendritic tree of neurons in the nucleus accumbens (Robinson and Kolb, 1997). A follow-up study by this same group found increased dendritic branching and spine density on both apical and basilar dendrites of layer V pyramidal cells in the prelimbic cortex consequent to repeated AMPH exposure (Robinson and Kolb, 1999). While the present analysis indicates that morphologic alterations are also associated with AMPH sensitization in the monkey, there are marked differences in the described dendritic changes in the two species. Several parameters of the experimental design might account for these discrepancies. One major difference between the experimental design in the present study as compared to the rodent studies is that the interval between discontinuation of repeated AMPH exposure and assessment of anatomical changes was much longer in the present study (36–42 months) than in the rat studies (1–3.5 months). Thus, the increase in dendritic branching and spine density observed at relatively short intervals following AMPH exposure might not persist for longer periods postsensitization. In fact, it is possible that the rodent findings represent the early changes in morphology that occur in response to a sensitizing regimen of AMPH exposure while those reported here represent the long-term consequences to a prolonged and seemingly permanent state of dopamine dysregulation in the prefrontal cortex. In addition, differences in analytic methodologies might also

have contributed to the disparity in findings. One of the prominent findings in our study is a reduction in spine density on the apical trunk, a parameter that was not analyzed in the rodent studies. In addition, spine density on apical dendrites was assessed throughout the first four orders of branches of the dendritc tree, and the reported densities represent averages of longer lengths of dendrites ($>250\,\mu m$) than in the rodent studies ($10\,\mu m$). However, we elected not to quantify spine density on the most distal branches ($>4^{\circ}$) of apical and ($>3^{\circ}$) of basilar dendrites because the number of these distal branches was limited and would not have provided a reliable sample between neurons. Therefore, increases in spine density on the most distal branches would not have been detected in this study. As the exact location of spine density assessment was not specified in the earlier rodent studies, it is possible that the increases in spine density were found on distal tips of dendrites.

Prominence of Morphologic Alterations in Layer II/IIIs

Layer II/IIIs pyramidal cells exhibited more pronounced changes in dendritic morphology following AMPH-sensitization than neurons in deeper layers, for example, reduced dendritic complexity and reduced spine density on the apical trunk. Immunocytochemical analysis of the dopamine projection to the prefrontal cortex in the macaque has revealed that dopaminergic fibers are distributed in a bilaminar pattern with the greatest density of fibers situated in superficial layers I, II, and IIIs and in deep layers V and VI (Williams and Goldman-Rakic, 1993). This pattern of innervation indicates that dopamine terminals are densely distributed within the same layers that house the dendritic arbor of layer II/IIIs neurons, and indeed, studies examining the density of tyrosine-hydroxylase (TH)-positive appositions on intracellularly filled neurons found a greater density of TH appositions on layer II/IIIs dendrites than on dendrites of neurons in layers IIId and V/VI (Krimer *et al*, 1997). Certainly, we cannot discount the possibility that chronic dysregulation of dopaminergic neurotransmission consequent to AMPH sensitization could have altered the dendritic architecture and spine density of the distal apical branches of more deeply situated pyramidal cells as these changes would not have been detected in our analyses. Nonetheless, morphologic alterations in the central core of the dendritic arbor were more pronounced for pyramidal cells in layer II/IIIs than for more deeply situated pyramidal neurons following AMPH sensitization, and the laminar-specificity of these effects corresponds to the anatomical distribution of dopamine fibers in the prefrontal cortex.

Prefrontal cortical area 9 is reciprocally connected with many other multimodal cortical areas, receives a prominent afferent input from the thalamus, and has projections to numerous subcortical areas, including the neostriatum, thalamus, and brain stem (Selemon and Goldman-Rakic, 1988, Giguere and Goldman-Rakic, 1988). Pyramidal cells in layer II/IIIs receive a balance of cortical/subcortical input that is distinct from that of pyramidal cells in other layers. Most long-tract cortical projections terminate in a columnar fashion in area 9, impinging on pyramidal neurons in all layers (Goldman and Nauta, 1977). Even those that have a more limited termination, as for example projections from the posterior parietal cortex which are concentrated mainly in layers I and IV (Goldman-Rakic, 1987), may still contact pyramidal cells in deep layers (V/VI) as all pyramidal neurons have apical dendrites that reach layer I. In contrast, subcortical projections from the thalamus terminate primarily in deep layer III and layer IV (Giguere and Goldman-Rakic, 1988) and therefore selectively terminate on the basilar dendrites of layer III pyramids. Likewise, subcortical projections arise mainly from pyramidal cells in infragranular layers (Jones *et al*, 1977; Arikuni *et al*, 1983; Arikuni and Kubota, 1986; Giguere and Goldman-Rakic, 1988). As a result, pyramidal cells in layer II/IIIs are more intimately linked to other cortical areas and less connected to subcortical sites than are more deeply situated pyramidal cells. Thus, the predominance of morphologic alterations in layer II/IIs pyramids suggests that amphetamine sensitization strongly impacts corticocortical communication.

Decreased Apical Trunk Spine Density and Implications for Alteration of Glutamate Neurotransmission

Analysis of spine density along the apical trunk revealed a lower peak spine density in layer II/IIIs pyramidal neurons in the AMPH-sensitized monkeys than in AMPH-naïve controls. Ionotropic glutamate receptors are distributed along the apical dendritic trunk of cortical pyramidal cells in rats (Petralia *et al*, 1994a, b) and monkeys (Huntley *et al*, 1993; Vickers *et al*, 1993), and excitatory glutamatergic terminals terminate on pyramidal dendritic spines and shafts in close approximation to dopaminergic terminals, many forming a triadic arrangement (Goldman-Rakic *et al*, 1989; Smiley *et al*, 1992). In addition, D1 dopaminergic receptors are located on presynaptic terminals, presumably glutamatergic, that are apposed to spines (Paspalas and Goldman-Rakic, 2005). As such, D1 receptors are in prime position to modulate recurrent excitation among neighboring pyramidal cells, the physiologic basis for mnemonic processing in the prefrontal cortex (Williams and Goldman-Rakic, 1995; Gao *et al*, 2001; Gao and Goldman-Rakic, 2003). In view of the proximity of dopaminergic variscosities and receptors to glutamatergic synapses, a long-term consequence of abnormal dopaminergic transmission in the prefrontal cortex might include alterations in the number, affinity, or activity of glutamatergic receptors.

Recent evidence has implicated glutamatergic receptors in the neurochemical mechanisms underlying the induction of behavioral sensitization (Wolf, 1998). For example, administration of a noncompetitive antagonist of the *n*-methyl-D-aspartate (NMDA) receptor has been shown to block induction of behavioral sensitization by cocaine or AMPH (Karler *et al*, 1989). More recently, direct injection of NMDA receptor antagonists into the ventral tegmental area (VTA) has been used to prevent the induction of sensitization by an intra-VTA injection of AMPH or cocaine, and these effects are mimicked by prefrontal lesions (Cador *et al*, 1999). The permissive effects of glutamate on sensitization have been attributed to the presence of excitatory, glutamatergic projections from the prefrontal cortex to the VTA. These prefrontal projections terminate directly on mesolimbic and mesocortical dopaminergic neurons (Carr and Sesack, 2000) and therefore are optimally positioned to modulate cortical dopaminergic release. The

present findings indicate that repetitive stimulation of dopaminergic receptors may alter glutamatergic transmission through this prefrontal-VTA circuit perhaps reducing glutamate induced excitation of cortical pyramidal cells and in turn decreasing prefrontal stimulation of mesocortical dopamine release. This might provide a critical mechanism by which AMPH sensitization induces a reduction in dopamine turnover in the primate dorsolateral prefrontal cortex (Castner *et al*, 2005).

Implications for the Neuropathology of the Prefrontal Cortex in Schizophrenia

The most prominent pathology in the dorsolateral prefrontal cortex in patients with schizophrenia is a deficit in interneuronal neuropil that has been interpreted as evidence for impoverished cortical connectivity (Selemon *et al*, 1995, 1998, 2003; Selemon and Goldman-Rakic, 1999). Studies examining the dendritic morphology of Golgi-impregnated pyramidal neurons in the prefrontal cortex of schizophrenic patients and have found reductions in spine density, dendritic complexity, and dendritic length that are consistent with the postulated reduction in cortical communication (Garey *et al*, 1998; Glantz and Lewis, 2000; Kalus *et al*, 2000; Broadbelt *et al*, 2002; Black *et al*, 2004). In the AMPH-sensitized monkey, we observed a reduction in complexity of dendrites in layer II/IIIs and a reduction in dendritic length in pyramidal cells across all layers. As the present findings in the AMPH-sensitized nonhuman primate are similar, albeit not identical, to the morphologic abnormalities described in schizophrenia, this raises the possibility that disturbances in dopaminergic transmission in the prefrontal cortex, or perhaps other monaminergic systems that are modulated by amphetamine, might contribute to the pathologic changes associated with the disease. Indeed, Lieberman *et al* (1997) have hypothesized that the onset of schizophrenia, which usually occurs in late adolescence and is often associated with stressful life experiences, may be characterized by neurochemical sensitization of the same circuits that are induced by psychomotor stimulants and that the neurochemical imbalance between dopaminergic and glutamatergic systems ultimately leads to a limited form of excitotoxicity. This hypothesis implies that reduction in cortical connectivity in schizophrenia may not be universal but rather selective to those synapses that are modulated by dopamine, a premise that is supported by the present findings. Moreover, one recent study has found that methamphetamine abuse can lead to schizophreniform psychosis in individuals with poor premorbid social function (Chen *et al*, 2003). Of course, unlike human subjects at risk for schizophrenia, the AMPH-sensitized monkeys in the present study did not have an underlying neurodevelopmental vulnerability and were not juveniles when neurochemical sensitization occurred. Differences such as these between the disease etiology and the model may account for the fact that the marked reduction of spine density on basilar dendrites of pyramidal cells observed in schizophrenic postmortem brains (Garey *et al*, 1998; Glantz and Lewis, 2000) was not reproduced in the non-human primate by the AMPH sensitizing regimen. Perhaps, more pronounced alterations in dendritic structure, including reduction of basilar spine density, might be produced by repeated amphetamine exposure during the adolescent period when the process of synaptic pruning is most active (Bourgeois *et al*, 1994; Anderson *et al*, 1995). Taken together, the present findings in conjunction with our previous report of profound cognitive deficits and reduced prefrontal dopamine transmission indicate that AMPH sensitization in the nonhuman primate is an invaluable model for providing insights into the etiology, as well as the treatment, of schizophrenia, particularly with regard to amelioration of working memory deficits.

ACKNOWLEDGEMENTS

We would like to thank Heather Findlay and JoAnn Coburn for excellent technical assistance, as well as Dr Ralitza Gueorguieva, Department of Biostatistics in the Yale School of Public Health, for statistical analysis of the data. This work was supported by NIMH Grants MH44866 and MH68789.

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