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Effects of neonatal thermal lesioning of the mesocortical dopaminergic projection on the development of the rat prefrontal cortex

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The role of dopamine (DA) in the development of the prefrontal cortex (PFC) was investigated by depleting the dopaminergic innervation of the PFC. A new stereotaxic procedure made it possible to produce small lesions in 1-day-old rats confined to the A₁₀ group of dopaminergic cell bodies in the ventral tegmentum, from which the dopaminergic projection to the PFC originates. The variety in the lesions revealed a clear topographical organization of the efferent connections of the ventral tegmental area (VTA) to the prefrontal cortex. As far as we know from the literature the data presented in this study are a first direct indication of a neurotrophic role for dopamine in the development of the prefrontal cortex. When the prefrontal cortex was depleted of the dopaminergic innervation from birth on, by lesioning the cells of origin on postnatal day 1, the cortical thickness in the medial PFC was reduced by about 6%. Although coagulative lesions in the ventral tegmentum cause also a depletion of cortical serotonin, cortical reduction seems to be rather the result of the absence of dopamine during its development. This is indicated by the absence of a significant cortical thickness reduction in the dysgranular part of the first somatosensory cortex, which receives a serotonergic but no dopaminergic innervation.

INTRODUCTION

An important role in early cortical development has been postulated for the afferent projections that reach the developing cortex^{13,18,30,31}. In the PFC monoaminergic as well as thalamocortical fibers are present in early development^{41,42,59,66}. The first presumed DA fibers arrive at the PFC on embryonic day 16 (ref. 65), focusing attention on a possible early neurotrophic role of the mesocortical dopamine system. In order to investigate such a role of DA in the development of the prefrontal cortex, attempts were made to effect an early depletion of the DA innervation of the PFC. Since a systemic or intraventricular injection of 6-OHDA, after pretreatment with DMI, seems to mainly deplete the striatal dopamine and, to a lesser extent the cortical DAergic fibers postnatal-

ly²⁵ or prenatally (unpublished results), it was necessary to lesion the cells of origin of the DA input. DA cells which project towards the PFC are localized mainly in the VTA^{16,34}. Therefore the DA content in the prefrontal cortex was depleted by thermal lesioning of the DAergic A₁₀ cell group in neonatal rats. Through a new stereotaxic procedure²⁴ it was possible to produce small lesions restricted to the VTA in 1-day-old rats. The variety in the lesions necessary to determine the optimum electrode placement enabled us to examine, in addition, the topographical organization of the efferent dopaminergic connections of the VTA.

MATERIALS AND METHODS

In this study 52 Wistar rats, both male and female,

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were analyzed, all bred at our own facility. The animals were housed in constant light:dark conditions (lights on 07.00–19.00 h), while food and water were available *ad libitum*. The day of birth was taken as PN0. On PN1 the pups were operated. After ether anaesthesia 40 animals received a bilateral thermal lesion in the VTA using a stereotaxic procedure adapted for neonatal rat pups²⁴. Lesions were made using a high-frequency lesion generator (Radionics, model RFG-4) with thermistor-type electrodes with an uninsulated tip of 1.5 mm and 0.7 mm in diameter. After positioning the electrodes they were heated to 65 °C for 1.5 min. Twelve sham-operated pups received bilateral electrode placement without heating the electrodes. Weaning occurred at PN21.

Histology

Both lesioned and non-lesioned animals were perfused with buffered 5% glutaraldehyde (pH 4) on PN35, when the prefrontal cortex had reached its maximum volume⁶². To control for possible sex differences⁶⁴ both male and female animals were used. Brains were removed from the skull, postfixed for 30 min and weighed immediately afterwards. In order to be able to compare the placement and extent of the lesion, together with the DA-depletion in the various projection areas, coronal Vibratome sections of 50 μ m, were stained immunocytochemically using a recently developed sensitive antibody to DA¹⁹. To control the effect of the lesions within the VTA on the DA innervation, the density of DA-containing fibers in different projection areas was estimated separately in a semi-quantitative manner distinguishing 4 categories: 0, almost no fibers; +, few fibers; ++, distinct reduction in the number of fibers; +++, no noticeable reduction in the number of fibers. Alternative sections (100 μ m) were stained with thionin to examine the cytoarchitecture of the PFC, using the Nissl staining procedure for Vibratome sections of our laboratory; (1) attach Vibratome sections to 0.5% gelatin-coated slides, and allow to dry at room temperature; (2) overnight in methanol (66 ml), chloroform (33 ml) and concentrated HCl (1 ml) solution (this step is to make sure that lipids are removed to brighten the Nissl staining); (3) let dry; (4) stain in thionine solution (1.5 g in 300 ml distilled water and 3 ml acetic acid) for 10–15 min; (5) rinse in distilled water, if necessary differentiate in alcohol

70% and 90%, dehydrate; (6) clear for 5 min in Histoclear (National Diagnostics, Sommerville) and mount sections. In some experiments alternative sections were also stained immunocytochemically for 5-HT or NA, using the 5-HT and NA antibodies, which Dr. M. Geffard (Bordeaux, France) was kind enough to donate.

Morphometry

In Nissl-stained sections of PFC DA-depleted and sham-operated animals, cortical thickness was measured at the curvilinear border lines of the cytoarchitectonic subareas of the medial PFC; viz., PL, ACd, PrCm and the borderline between the AI_d/AI_v (Fig. 1). The criteria for identification of the different PFC subareas were derived from Van Eden and Uylings⁶¹ and Krettek and Price²⁹. To indicate whether or not the changes in the PFC thickness are related with DA innervation cortical thickness was also measured in a dysgranular part of the SI¹⁵, which contains no clear DAergic innervation^{5,16,54}. Measurements were per-

Fig. 1. Nissl-stained coronal section of the right frontal pole of the rat brain. Arrows indicate curvilinear borderlines where cortical thickness was measured. *, Dysgranular part of the first somatosensory cortex.

formed with the aid of a computerized digitizing tablet attached to a research microscope, equipped with plan objectives. The thickness measurements were done, in the most rostral coronal section that contained the nucleus accumbens. For the selection of this section, the criteria described in Uylings et al.⁵⁸ were applied to avoid too asymmetrical or too obliquely cut brains in order to ensure a correct comparison of cortical thickness in homological areas. Also due to the lateralization of different PFC subareas⁶⁴, those brains that had been cut too asymmetri- cally were excluded from the thickness analysis. Mean cortical thickness in experimental and control groups was tested with the non-parametric Mann-Whitney *U*-test. To control for any interfering effects of differences in brain weight¹² regression lines were computed for experimental and control animals according to the standard major axis method, since both variables (brain weight and cortical thickness) are subject to biological variation and measurement error^{23,48}. To compare experimental and control values, for each bivariate point the product of the deviations in the *x* and *y* directions from the significant bivariate linear relation of the control group was determined (see Fig. 6). These products are regarded as positive if the bivariate point lies above the regression line, and as negative if it lies below this line. This procedure developed by Uylings et al.⁵⁷ presupposes a binormal distribution of cortical thickness and brain weight and may only be applied when there is a significant correlation between these variables for the control group. The samples of products of deviation in *x*- and *y*-direction were tested with a *t*-test.

RESULTS

Topographic organization of the dopaminergic projections within the ventral tegmental area

In order to determine the most suitable electrode placement for depleting the DAergic projection to the prefrontal cortex, the DAergic projection areas were compared with control series of DA-stained sections. The various lesions revealed a clear topographical organization of the VTA projections, as indicated in Fig. 2. Roughly 3 subareas within the VTA could be distinguished; a ventrolateral, a dorsolateral and a medial part. In most cases lesioning of the ventrolateral part of the VTA affected also the medi-

	I	II	III	IV
PFC-med	+++	+++	0	++
PFC-orb	++	0	++	+++
PFC-sg	0	+++	+++	+++
LS	+++	+++	+	0
Acc	+++	+++	+	+++
Striatum	++	++	+++	+++
Ent cortex	+	+++	+++	+++
Amygdala	+++	+++	+++	+++
Olf tub	+++	+++	+	+++

Fig. 2. Topographical organization of the DAergic VTA projections as indicated by disappearance of DA-positive fibers after neonatal VTA-lesions. 0, Almost no fibers; +, few fibers; ++, clear reduction in the number of fibers; +++, no noticeable reduction in the number of fibers.

al part of the SN. This combined VTA/SN lesion resulted in a depletion of DA-containing fibers in only the supragenual part of the prefrontal cortex and, depending on the severity of the SN lesions, also in depletion of the entorhinal cortex. A lesion in the dorsolateral part mainly caused depletion of DA-positive fibers in the orbital part of the prefrontal cortex. Lesioning the medial part of the VTA, dorsal to the interpeduncular nucleus, caused more widespread depletions; e.g., the medial part of the PFC as well as the lateral septum, nucleus accumbens and the olfactory tubercle were affected. These medial VTA lesions never caused a complete depletion of the DAergic innervation in the nucleus accumbens and/or olfactory tubercle. For a total depletion of the lateral septum a small lesion near the midline was sufficient (Fig. 3). Total depletion of the dopaminergic innervation in the medial PFC was achieved only when the lesion comprised the medial part of the VTA along nearly its entire rostrocaudal extension. After VTA

Fig. 3. Effect of a small unilateral lesion in the medial VTA. a: intact dopaminergic innervation in the supragenual part of the PFC. b: almost complete unilateral depletion of the DAergic innervation in the lateral septum. c: small unilateral lesion in the medial VTA (asterisk, bregma -5.0 , ref. 37). Arrows indicate borderlines of the PFC subareas.

lesions no depletion of the DAergic innervation could be detected immunocytochemically in the following nuclei: the dorsal striatum, amygdala, dorso-medial nucleus of the hypothalamus and the lateral habenula. Consequently, for a total depletion of the

Fig. 4. Effect of a unilateral lesion of the DAergic A_{10} cell group on the cortical 5-HTergic innervation. a: complete unilateral depletion of 5-HT. b: unilateral VTA lesion, notice that the SN is not affected (bregma -5.3 , ref. 37). Arrows indicate borderlines of the PFC subareas.

dopaminergic innervation in both the medial and orbital PFC it was necessary to lesion nearly the whole VTA. However, as shown by sections stained with the anti-5-HT serum, successful depletion of DA in the prefrontal cortex was accompanied by a total loss of cortical 5-HTergic innervation (Fig. 4). Immunocytochemical staining for NA showed no depletion.

Analysis

Fourteen animals showed total or near-total depletion (categories 0 and +) of the DAergic innervation in both prefrontal subareas (Fig. 5). After controlling for asymmetrical and/or oblique cutting 6 experimental (4 males and 2 females) and 8 controls (6 males and 2 females) remained for further analysis of the cortical thickness. Lesioning the VTA had no significant effect on body weights or brain weights although body weights and brain weights of the experi-

Fig. 5. Effect of a unilateral lesion of the DAergic A₁₀ cell group on the DAergic innervation of the prefrontal cortex. a: almost complete unilateral depletion of the DAergic innervation in the medial PFC (section adjacent to the one shown in Fig. 4a). Orbital part of the PFC: (b) contralateral no depletion; and (c) ipsilateral depletion of the dopaminergic innervation. Arrows indicate borderlines of the PFC subareas.

mental animal tended to be smaller (Table I and Fig. 6).

The effects of VTA lesions on cortical thickness are shown in Table I. Compared with control animals, in

the experimental animals cortical thickness was significantly reduced at the dorsal border of the PrCm, the ACd, and the PL. At the dorsal border of the IL and the borderline between AId/AIv (orbital) no significant alterations were observed. Also measurement of the cortical thickness of the control area in the somatosensory cortex showed no significant effects for the VTA lesions (Table I). A significant correlation between cortical thickness and brain weight was found in the control group at the curvilinear borderlines of PrCm, ACd, orbital PFC and the dysgranular somatosensory cortex (Table I) but not in the experimental group. Taking into account the significance of the cortical thickness–brain weight relation for the control animals the VTA-lesioned group is also compared with the control group by testing the residual products of both groups relative to the control regression line. The medial prefrontal cortex revealed still a significant effect of DA depletion (PrCm, $P < 0.05$; ACd, $P < 0.01$; Student's t -test). The cortical thickness in the dysgranular SI area again was not significantly different, as is the difference in the orbital PFC. In this study the results are similar in that those comparisons which differed sig-

Fig. 6. Relation between brain weight and cortical thickness at the curvilinear borderline between PrCm and ACd for experimental animals and controls. Correlation coefficient for control animals is $r = 0.67$ (Spearman rank correlation). d_x , d_y : deviation for a bivariate point in the x and y direction from the significant bivariate linear relation of the control group.

TABLE I

Influences of VTA lesions on cortical thickness, brain weight and body weight

<i>Cortical thickness (mm)</i>	<i>Control</i>		<i>Experimental</i>		<i>% Change^a</i>	<i>r^b</i>
	<i>Mean</i>	<i>S.E.M.</i>	<i>Mean</i>	<i>S.E.M.</i>		
PrCm	2.41	0.07	2.27	0.07	-5.9***	0.62**
ACd	2.49	0.08	2.32	0.09	-6.9***	0.67**
PL	1.78	0.10	1.67	0.04	-6.5**	0.17 n.s.
IL	1.18	0.06	1.14	0.09	-3.4	-0.25 n.s.
Orbital	2.71	0.17	2.60	0.14	-4.2	0.45*
SI	2.54	0.18	2.49	0.07	-1.9	0.67**
Brain weight (g)	1.601	0.030	1.555	0.020	-2.9	
Body weight (g)	126	5	123	5	-2.2	

^a Differences were tested with the Mann-Whitney *U*-test, ***P* < 0.01, ****P* < 0.001.^b Correlation between brain weight and cortical thickness in controls according to the Spearman rank correlation test, **P* < 0.05, ***P* < 0.01, n.s., not significant.

nificantly in Table I remain significant, thus strengthening the significant findings. No clear-cut sex differences or left-right asymmetries could be detected with the number of animals examined.

DISCUSSION

Initially an attempt was made to inject rats intraventricularly with 6-OHDA, after pretreatment with DMI both in utero on day E17 and neonatally on PN1 and PN3. However, no depletion of the prefrontal cortical dopaminergic fibers was found, whereas the dopaminergic innervation of the nucleus accumbens and the nucleus caudatus was almost completely lost (unpublished results). These results confirm other studies^{25,40,43,49}. It therefore seems plausible that 6-OHDA might be useful for studying the effect of DA depletion of the nucleus accumbens or caudate putamen on the development of these areas. But these neurotoxins, administered either intraventricularly or i.p., seem to be unsuitable for studying the effect of DA depletion on the development of the PFC. Although several of the known monoamine neurotoxins, 6-OHDA, 6-OH-DOPA, DSP4, are in principle also suitable for developmental studies²⁵ none of these seem suitable for selective depletion of cortical dopamine. Therefore, in the present study stereotaxic lesions in the VTA were made on PN1 by applying radiofrequency energy to examine the effect of DA depletion on the cytoarchitectural development of the PFC. The variety of the lesions and their effect on the DA innervation pattern suggest a topographic or-

ganization of the projection neurons in the VTA. Since the discovery of the mesolimbocortical DA system there have been several studies examining the efferent connections of the VTA^{2,4,10,17,34,45,46,53}. Some of these also proposed a topographical organization^{4,10,17,45}. The results of our lesion experiments in neonatal rats, using the DA-specific antibody staining, largely confirm and extend these earlier studies. As shown also in earlier studies, the DA projection to the supragenual part of the PFC arises from neurones in the area of the lateral VTA and medial SN (Fig. 2). The position of VTA cells projecting to the orbital PFC is not mentioned, however^{2,46}, or has not been found⁴⁵. Our lesion experiments show that these cells are situated in the more dorsolateral part of the VTA, dorsal to the medial SN (Fig. 2). This result is complementary to the one of Gerfen and Clavier²⁰ in their HRP study on neural inputs to the prefrontal agranular insular cortex in the rat. In the medial part of the VTA neurons are situated that project to the nucleus accumbens, the olfactory tubercle, the lateral septum and the medial PFC (Fig. 2). The partial depletion of nucleus accumbens and olfactory tubercle after VTA lesions can be explained by an additional DA projection to these areas from the SN (e.g. refs. 5, 10, 46). The projection to the lateral habenula is reported to originate from cells situated at and near the midline in the VTA^{5,38}. In this study no signs of depletion of DAergic fibers were found after medial VTA lesions. However, as the main part of the habenular projection from the VTA is non-DA⁵³ this might indicate that there is yet another DA projec-

tion to the lateral habenula, probably from more posterior regions in the A₁₀ cell group⁴⁵. As expected, the DA innervation to the dorsomedial nucleus of the hypothalamus was not affected after VTA lesioning, since this projection arises in the diencephalic A₁₁ cell group⁵. The reports on the origin of the DA innervation to the amygdala are still not fully in agreement. Generally this projection is assumed to originate from the VTA and medial SN^{2,5,16,53}. However, in this study no signs of depletion were found after VTA lesions, even when these involved the medial part of the SN. This confirms the study of Simon et al.⁴⁶. A more detailed inspection of the tracer studies reported in the literature shows that the cells projecting to the amygdala are located in the transition zone of VTA and SN at the most rostral part of the VTA (bregma -4.8, ref. 37)^{2,10,53}. This location was not studied by Simon et al.⁴⁶, as is illustrated by their figures. In this study the lesions were aimed at the portion of the VTA with the highest density of DA cells (bregma -5.3 to -5.8; see Figs. 3, 4), which might be the reason for sparing the DA amygdala projection.

It is evident from our immunocytochemical results that successful thermal lesioning of the VTA affects not only the mesocortical DA system but also the cortical serotonergic innervation (Fig. 4a). It is very difficult, therefore, to lesion the VTA thermally without damaging the 5-HTergic fibers in the neocortex. As expected, the cortical NAergic innervation was not affected because these fibers are located in the dorsal NAergic bundle. The ventral NAergic bundle, which mainly projects to the hypothalamus, is more likely to be damaged. Especially in behavioural studies these lesion effects on monoamines should be considered^{50,51,55}. Although immunocytochemical staining gives no quantitative information about DA levels, the method used in this study seems to give a reliable indication of the areas of DA depletion. This is indicated by a parallel study in which both methods, immunocytochemical and biochemical, were used to evaluate the effect of VTA lesions on the DAergic innervation in the adult prefrontal cortex. A clear reduction in the number of DA-positive fibers indicated by the immunocytochemical staining (category ++) corresponds with a 60% depletion of DA in the medial and orbital PFC according to the biochemical measurement, in animals with a comparable VTA le-

sion⁵⁰. Neonatal lesioning of the DA A₁₀ cell group in the VTA resulted in a significantly thinner medial PFC as compared to their sham operated littermates with an intact mesocortical system. In the dysgranular part of the somatosensory cortex no significant alterations in cortical thickness were detected. The somatosensory cortex contains a clear 5-HTergic innervation³³, but no clear dopaminergic innervation can be found in this somatosensory cortical area in sham-operated control rats^{5,16,54} and biochemical measurements detect only low levels of DA, especially in rodents^{9,21,36}. Most of this DA is concentrated in ascending axons in the deep layers³⁶. Only with indirect methods Berger et al.³ were able to visualize a minor DAergic innervation in a part of the motor and visual cortex and to discrete zones of the retrosplenial areas. So this indicates that serotonin depletion alone does not significantly affect the cortical thickness. This is in agreement with the relative late development of the cortical 5-HTergic innervation as opposed to the DA- and NAergic innervation^{14,33}. Although Lauder and Krebs³¹ found evidence for a 5-HTergic role in the early differentiation of target neurons, there was no effect on cortical neurons after prenatal 5-HT depletion. Also transmitter systems other than the 5-HTergic system in the PFC might be affected by the VTA lesions, although the selective reduction in cortical thickness of the medial PFC suggests an effect of the DA depletion, or might be a combined effect of DA depletion and a change in other transmitter levels, e.g. 5-HT or cholecystokinin⁵². Qualitative inspection of the Nissl sections indicates that the greatest changes occur in layers V and VI of the medial PFC. In these deeper layers normally also the greatest density of DA fibers is found^{16,36,54,60}. At the ventral border of the PFC (IL, Table I) the decrease in cortical thickness does not reach the level of significance, which is probably a result of the fact that the layers with the greatest density of DA fibers, viz., V and VI, are relatively small at this place. In the orbital part of the PFC no significant alterations were detected. This lack of size differences in the orbital PFC might be caused by two factors. The most important factor might be the smaller content of DA (e.g. refs. 16, 47, 54), while in addition the greater measuring error in the orbital area⁶² could play a role.

Although a number of studies has shown altera-

tions in cortical development as a consequence of monoamine (mainly only of supposed NA) depletion^{1,6-8,18,22,27,28,44} several of these studies yielded conflicting results, viz., no effect^{26,32,35}, an increased cortical thickness²⁷ or a decreased cortical thickness were reported⁸. Besides methodological differences and the different places of thickness measurements, also the relation between brain weight and cortical thickness (Fig. 6, Table I) might be a factor in confounding the results. As reported earlier, this correlation is especially apparent in young animals¹². In the present study the differences in brain weight were too small for the differences in cortical thickness to interfere with a decrease due to DA depletion. However, this effect can be of importance in other studies, especially when the deviation in brain weight values is large, as might be the case after monoamine depletion (e.g. refs. 1, 7).

In the present study a clear-cut effect of DA depletion on the development of the cortex has been shown for the first time, though a combined effect with other transmitters cannot be excluded. What exactly causes the reduction of the cortical thickness is poorly understood, however. As pointed out by Pinto Lord and Caviness³⁹ the dendrite-axon inter-

actions provide an important stimulus to growth and differentiation of the dendritic arbors of pyramidal cells^{11,39}. Preliminary results of neonatal lesioning of the mediodorsal nucleus of the thalamus strengthen this idea, as they also result in a reduction of the prefrontal cortex⁶³. The results of Tennyson et al.⁵⁶ who showed less mature striatal perikarya in the rabbit after DA depletion by reserpine or α -methyl-*p*-tyrosine, indicate a delayed development. This retarded development of cortical neurons together with a possible reduction in cellular density³⁵ might lead to a diminished cortical thickness. In addition, it will be necessary to determine whether the effect of DA depletion on the PFC thickness is a transitional delayed development or a permanent effect.

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ABBREVIATIONS

Acc	nucleus accumbens
ACd	dorsal anterior cingular cortex
ACv	ventral anterior cingular cortex
AId	dorsal agranular insular cortex
AIV	ventral agranular insular cortex
cc	corpus callosum
DA	dopamine
DMI	desmethylinipramine
Ent	entorhinal
5-HT	serotonin
IL	infralimbic cortex

LS	lateral septum
ml	medial lemniscus ³⁷
NA	noradrenaline
6-OHDA	6-hydroxydopamine
Olf tub	olfactory tuberculum
PFC	prefrontal cortex
PL	prelimbic cortex
PN	postnatal day
PrCm	medial precentral cortex
rs	rhinal sulcus
SI	somatosensory cortex I
SN	substantia nigra
VTA	ventral tegmental area

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