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Atonia after Carbachol Microinjections Near the Locus Coeruleus in Cats

P. A. M. VAN DONGEN, C. L. E. BROEKKAMP AND A. R. COOLS

Department of Pharmacology, University of Nijmegen, Geert Grooteplein N 21, Nijmegen, The Netherlands

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VAN DONGEN, P. A. M., C. L. E. BROEKKAMP AND A. R. COOLS. *Atonia after carbachol microinjections near the locus coeruleus in cats.* PHARMAC. BIOCHEM. BEHAV. 8(5) 527–532, 1978. – The effect of microinjections of carbachol into the dorsolateral pontine tegmentum on the behaviour of cats is investigated. Injections of small amounts (50 and 500 ng) of carbachol into the pontine reticular formation induced muscular atonia in otherwise awake animals. The atonia is not due to cholinergic stimulation of the noradrenergic cells of the locus coeruleus or the dorsolateral pons, since the most effective sites were situated ventrally to the locus coeruleus and alpha- and beta-adrenergic blocking agents did not affect the atonia. The results are discussed in view of the postulated role of the locus coeruleus in paradoxical sleep.

Locus coeruleus Pontine reticular formation Noradrenergic cells Cholinergic stimulation Atonia Paradoxical sleep

THE NOREPINEPHRINE (NE)-containing cells of the area of the locus coeruleus (LC) [9, 25, 33] are said to be involved in paradoxical sleep (PS) [10, 21, 26, cf. 24], activation of the hippocampal and neocortical EEG [19, 23, 32, cf. 24], learning [15, 51, cf. 43, 47], reward [14, cf. 11], vegetative [17, 39, 41, 44, 50] and other functions [36, 40, 42, 46]. The involvement of the LC, other pontine NE-containing cells and the pontine reticular formation in PS is still under discussion [1, 6, 10, 20, 21, 23, 24, 26, 27, 32, 44].

The LC is a cholinoceptive nucleus [5, 8, 30], so the role of this area can be investigated by cholinergic stimulation. Cholinergic stimulation of the pontine tegmentum (i.e., the area in which the LC is situated) induces a state of postural atonia or a state resembling PS [1, 4, 13, 18, 37]. But due to (1) insufficient documentation of the sites of injection, (2) few injections into the LC, and (3) high doses of cholinergic drugs, the involvement of the LC or other pontine NE-cells in cholinergic-induced effects is still uncertain.

In this study, the effects of low doses of carbachol injected in and around the LC were investigated. A study on the effectiveness of systematically given alpha- and beta-noradrenergic blocking agents on the carbachol-induced effects was included in order to investigate whether the effects were mediated via cholinergic stimulation of noradrenergic cells. The present report shows that the carbachol-induced atonia cannot be ascribed to stimulation of NE-cells of the LC or the subcoeruleus area. The implications of these data are discussed in relation to the role of the LC.

METHOD

Animals and Surgery

In this study 20 adult cats of both sexes were used. The animals were selected from a large stock for being easy to handle and non-aggressive. Under pentobarbital (Nembutal®) anaesthesia (30 mg/kg, IP; if necessary a supplementary dosage was given), two cannulas [12] were implanted aimed at the LC (Horseley-Clarke coordinates P 2.5, L 2.5, H −2.0). The diameter of the guide cannula and the injection cannula was 0.8 and 0.5 mm, respectively. The cannulas were placed in a parasagittal plane under an angle of 30° (Fig. 1). They pierced the visual cortex, superior colliculus and the central grey. This orientation was preferred to prevent diffuse of the drug to the ventricle [cf. 4, 29, 45].

Procedure

The animals were allowed to recover from the operation for at least two weeks. By gross inspection no behavioural signs of brain destruction by the cannulas could be detected after one week. Two days before the experiments, the cats were familiarized with the observation box (80 × 80 × 80 cm). Before the intracerebral administration of drugs, the animals were placed in the observation box and left undisturbed for 30 min, the last 15 of which the behaviour was observed via a closed TV circuit and recorded on tape in order to provide control recordings of behaviour. Then they received a unilateral intracerebral injection (0.5 μl) with a 5 μl Hamilton syringe, the needle of which extended 2 mm beyond the tip of the inner cannula. In four cats successive injections, each of them separated by two days, were made along a needle track from 2 up to 8 mm below the tip of the cannula. The drugs (dissolved in saline) were manually injected over a period of 1 sec. In none of the 42 histologically examined sites of injection we observed brain damage due to the injections (Fig. 1).

The behaviour was observed by means of a detailed ethogram [12] during at least 1 hr, the first quarter of

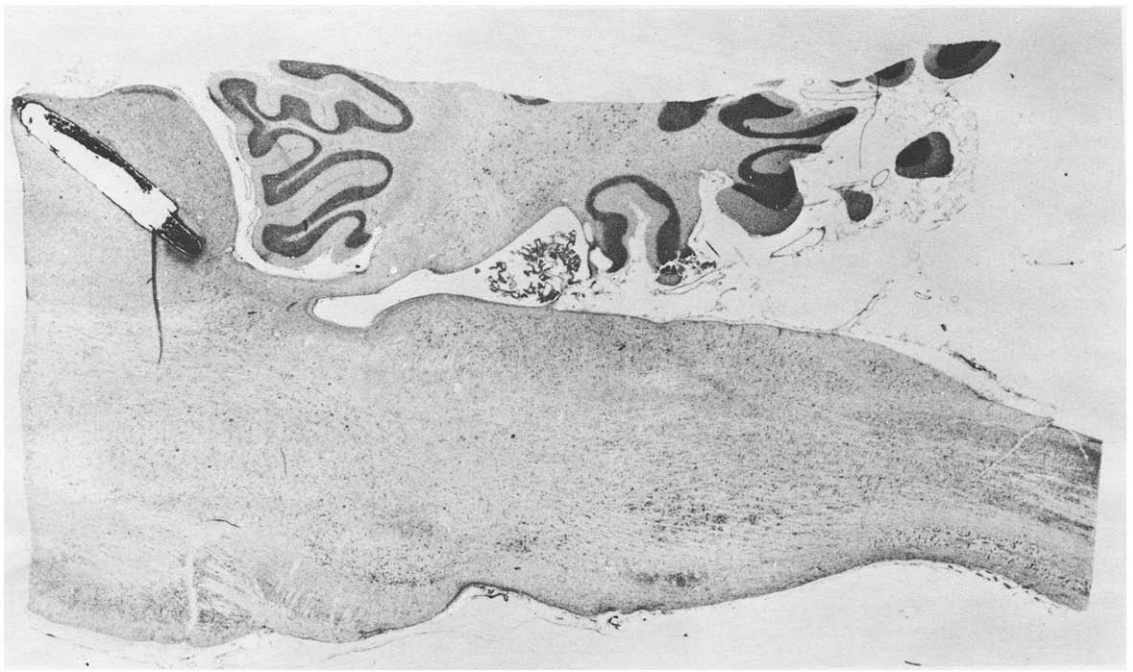

FIG. 1. Nissl-stained parasagittal section showing the track of the cannula to LC. This site of injection has been used for 9 times; note the absence of tissue damage due to the injections.

which the animals were left undisturbed. When the carbachol-induced effect consisted in the appearance of a posture indicating muscle relaxation (see RESULTS), additional behaviour assessments were made in order to characterize the nature of this phenomenon: (A) Measurement of the animal's ability to react to a) visual stimuli (a moving object in front of the animal), b) auditive stimuli (clicks or hissing noises behind and above the animal), and c) tactile stimuli (touching the animal's limbs, flanks and ears by the hand); special attention is paid to reactions with the eyes and ears. B. Measurement of the animal's motor ability by lifting a single limb, the head or the whole body from the floor and letting loose; attention is paid to resistance of the legs to movements, active flexion, raising the head and ability to stand, walk and jump. These tests were done 15 min after the injection or − in cases with longer latency − 5 min after the animals laid immobile.

Two days after the effectiveness of carbachol (0.1 μg) to elicite atonia was established, the drug (0.1 μg) was given to cats pretreated with phenoxybentzamine (20 mg/kg, N = 2) or DL-propranolol (2 mg/kg, N = 2) intravenously, one hr before the injection of carbachol. The rate of injection of phenoxybenzamine was 12 mg/min to prevent severe decrease of the blood pressure.

At the end of the experiments the animals were anaesthetized with nembutal and transcardially perfused with Susa-solution. The brains were sectioned in a parasagittal plane and stained with Nissl's stain. Fifty-nine sites, 47 of which were histologically verified, were used. As the distance between the tip of the needle and the tip of the cannula, which was clearly visible in the histological material (Fig. 1), was a given parameter, it was possible to determine the site of injection, when the amount of tissue shrinkage is known. This correction factor was calculated on the basis of 1) the actual size of the cannula and 2) the size of the cannula-induced lesion which was determined by means of a calibrated ocular-micrometer.

The following drugs were used: carbachol–HCl (Sigma), atropine-SO_4 (Ned. Farm.), mecamylamine-HCl (Merck, Sharp and Dohme), physostigmine-SO_4 ("De Onderlinge Pharmaceutische Groothandel"), L-noradrenaline-HCl (Fluka), clonidine-HCl (Boehringer, Ingelheim), oxymetazoline-HCl (Ciba Arnhem, The Netherlands), isoprenaline-bitartrate (Sigma), piperoxane-HCl (Brocades), L-propranolol-HCl (ICI, U.K.), DL-propranolol-HCl (Sigma), phenoxybenzamine-HCl (Dibeniline®; Smith, Kline and French), 6-hydroxydopamine-HBr (Sigma) and 5-hdyroxytryptamine-creatinine-SO_4 (Koch-Light Laboratories).

RESULTS

The induction of the needle sometimes produced a sudden twitch of the head or shaking of the head. Apart from this injection artifact, administration of saline did not affect the behaviour. After unilateral injections of carbachol (50 or 500 ng) different symptoms were observed; only one of them will be described in detail in this report: postural atonia.

Postural Atonia

Phenomenology. In 36 out of 59 sites of injection (in 13

cats), application of 0.5 μg of carbachol induced a state of muscle relaxation. Before the muscle relaxation was apparent, the animals adapted a sitting posture, bended down in a prone position. Then their heads gradually fell until they came to rest with their nose or cheek on the floor (latencies ranging from 20 sec to 32 min in an extreme case). Finally, the animals lied down in an abnormal position: they were never curled up, but were lying on their flank or belly with their head on the floor.

When the animals were left undisturbed, they stayed immobile in this position for 7 to 19 min. Then small movements with the head or legs were made for 10 to 30 sec. In eight animals, a small tremor was superimposed on these movements. Again a period of immobility lasting up to 16 min appeared. Short periods with movements and longer periods of immobility alternated. The tremor could generalize over the whole body, especially when the animals apparently made attempts to move. After 25 to 95 min, the animals stood up, made a few staggering steps, sat down, and again their head gradually sank to the floor. Up to 140 min after the injection, the animals remained immobile in the above-mentioned position for several minutes. Even 4 hr after the injection, the locomotion was not fully normal.

Behaviourally, the animals were not asleep. The eyes were open all the time. The pupils were normal, and slightly miotic in two cases. The nictitating membrane remained retracted, except in certain cases (see localization). The eye movements resembled those of awake animals. Although particular attention was paid to phasic behavioural signs of PS such as rapid eye movements, sudden movements of the ears, the whiskers and the facial muscles, these movements were not observed.

When lifted from the floor, the animals were slackly hanging in the hand of the experimentator. The big saline-treated animals: sometimes only with eyes and ears and sometimes also by lifting the head, and turning to the stimulus. An initially relaxed nictitating membrane was retracted.

When the animals lifted from the floor, they were slackly hanging in the hand of the experimentator. The big postural muscles of the neck, the trunk and the legs were atonic. The animals could not stand or move, but sagged under their weight. Only small movements with the paws were made. Although the drug was unilaterally applied, the animals showed bilateral muscle relaxation. At two sites of injection, only at near threshold doses, the ipsilateral legs were more affected than the contralateral ones. The carbachol-induced effect is called atonia, when the animal is unable to stand (other authors described a flat EMG after local injection of carbachol into this area, demonstrating real atonia [1, 18, 37]). The latency of the atonia is defined as the moment that the head of an animal with atonia touched the floor and remained immobile for at least 5 min.

Effectiveness. Two days after an effective injection of 0.5 μg carbachol, a subsequent injection of 50 ng was always effective (13 sites tested). In a single cat still lower doses were tested: 20 ng induced atonia, while 10 ng caused some motor disturbances when the animal walked or jumped.

Antagonism. Atropine (1.0 μg, $1{,}5.10^{-9}$ mol), locally injected 5 min after the beginning of the carbachol-induced atonia, completely disrupted the atonia (N = 4). The latency of this disruption was of the same order of magnitude as the latency of the atonia. On the contrary, a local injection of mecamylamine (0.3 μg, $1{,}5.10^{-9}$ mol) had no or a very weak transient effect on the carbachol-induced atonia (N = 4).

Localization. The localization of the sites of injection inducing atonia is plotted in Fig. 2 and indicated in Table 1. Compared to sites of injection into and dorsal to the LC, sites ventral to the LC were more effective in eliciting atonia ($\chi^2(1) = 12.4$, $p<0.001$). Moreover, when an injection into the LC induced atonia, the latency of the atonia was medium to long, while in ventral sites atonia with a short latency was elicited. After injections in ventral parts of the nucleus pontis centralis caudalis relaxation of the nictitating membrane is observed. Additionally, local injections of carbachol into the fourth ventricle did not induce atonia (N = 5, not shown).

Atonia and NE-transmission. High doses of phenoxybenzamine (20 mg/kg) and DL-propranolol (2 mg/kg) made the animals behaviourally sedated and tranquilized [cf. 28, 38], while after phenoxybenzamine the nictitating membrane was relaxed and the eye-lids were half closed; still, they did not show any effect comparable to that elicited by carbachol alone.

Yet, the carbachol-induced atonia remained unaffected by these drugs in the animals tested: the animals were sitting, but only after a microinjection of carbachol they lied slackly with their head on the floor.

Other drugs. The carbachol-induced atonia was not mimicked by local injections of physostigmine (2 or 20 μg, N = 6, see DISCUSSION), L-noradrenaline (10 μg, N = 15), clonidine (5 μg, N = 18), oxymetazoline (5 μg, N = 8), isoprenaline (5 μg, N = 10), piperoxane (5 μg, N = 8), L-propranolol (10 μg, N = 10) or 5-hydroxytryptamine (10 μg, N = 3).

Miscellaneous

Behavioural asymmetry and rotations were elicited at 24 sites of injection. Microinjections into and dorsal to the LC caused contraversive rotations, while in the pontine reticular formation ipsiversive rotations were elicited. Also other behavioural effects were incidentially observed after unilateral injections of 0.5 μg of carbachol. At five sites, the respiration was accelerated or irregular. The facial expression was disturbed after injections at two sites. After injections at three sites, the period of postural atonia was followed by real and reproducible aggression. The cats, having sidewards turned ears and small pupils characteristic for attack behaviour [31], attacked the experimentator. The involvement of NE-cells in these effects is the subject of further investigation.

DISCUSSION

Specificity

The atonia induced by injection of carbachol in the dorsolateral pons appeared to be due to specific interaction between carbachol and muscarinic acetylcholine receptors, as suggested by the outcome of the experiments with muscarinic and nicotinic acetylcholinoreceptor blocking agents [cf. 18, 37]. Still, physostigmine remained ineffective. In order to understand this seemingly conflicting result, one has to realize that physostigmine will only be effective on the conditions that there is a sufficient release of endogenous acetylcholine. We suggest this is not the case

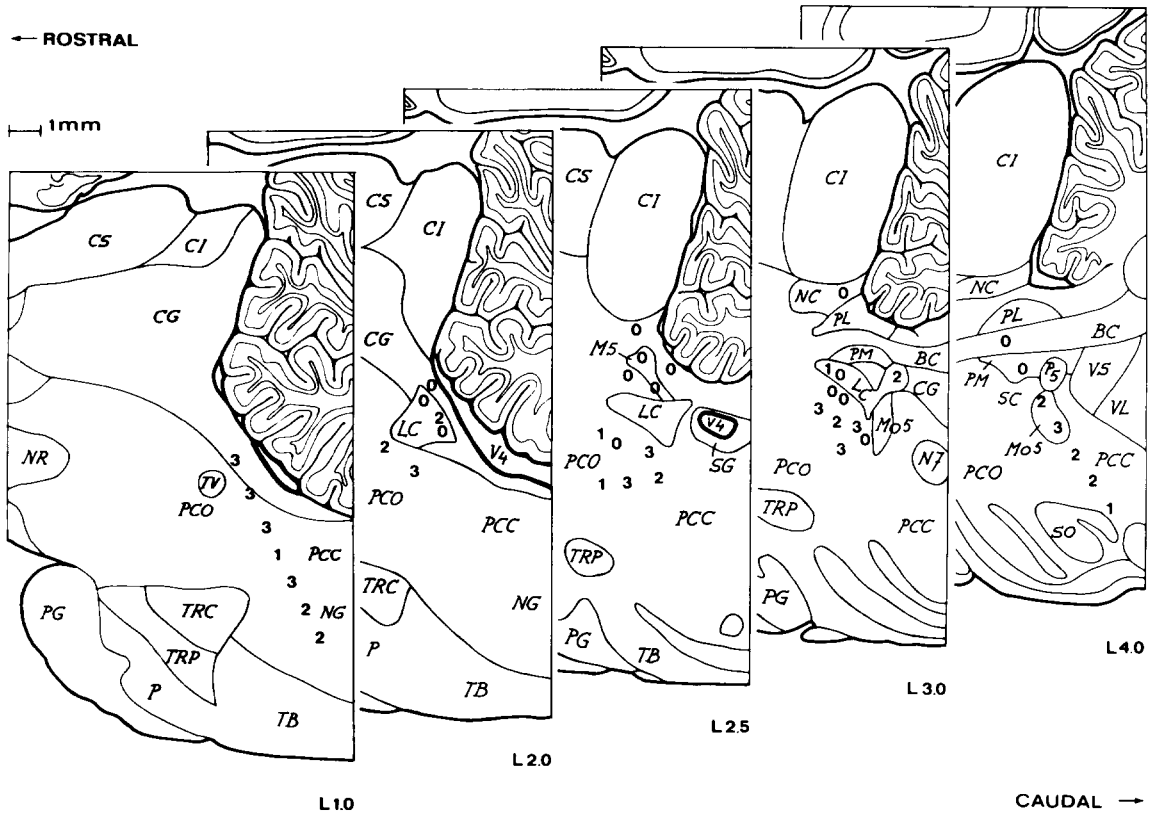

FIG. 2. Localization of the sites at which 0.5 μg of carbachol induced postural atonia, in parasagittal plance modified from Berman [7]. 0 = no atonia; 1 = atonia with a latency longer than 10 min; 2 = atonia with a latency between 5 and 10 min; 3 = atonia with a latency shorter than 5 min. Abbreviations: BC = brachium conjunctivum; CG = substantia grisea centralis; CI = colliculus inferior; CS = colliculus superior; LC = locus coeruleus; M5 = nucleus tractus mesencephalici nervi trigemini; Mo5 = nucleus motorius nervi trigemini; N7 = nervus facialis; NC = nucleus cuneiformis; NG = nucleus gigantocellularis; NR = nucleus ruber; P = tractus pyramidalis; P5 = nucleus gigantocellularis; NR = nucleus ruber; P = tractus pyramidalis; P5 = nucleus sensorius superior nervi trigemini; PCC = nucleus pontis centralis caudalis; PCO = nucleus pontis centralis oralis; PG = pontine grey (nuclei pontis); PL = nucleus parabrachialis lateralis; PM = nucleus parabrachialis medialis; SC = subcoeruleus area; SO = oliva superior, TRC = nucleus reticularis tegmenti pontis, pars centralis; TRP = nucleus reticularis tegmenti pontis, pars pericentralis; TV = nucleus tegmenti ventralis; VL = nucleus vestibularis lateralis; VS = nucleus vestibularis lateralis; VS = nucleus vestibularis superior.

TABLE 1

LOCALIZATION OF SITES OF INJECTION AND OCCURRENCE AND LATENCY OF MUSCULAR ATONIA

Effect	Dorsal to LC	LC	Ventral to LC	Into Ventricle
no atonia	8	3	6	5
atonia	0	3	22	0
latency 5 min	0	0	11	0
latency 5–10 min	0	2	7	0
latency 10 min	0	1	4	0
Total	8	6	28	5

in our awake cats [cf. 22, 48]. The carbachol-induced atonia was not caused by diffusion of the drug to the ventricular system [cf. 4, 45], since local injections of carbachol into the fourth ventricle did not induce atonia and most of the sites of injection near the ventricular wall were not effective, while more remote sites were (Fig. 2). Carbachol not only influenced neurones but also cerebral blood vessels via muscarinic receptors. Low doses of carbachol cause vasodilation like β-adrenoreceptor stimulating agents, while high doses cause vasodilatation like α-adrenoreceptor stimulating agents [16]. Yet, neither α-adrenoreceptor stimulating drugs (clonidine, oxymethazoline) nor β-adrenoreceptor stimulating drugs (isoprenaline) nor L-noradrenaline itself induced atonia. So, the carbachol-induced atonia was not due to influences of carbachol on the cerebral circulation.

Structures Involved in Muscular Atonia

The present study shows that the carbachol-induced atonia was mediated by pontine reticular structures such as the nucleus pontis centralis caudalis and oralis and the nucleus gigantocellularis. Neither the noradrenergic cells of the LC nor noradrenergic cells of the pontine tegmentum were involved, as is shown by the following findings:

(1) The most effective sites of injection inducing muscular atonia were situated in pontine reticular fields medial and ventral to the LC and SC. Only three out of six sites in the LC were effective. The atonia following injections into the LC appeared with medium to long latencies, suggesting diffusion of the drug to more ventral or medial sites, at which short latency responses could be evoked.

(2) Blockade of the alpha- and beta-noradrenergic transmission did not disrupt the carbachol-induced atonia. Yet, the alpha- and beta-blocking agents used are capable of penetrating the brain [34,35] and the doses used were high enough to diminish spinal and cortical NE transmission [2,3].

(3) In two animals the LC was destructed by a unilateral local injection of 6–OHDA (3 μg into 3 μl nitrogen-bubbled saline solution, with 1.0 μg ascorbic acid, injected with a rate of 1 μl/min). Histologic examination revealed great unspecific brain damage with a diameter of 3 mm. All of the LC cells were destructed. Carbachol (0.5 μg) injected at the same site as 6–OHDA, 4 and 14 days after the injection of 6–OHDA, was as effective as before in eliciting atonia (data not shown). In this context, it should be noted that even a low dose of 6–OHDA did not produce any specific lesion as it could be expected on the basis of its selective effect in rat [49]. Similar observations as above mentioned in cats have been done by others (Ungerstedt, personal communication).

(4) Although both acetylcholine and piperoxane increase the firing rate of LC cells [5,7], piperoxane was unable to mimic the carbachol-induced atonia, suggesting that other cells than those of the LC were involved.

In conclusion, the present study dealing with a large number of tested brain sites and measurement of the effectiveness of low doses of carbachol gives harder evidence in favour of Amatruda's suggestion concerning the involvement of non-noradrenergic pontine reticular structures in the carbachol-induced atonia [1].

Implications: Muscular Atonia and Paradoxical Sleep

Cholinergic stimulation of the pontine reticular formation induces a state of muscle atonia in cats, while the animals remain awake and have no behavioural signs of paradoxical sleep (PS). On the other hand, after electrolytic lesions in this area a normal amount of PS is observed, with the phasic and tonic characteristics of PS, except muscular atonia which is completely absent (e.g., [20,24]). So, it is assumed that conclusions with regard to carbachol-induced atonia also apply to atonia during PS:

(1) Cholinergic stimulation of non-noradrenergic cells in the pontine reticular formation generates the atonia during PS (cf. [6, 24, 27]).

(2) These cells generate just the atonia and not the whole pattern of PS (cf. [20, 24, 37]).

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