ONTOGENY OF TWO CHOLINERGICALLY MEDIATED CENTRAL EFFECTS: STEREOTYPED YAWNING AND POTENTIATION OF HEAD-SHAKING

Björn HOLMGREN and Ruth URBA-HOLMGREN
Departamento de Neurofisiologia, Centro Nacional de Investigaciones Científicas Habana, Cuba

Abstract. The ontogenetic course of two cholinergically mediated central neuropharmacological effects, yawning and potentiation of head-shaking induced by D-amphetamine (5 mg/kg), was explored in developing rats. Physostigmine (0.1 mg/kg) and pilocarpine (4 mg/kg) evoke stereotyped yawning in neonatal rats, the effect declining in the middle of the second week of life. Both cholinomimetic drugs strongly potentiate amphetamine induced head-shaking between the 4th and 10th postnatal days. Pilocarpine per se is capable of inducing head-shaking, in the absence of amphetamine, in rats from 8 to 12 days. Infant rat yawning and head-shaking are blocked by scopolamine (5 mg/kg). Nicotine (0.1 mg/kg) potentiates head-shaking but inhibits yawning. Yawning is also depressed by D-amphetamine. The early maturation of these cholinergic effects is discussed in comparison to the later maturation of several forebrain cholinergic systems.

INTRODUCTION

It is generally accepted that in the course of ontogenetic development different structure-functional systems in the brain mature heterochronously (2, 12).

Nevertheless, the exact timing in maturation of functionally interrelated systems, as, i.e., the monoaminergic and cholinergic pathways or
structures in the brain, remains as an open question, and an active field of research, in which many relevant morphological, physiological, biochemical, neuropharmacological and behavioral contributions have appeared in recent years (1, 3, 6, 8, 11, 17, 21, 24, 26, 28, 30, 31).

McGeer et al. (28) have made an effort to correlate the differential development of caudate enzymes with behavioral effects induced by amphetamine and scopolamine in neonatal rats. As amphetamine was able to induce stereotyped gnawing behavior already in 10-day-old rats, while a synergistic action of scopolamine on this behavioral pattern was only significant around the 30th postnatal day, they concluded that “10-day-old rats have no functioning cholinergic mechanisms to counteract the dopaminergic effects initiated by amphetamine”. Quite comparable observations and conclusions regarding the ontogeny of adrenergic arousal and its cholinergic modulation had previously been made in the same species by Campbell et al. (8) and Fibiger et al. (11), who demonstrated that scopolamine and pilocarpine, which depend upon cholinergic mechanisms, did not influence arousal and locomotor activity until the 20th day, while amphetamine was active already 10 days after birth.

Other central cholinergic mechanisms seem to mature earlier. Studying the ontogenesis of drug-induced tremor in the rat, Henderson and Woolley (17) observed that the tremorogenic effects of tremorine and oxotremorine do not appear in rats until the ninth postnatal day. As these drugs exert their action upon cholinergic systems, it is reasonable to presume that the responsible cholinergic synapses, critical for this tremor to appear, begin to be functional around this day.

We have studied in detail the ontogeny of a particular motor item, D-amphetamine induced head-shaking (19) and disclosed the relative contributions in it of both dopaminergic and noradrenergic elements (20). Early maturing synergistic cholinergic mechanisms seem also to be involved in this motor pattern because, in 9-day-old rats D-amphetamine induced head-shaking is blocked by scopolamine, intensely potentiated by physostigmine or pilocarpine, and this last drug is capable by itself, in the absence of amphetamine, to evoke the same motor item (18). Both abovementioned centrally active cholinomimetic agents also induce frequent or stereotyped yawning in infant rats (38). Neostigmine, which passes the blood–brain barrier with difficulty, does not potentiate head-shaking (18) and has only a negligible yawning inducing effect (28).

Two different, early maturing, cholinergically mediated central effects, potentiation of D-amphetamine induced head-shaking (H-S) and yawning, both of which are easily quantifiable, are thus available for a comparison of their ontogenetic course, which is the aim of the present study.
MATERIAL AND METHODS

Our observations and experiments have been done in infant albino rats, born in the laboratory from pregnant females of a Wistar strain, obtained from the Animal House of the Ministry of Public Health, Cuba. The age of the experimental litters was estimated, in general, with an approximation of $\pm 8$ h. For experiments with rats younger than 4 days only animals in which the birth time was known with a precision of $1\pm h$ were used. Litters were regularly reduced to eight pups between 24 and 36 h after birth. The rats weighing below or above 1 s.d.m. from the corresponding mean weight were excluded from the experiments. All experiments were performed in early morning hours (8 to 10 AM) because important circadian variations have been observed in D-amphetamine induced H-S (37). Each litter was equally distributed, at random, between experimental and control groups. The animals were tested only once. For the behavioral observations, the animals, after being injected, were placed in groups of two, in transparent glass cylinders (diam., 18.5 cm; height, 9 cm) the floor of which was covered with a sheet of

Fig. 1. Cholinomimetic-induced yawning in a 9-day-old rat.
filter paper. The only motor items that were attentively observed for quantitative evaluation were the H-S episodes and yawning. The duration of the former was measured with stopwatches and yawns were simply counted.

Head-shaking in infant rats, as formerly described in detail (19), consists of regular rotatory shaking or rocking movements of the head, at a frequency varying from 4 to 10 Hz, the frequency increasing with age. Yawning is one very obvious motor item in a set of cholinergic effects. Generally preceded by salivation, licking of the forepaws or cleaning movements of the snout, "gumming" or sucking movements, and sometimes by a jerky extension of the forelimbs, the yawn itself is a slow wide opening of the mouth which lasts around 3 s (Fig. 1).

Freshly prepared solutions of the following drugs were used: D-amphetamine sulphate (Rhone-Poulenc), physostigmine salicylate (Sigma Chemical Co.), scopolamine hydrobromide (Merck), pilocarpine hydrochloride (C. H. Boehringer) and nicotine (BDH Chemicals Ltd.). All drugs, except D-amphetamine sulphate, are expressed in mg/kg body weight, of the free base. The drugs were dissolved in saline (0.9% NaCl) so that the total volume to be injected i.p. was always equivalent to 0.01 ml/g body weight. Controls were injected with saline.

Statistical procedures are mentioned with the results. Two standard nonparametric tests were generally used: the Kruskal–Wallis Test for variance, and the Mann–Whitney U Test (34).

RESULTS

Ontogeny of cholinergic potentiation of head-shaking

Figure 2 presents the results of comparing the total duration of the head-shaking episodes, during an observation period of 1 h, in five groups of animals, from 4 to 16 days old: one control group, injected with saline, and four groups under different pharmacological conditions. The curves corresponding to the ontogenetic evolution of spontaneous and D-amphetamine induced H-S follow the general trend previously described (19). It may be observed that rats from 7 to 10 days old, injected with D-amphetamine, rock their heads for more than 400 s, as a mean value, during the observation period, that is to say, more than a 100% of the time.

While physostigmine per se (in doses equivalent to 0.1 and 0.2 mg/kg) does not induce H-S, when injected together with D-amphetamine (5 mg/kg), it exerts a clear potentiating effect, which is statistically
significant from 7 to 12 days. On the other hand, pilocarpine, which by itself induces H-S in rats from 8 to 12 days, potentiates the D-amphetamine action already in 4-day-old rats. On the 9th day the cholinergic potentiating effect is so intense that the animals shake their heads, as an average, for approximately two thirds of the observation period.

![Graph](image)

Fig. 2. Ontogeny of cholinergic potentiation of head-shaking. H-S time recorded during 1 h of observation. N = 10–14 animals for each age and drug tested. Drug doses: D-amphetamine sulphate, 5 mg/kg; Pilocarpine, 4 mg/kg; physostigmine, 0.1 mg/kg. Controls injected with saline. Kruskal-Wallis tests (34) for the analysis of variance, applied to the H-S data obtained at all ages for each particular drug situation showed significant differences (P < 0.05). Comparison between controls and experimental groups by the Mann-Whitney U test shows the following results: amphetamine, from 7 to 10 days, P < 0.01; pilocarpine, from 8 to 11 days, P < 0.02; 12 days, P < 0.05; amphetamine + physostigmine, from 7 to 12 days, P < 0.01; amphetamine + pilocarpine, from 6 to 11 days, P < 0.01; 4 and 12 days, P < 0.05.

**Evolution of cholinergic stereotyped yawning**

The results presented in Fig. 3 confirm preliminary experiments performed with physostigmine salicylate (0.1 mg/kg) injected i.p. in neonatal and infant rats, in which frequent yawning was a most relevant behavioral feature (37). It includes similar observations with pilocarpine (4 mg/kg) in rats from 2 to 16 days. In its period of maximal expression, around the 4th postnatal day, cholinergic yawning reaches an average level of around 1 yawn/min, the effect declining rather abruptly with age, to a level of 4–6 yawns/h from the 11th day onwards. Control rats practically do not yawn at all, except on the first postnatal days, when this motor item is occasionally observed. If rat pups are simultaneously
Fig. 3. Ontogeny of cholinergic stereotyped yawning. Observation begun 5 min after drug injection. N = 12 rats for each age and drug tested. Drug doses: pilocarpine, 4 mg/kg; physostigmine, 0.1 mg/kg controls injected with saline. The quantitative differences in the yawning responses at the different ages with each of the drugs studied are significant (Kruskal–Wallis Test, P < 0.05) Experimental results significantly different from the controls at the level of P < 0.01 or less (Mann–Whitney U test).

Fig. 4. Depressive effect of amphetamine on pilocarpine-induced yawning. N = 12 rats for each age and drug tested. Drug doses: D-amphetamine sulphate, 5 mg/kg; pilocarpine, 4 mg/kg. Controls injected with saline. Drug effects statistically different from the controls at the following levels: Pilocarpine from 2 to 9 days, P < 0.001; 12 days, P < 0.01; 11 and 16 days, NS; pilocarpine + amphetamine: 2 and 9 days, P < 0.05; 4 and 6 days, P < 0.01; other days, NS. Pilocarpine vs. pilocarpine + amphetamine, from 2 to 8 days, P < 0.001; 9 days, P < 0.01; 12 days, P < 0.05; 11 and 16 days, NS (Mann–Whitney U test).
injected with pilocarpine and D-amphetamine, as is illustrated in Fig. 4, a strong depressing effect of amphetamine on pilocarpine-induced yawning is clearly observed.

**Cholinergic receptors involved in head-shaking and yawning**

As pilocarpine per se induces both yawning and head-shaking in neonatal rats, it was thought of some interest to try to establish if these effects are exerted through the same type or through different cholinergic mechanisms. A comparison of the ontogenetic courses of pilocarpine-induced yawning and head-shaking, as illustrated in Fig. 4 and 2 respectively, shows that both curves are quite different. But a simultaneous study of the dose-effect curves of the two abovementioned pharmacological actions, performed in a group of 9-day-old animals, shows, nevertheless, striking similarity between both curves in the dose range studied (Fig. 5).

![Graph showing dose-effect curves of pilocarpine-induced yawning and head-shaking](image)

**Fig. 5.** Dose-effect curves of pilocarpine-induced yawning and head-shaking. *N* = 12 9-day-old rats for each dose tested. Controls (0 mg/kg), injected with saline. Dose-effect curves significant according to Kruskal–Wallis test for variance, *P* < 0.05. Differences between pilocarpine injected animals and controls statistically significant (Mann–Whitney U test) as follows: (i) Yawning: at all doses, *P* < 0.001; (ii) H-S: 1 mg/kg, *P* < 0.025; 2, 5 and 10 mg/kg, *P* < 0.01; 4 mg/kg, *P* < 0.001.

The rapid suppression of pilocarpine-induced yawning by the administration of a cholinolytic agent that passes the blood–brain barrier, as scopolamine hydrochloride, as is shown in the experiment illustrated in Fig. 6, suggests that “muscarinic” receptors are responsible for the yawning effect. A similar conclusion had formerly been reached regarding the cholinceptive receptors involved in head-shaking (18).
The possible participation of "nicotinic" cholinceptive receptors, at some central synaptic links of the neuronal circuits involved in H-S or yawning, was explored by the simultaneous administration of nicotine to rats receiving amphetamine or pilocarpine. Nicotine, in doses of 0.1 or 0.2 mg/kg, induces H-S at a level slightly above the controls, in 9-day-old rats (Fig. 7), even in the absence of amphetamine, but clearly potentiates the action of the latter drug, although not reaching the values already described for physostigmine or pilocarpine.

If experiments are performed with pilocarpine and nicotine on 6 or 7-day-old rats, in which the first drug is able to induce both H-S and yawning at quite a high level, results as illustrated in Fig. 8 are obtained.
While nicotine acts synergically with pilocarpine in the induction of head-shaking, its action is antagonistic with the latter drug in relation to its yawning-inducing effect.

Fig. 8. Nicotine action on pilocarpine induced head-shaking and yawning. H-S time recorded during 1 h observation. N = 12-14 animals for each group. Drug doses: pilocarpine, 4 mg/kg (P); nicotine, 0.1 mg/kg (N). Differences between pilocarpine and pilocarpine + nicotine injected animals as follows: head shaking $P < 0.05$; yawning $P < 0.001$ (Mann-Whitney U test).

**DISCUSSION**

In the preceding results we have described two early maturing behavioral items (32), observable in the neonatal rat, in which central cholinergic synaptic links seem to be involved. One of these motor patterns, yawning, is evokable in a stereotyped fashion, by i.p. injection of physostigmine as early as 6 h after birth. The yawn-inducing effect of physostigmine declines rather abruptly, from a relatively high rate (1 yawn/min) to a low level, between the 8th and 12th day. Pilocarpine-induced yawning follows practically the same ontogenetic evolution (Fig. 3). Precisely because of its early appearance, and based on the classical Jacksonian principle of a caudo-rostral maturational gradient of the CNS, it may be assumed that a hypothetical diffuse or localized command center (32), responsible for the triggering and/or coordination of yawning, might be localized in caudal brainstem structures. Some evidence in support of this assumption may be found in the demonstration by Mc Caman and Aprison (27) that both acetylcholinesterase and cholineacetylase reach earlier maxima in the rabbits medulla than in the thalamus or the caudatum. While on the 3rd postnatal day these enzymes reach in the medulla levels around 2/3 of those measured at the 32nd day, in the n. caudatus, acetylcholinesterase level is only 1/4 and cholineacetylase only 1/6 of their respective levels assayed in 1-mo-old rabbits.
Although we are willing to comply with Bureš’ (5) assertion that: “no definite behavior can be claimed to be specifically dependent on cholinergic transmission, which diffusely participates in activities of different CNS structures”, our experimental data point towards a predominant weight of “muscarinic” cholinergic influences in the induction of yawning (induction by physostigmine or pilocarpine, and blocking by scopolamine). Catecholaminergic effects, as evoked by D-amphetamine, are in this case clearly antagonistic (Fig. 4). Nicotine, a cholinergic drug acting on “nicotinic” receptors is also a yawning inhibitor (Fig. 8), effect which well could be due to the catecholamine liberating action of nicotine, as has been suggested by some authors (9, 13, 22, 33). We have not yet explored possible participation of serotoninergic mechanisms in yawning.

The maturation of D-amphetamine induced head-shaking follows a different chronological course. Not evident under any circumstances before the 4th postnatal day (Fig. 2), H-S increases to its highest level on the 8th or 9th, is reduced abruptly by the 10th, and is scarcely apparent by the 11th or 12th day. The different concurrent synaptic mechanisms underlying this motor item (18, 20) seem to mature very synchronously, with only slight temporal deviations. On the 4th postnatal day amphetamine alone induces H-S only by exception. But apomorphine, which stimulates directly post-synaptic dopaminergic receptors (10,25), or particularly, a combined i.p. injection of apomorphine and D-amphetamine, are capable of inducing H-S at this early period (20). This suggests that postsynaptic dopaminergic receptors are functionally mature about 1 day before the presynaptic structures are sufficiently developed so as to be able to evoke the former’s full potential activity, by transsynaptic action. A slight synergic effect on H-S in 4-day-old rats is also evident between D-amphetamine and pilocarpine (Fig. 2), synergism which rapidly grows into an intense potentiation, with a maximum effect on the 9th day. We have elsewhere (18) extensively discussed the different CNS levels at which cholinergic structures might exert this potentiating action on D-amphetamine induced H-S. It could be directly on the dopaminergic neurons activated by D-amphetamine, as it has been demonstrated that neurons in the pars compacta of substantia nigra contain acetylcholinesterase (7,35) thus suggesting their cholinceptive character, and their possible activation by cholinergic inputs (4). But it could alternatively or concurrently take place on cholinergic interneurons in the striatum (4, 14, 15, 29) or on the cholinergic pallido-mesencephalic efferent neurons (16). This last possibility seems attractive, because mesencephalic cholinceptive structures are functional in the rat around the 9th postnatal day, as judged by oxotremorine induced tremor (17), the
neuroanatomical substrate of which is considered to be located at this particular level of the brain stem (36). It is not discarded that more caudally placed cholinergic neurons in the rhombencephalic reticular formation might also be contributing to head-shaking, as it has been demonstrated that some reticular influences on vestibulospinal neurons include a cholinergic synaptic link (23). The strikingly similar dose-effect curves of pilocarpine evoked H-S and yawning when explored simultaneously in 9-day-old rats (Fig. 5) are suggestive of at least the same type of receptor being involved (if not of similarly anatomically situated receptors). The early concurrent effect of pilocarpine on d-amphetamine induced H-S, already evident on the 4th postnatal day, is also an argument in favor that cholinceptive structures, caudally placed in the brain stem, might be implicated in H-S. The facilitating effect of nicotine on head-shaking is coherent with the idea that “nicotinic” cholinergic receptors might act through the release of catecholamines (9, 13, 22, 23). We have demonstrated that very early postnatal behavioral effects may be induced in rats by pilocarpine and/or physostigmine, and may be blocked by scopolamine, thus strongly suggesting that some central cholinergic mechanisms are functionally mature, in the case of yawning some hours after birth, in that of head-shaking, on the fourth postnatal day.

These results, as well as those already mentioned in relation to oxotremorine tremor (17), stand in sharp distinction with pharmacological evidence showing that several forebrain cholinergic systems, as those functionally balancing the catecholaminergic mechanisms involved in arousal or locomotory activity (8, 11) in stereotyped gnawing, licking or sniffing behavior (28), or those participating in neuroleptic-induced catalepsy (3), do mature much later.

REFERENCES

5. BURES, J. 1968. The effect of physostigmine and atropine on some behavioral and electrophysiological functions in rats. In P. B. Bradley and M. Fin


Accepted 19 September 1977

Björn HOLMGREN and Ruth URBÁ-HOLMGREN, Centro Nacional de Investigaciones Científicas, Apartado 6990, Habana, Cuba.