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Age-Dependent Effects of NGF and Scopolamine on Suckling Behavior of Neonatal Mice

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CALAMANDREI, G. AND A. VALANZANO. *Age-dependent effects of NGF and scopolamine on suckling behavior of neonatal mice.* PHARMACOL BIOCHEM BEHAV **49**(4) 1043–1048, 1994.—Nerve growth factor (NGF) influences the neurochemical differentiation of central cholinergic neurons of developing rodents. In this study, NGF was given intracerebrally to mice on different postnatal days (days 5 and 7, or days 8 and 10). Pups were tested for suckling behavior 24 h after the second NGF injection, following systemic administration of either the muscarinic cholinergic antagonist scopolamine or saline solution. Scopolamine significantly impaired nipple attachment on day 11 but not on day 8, and decreased locomotor activity in 11-day pups. NGF given on days 5 and 7 increased paddling and treading on day 8, and this effect was more pronounced in scopolamine-injected pups. Pretreatment with NGF on days 8 and 10 decreased activity levels in 11-day pups. The differences in the effects of scopolamine at successive ages suggest that distinct portions of the cholinergic system mature at different rates and that sensitivity to NGF is age dependent. NGF appears to influence functional maturation of that portion of the cholinergic system involved in the regulation of locomotor activity.

Cholinergic system development Nerve growth factor Scopolamine Suckling Locomotor activity Neurobehavioral ontogeny Mouse

NERVE growth factor (NGF) is a specific neurotrophic factor of sensory and sympathetic neurons of the peripheral nervous system, which require NGF for development, survival, and maintenance of function (17,26). Within the mammalian brain, NGF exerts its trophic action on basal forebrain cholinergic neurons (14,15,18,25). Specifically, high NGF levels have been found both in regions innervated by the magnocellular cholinergic neurons of basal forebrain (hippocampus, olfactory bulbs, neocortex) and in regions containing the cell bodies of these neurons, such as septum (16,28).

Forebrain cholinergic neurons appear to be the targets for NGF even in the very early postnatal phases. Both NGF mRNA expression and NGF protein synthesis reach the adult levels at the end of the third postnatal week in rats, that is, at a time when some portions of the cholinergic system undergo a rapid maturational transition (28). NGF accumulation in the basal forebrain parallels that in the hippocampus and neocortex, and precedes an increase in the activity of choline acetyltransferase (ChAT), the enzyme involved in the synthesis of acetylcholine, suggesting that the neurochemical differentiation of magnocellular cholinergic neurons is regulated by retrogradely transported NGF (16). A marked increase in ChAT activity as well as in acetylcholine concentration is observed in septum, hippocampus, nucleus basalis, neocortex, and caudate-putamen of neonatal rats after a single intracerebroventricular (ICV) NGF administration (13,18,19). Conversely, ChAT activity and expression are reduced in these same areas by anti-NGF administration (27).

The central cholinergic system is generally thought to attain functional maturity around the end of the third postnatal week in altricial rodents (23), when animals start responding to cholinergic antagonists such as scopolamine with a dramatic increase in locomotor activity (1). Some cholinergic functions, however, appear to be functional already at an earlier postnatal stage. Suckling behavior, latency to choice in a T-maze, spontaneous alternation, and conditioned response suppression are some of the different behavioral endpoints affected by pharmacological manipulation of cholinergic

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transmission as early as the first postnatal week (9,21,22). Several recent results indicate that early NGF administration can potentiate, or anticipate, the emergence of behavioral responses that are deemed to be under cholinergic control. Around the weaning period, a single ICV NGF administration given 24 h prior to the activity test enhances the hyperkinetic effect of scopolamine in 21-day-old mice (4). A single intrahippocampal injection of NGF (postnatal day 8 or 13) accelerated the development of spontaneous alternation by 5 days in rats (12). Recently, we were able to anticipate the appearance in mice of scopolamine-induced hyperactivity (which normally sets in at the end of the third postnatal week in rodents) to postnatal day (pd) 5, by injecting NGF ICV on pds 2 and 4. In addition, NGF pretreatment enhanced the scopolamine blocking effect on nipple attachment recorded on pd 5, without altering any other components of the suckling behavior (7).

It has been hypothesized that the NGF-mediated increase in cholinergic neuron metabolism is associated with an accelerated formation of cholinergic synaptic contacts or with an earlier onset of synaptic function (13). Indeed, the behavioral findings are supported by recent in vitro evidence, indicating that exposure to NGF of telencephalic neurons in primary cultures affects the density of muscarinic cholinergic receptors (11). Quantitative and/or qualitative changes in brain muscarinic receptor populations upon early exposure to NGF could explain both the enhancement of some scopolamine effects that normally occur at an early postnatal stage, and the dramatic anticipation of the hyperactivity response to the cholinergic blocking agent.

Our aim was to verify the existence of age-specific developmental changes in the cholinergic system sensitivity to NGF. In the present study, suckling behavior and its modifications upon administration of the muscarinic blocking agent scopolamine were investigated at different postnatal ages (pd 8 or 11) in mouse pups previously treated with NGF.

METHOD

Subjects

Mice of an outbred Swiss-derived strain (CD-1), weighing 25–27 g, were purchased from Charles River Italia (I-22050 Calco, Italy). The animals were kept in an air-conditioned room at 21 ± 1°C and 60 ± 10% relative humidity, with lights on from 2130 to 0930 h. Males and multiparous females (one to two deliveries) were housed separately in groups of 10 in 42 × 27 × 14 cm Plexiglas boxes with sawdust as bedding and a metal top. Pellet food (Enriched standard diet purchased from Piccioni, I-25100 Brescia, Italy) and water were continuously available. Breeding pairs were formed and housed in 33 × 13 × 14 cm boxes and the females were inspected daily at 0900 h for the presence of vaginal plug and for delivery (pd 1). The stud was removed 15 days after the discovery of the plug. Eighteen litters were used and culled at birth to six males. Two pups of each litter were randomly assigned either to NGF or to cytochrome (control) treatment, while the remaining two pups were not used and served to maintain an appropriate litter size for the duration of the experiment.

Procedure

On pds 5 and 7, or 8 and 10, NGF pups received under hypothermic anaesthesia an ICV injection of 30 μg NGF (injection volume 3 μl), while control littermates received a similar injection of cytochrome *c* (Sigma Chemical Co., St. Louis, MO). A glass needle was placed approximately 1.5 mm anterior to the interaural line along the sagittal suture, and the ICV injection was made after direct transcutaneous puncture. A pilot study, carried out to evaluate the potential consequences of the injection procedure on the behavioral test, showed that the suckling performances of 8-day animals injected with different volumes of vehicle on days 2 and 4 did not significantly differ from those of noninjected animals. Cytochrome *c* was selected as control treatment because it is physicochemically similar to NGF, but lacks its neurotrophic activity.

NGF was prepared using the Bocchini and Angeletti method (5), and further purified to eliminate renin-like activity by additional carboxymethilcellulose chromatography (8). NGF was dissolved in physiological saline (pH 7.4) at a concentration of 10 mg/ml. Injections were given between 1100 and 1300 h.

Testing Procedure

On pd 8 or 11, the four ICV-treated pups of a litter were removed from the home cage 1 h before the beginning of the suckling test and housed in a temperature- and humidity-controlled incubator (Elmed Ginevri, Italy), maintained at 34 ± 1°C and 60% humidity. Ten minutes prior to the testing, pups were numbered on their backs with a marking pen and given either a scopolamine or a saline treatment. One NGF-treated pup and one cytochrome-treated pup were injected IP with 2 mg/kg scopolamine hydrobromide (Sigma), while the remaining two pups received an equal volume of 0.9% saline solution. This particular scopolamine dose was chosen on the basis of pilot experiments, which showed that it produced the clearest effect on pups' suckling performances on pd 5. Specifically, although all four doses tested (0.2, 1, 2, and 3 mg/kg) were effective in impairing nipple attachment, the two lower doses yielded a larger scatter of the data.

The suckling test was performed following to the procedure described by Ristine and Spear (21) and by Calamandrei et al. (7). Suckling is a typical behavioral pattern of neonatal mammals, and consists of a sequence of different responses that are amenable to quantitative analysis. Specifically, when placed in contact with the dam, the pup first begins to search for the nipple; once close to it, the pup starts to probe around it, paddling with the forelimbs and treading with the hindlimbs, until attachment is achieved. The pups' own dam was used as a suckling stimulus, after anesthesia with 3 mg/kg of sodium pentobarbital IP, which is effective in blocking milk letdown. The anesthetized dam was placed on her side and tilted onto her back at an angle of about 45° in a test chamber (33 × 13 × 14 cm) with a layer of paper towels on the floor. Tests were carried out at ambient room temperature (approximately 23°C). At the beginning of the suckling test, pups were placed in close proximity to the nipples against the ventrum of their dam, and were checked for attachment to the nipple and other behavioral responses at the beginning of the test and every 1 min thereafter, for a total duration of 60 min. The behavioral responses included probing (with the snout against an object, predominantly the dam), paddling with forelimbs, treading with hindlimbs, lying still (at rest when nonattached to the nipple) and forward locomotion (movements translocating the body). Pups that were found to be unattached were prompted to suckle at 5-min intervals by returning them in proximity to it. The entire session was videorecorded using a Sony VO-5360 apparatus equipped with CH-1400CE videocameras for red lights. Recordings were scored by an observer blind to the treatments received by each animal.

Statistical Analysis

The data were analyzed by appropriate mixed model parametric ANOVAs. The design was a factorial 2 × 2 (NGF pretreatment × scopolamine challenge) with litter as random blocking factor. Post hoc comparisons were performed using the Tukey HSD test. Because scopolamine treatment was expected to decrease the amount of time pups spent attached to the nipple, the number of time periods pups exhibited behaviors mutually exclusive with attachment (i.e., probing, immobility, and forward locomotion) was calculated as a percentage of nonattachment time. Conversely, the number of time periods spent paddling and treading was calculated as a percentage of total test time.

RESULTS

On pd 8, no main effect of scopolamine on time spent suckling and latency time to suckle was found. Post hoc comparisons showed a significant reduction ($p = 0.05$) in nipple attachment time only in cytochrome-treated pups receiving scopolamine, when compared to the corresponding controls (Fig. 1, top panel). Probing, immobility, and forward locomotion were not modified by scopolamine challenge (see Table 1). NGF-treated animals did more paddling and treading than cytochrome-treated animals, $F(1, 9) = 6.84$, $p < 0.05$; $F(1, 9) = 10.88$, $p < 0.01$, respectively. Post hoc comparisons yielded a significant difference in treading only between cytochrome- and NGF-scopolamine--injected pups ($p < 0.05$).

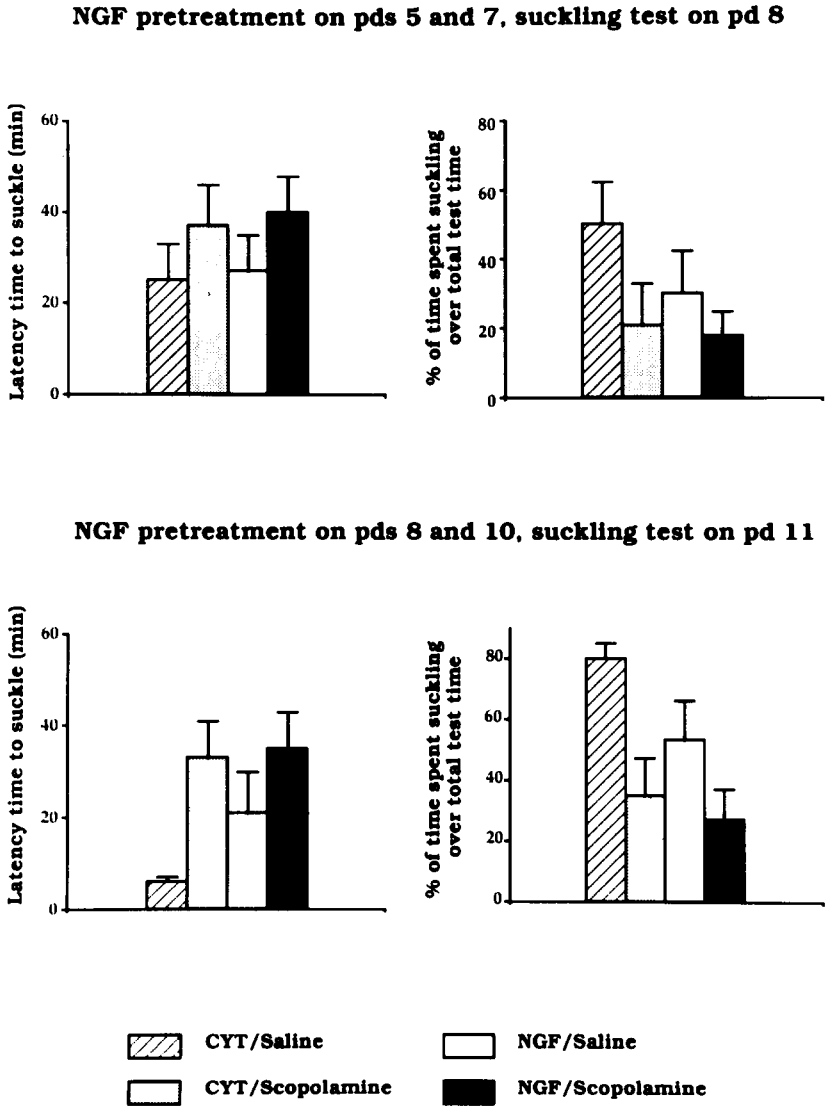

FIG. 1. Latency time to attach to a nipple (left) and percentages of time periods spent suckling (right) by male mouse pups pretreated with either NGF (ICV, 30 μg) or cytochrome *c*, and given either scopolamine (IP, 2 mg/kg) or saline 15 min before the suckling test. Values are means ± SEM. $N = 10$ pups (pd 8) and 8 pups (pd 11) in each final group.

TABLE 1

PERCENTAGE OF TIME PERIODS SPENT IN DIFFERENT BEHAVIORAL ITEMS BY 8-DAY MICE, INJECTED ICV WITH EITHER CYTOCHROME OF NGF ON PDS5 AND 7

	Probing	Paddling*	Treading*	Immobility	Locomotion
CYT/Saline	35 ± 9	12 ± 3	12 ± 3	66 ± 9	23 ± 5
CYT/Scopolamine	41 ± 7	17 ± 3	15 ± 3	58 ± 7	22 ± 5
NGF/Saline	30 ± 8	17 ± 7	20 ± 5	63 ± 8	20 ± 3
NGF/Scopolamine	42 ± 7	27 ± 5	28 ± 5	52 ± 8	27 ± 6

Scopolamine or saline were given IP 15 min prior to the suckling test.
*Significant main effect of NGF treatment, $p < 0.05$.

The interaction between NGF pretreatment and scopolamine challenge produced no evident effect on suckling behavior and activity levels. Body weight gain was significantly retarded by NGF treatment, $F(2, 18) = 33.64, p < 0.001$.

On pd 11, suckling behavior was clearly impaired by scopolamine. As shown in Fig. 1 (bottom), latency to nipple attachment was higher in scopolamine pups, $F(1, 7) = 18.29, p < 0.01$, while time spent suckling was correspondingly decreased by the drug, $F(1, 7) = 15.6, p < 0.01$. As for the other behavioral categories (Table 2), a main effect of scopolamine was evident only for locomotor activity, $F(1, 7) = 12.83, p < 0.01$. A significant NGF × scopolamine interaction, $F(1, 7) = 6.57, p < 0.05$, revealed that the depressant effect of the drug was evident only in cytochrome-treated animals, being NGF/saline animals significantly less active than cytochrome/saline controls ($p < 0.05$). NGF per se did not influence any of the categories under score. Though a tendency toward an increase in latency time to suckle was detectable in NGF/saline pups, however, they did not differ significantly from the corresponding controls. Weight gain was significantly reduced in NGF-treated pups, $F(2, 14) = 10.59$, $p < 0.01$.

DISCUSSION

The results of the present study confirm previous findings indicating the involvement of the cholinergic regulatory systems in the suckling response of mice. The quantitative and qualitative differences in the effects of the muscarinic blocker at successive ages support the view that distinct portions of the cholinergic system have different rates of maturation (22), and that sensitivity to NGF is age dependent. Furthermore, it appears that this growth factor is involved in the functional maturation of at least some of these cholinergic subsystems.

The extent of the blocking effect of scopolamine on nipple attachment behavior was found to vary with age. Specifically, no main effect of scopolamine on suckling was found on pd 8, while on pd 11 both time spent suckling and latency to attach were significantly affected. Because previous data on mice indicated that the drug is highly effective in impairing nipple attachment on pd 5, the present results suggest that the same behavioral endpoint can be differentially affected by the same drug at successive ages in developing rodents. A similar trend has been observed in rats by Ristine and Spear (21), who reported a scopolamine-induced impairment in suckling on pds 3–4, no effect on pds 8 and 11, and again an impairment on pds 14–15.

On pd 11 scopolamine was found to exert a depressant effect on forward locomotion. This is in accordance with existing data showing that the earliest ontogenetic responses observed following administration of cholinergic antagonists is a decrease in locomotor activity rather than the increase in activity seen later in life (22). A number of drugs have been found to produce these so called "paradoxical" responses in immature animals (10,21,24). To date conventional locomotor activity tests [i.e., open-field arena; see (1)] have failed to evidence a significant depressant effect of scopolamine before weaning. Pups younger than 14–15 days show very low baseline levels of spontaneous activity when tested in isolation in an unfamiliar environment. In the present experiment, the presence of mother and siblings appeared to favor the expression of active locomotion, thus making it possible to detect drug effects on activity levels at an earlier developmental stage.

As previously mentioned, pretreatment with NGF on pds 2 and 4 has been shown to exert marked proactive effects on reactivity to scopolamine on pd 5 (7). The rise in ChAT activity in septum and caudate putamen after a single injection of NGF on pd 2 is maximum 48 h following administration (19).

TABLE 2

PERCENTAGE OF TIME PERIODS SPENT IN DIFFERENT BEHAVIORAL ITEMS BY 11-DAY MICE, INJECTED ICV WITH EITHER CYTOCHROME OR NGF ON PDS 8 AND 10

	Probing	Paddling	Treading	Immobility	Locomotion*
CYT/saline	58 ± 9	5 ± 1	4 ± 1	63 ± 12	26 ± 5
CYT/scopolamine	54 ± 9	12 ± 2	12 ± 2	50 ± 12	10 ± 3
NGF/saline	53 ± 11	12 ± 3	12 ± 3	40 ± 7	14 ± 3
NGF/scopolamine	43 ± 7	13 ± 2	13 ± 2	50 ± 7	13 ± 3

Scopolamine or saline were given IP 15 min prior to the suckling test.
*Significant main effect of scopolamine, $p < 0.01$; interaction between scopolamine and NGF treatment, $p < 0.05$.

Unfortunately, no data on ChAt activity levels on pd 8 and pd 11 after a schedule of two NGF injections are available, for comparison with those reported on pd 5. The absence of proactive NGF effects on suckling at these ages, notwithstanding the proximity of NGF pretreatment and scopolamine administration, suggests that the age of exposure to exogenous NGF is an important factor. Indeed, it appears that the sooner the developing brain is exposed to exogenous NGF, the more consistent the effect on ChAT activity (13,18,19). However, pretreatment with NGF on pds 5 and 7 increased the frequency of paddling and treading behavior. Paddling and treading are important components of the suckling behavior pattern, as they help pups to maintain contact with the dam ventrum (21). Coordinated limb movements during the first week of life reflect the development of greater motoric competence; they are also used as an index of the level of pup activation. The increase in paddling and treading frequency was amplified in NGF/scopolamine animals. This effect partially resembles the appearance of scopolamine-induced hyperactivity in 5-day pups pretreated with NGF, though at that age baseline activity levels were not increased by NGF per se. On pd 11, NGF seemed to act in the same direction as for scopolamine, significantly reducing the levels of forward locomotion in NGF control animals in comparison to cytochrome controls.

The effect of NGF on body weight gain was similar at the two different ages considered, regardless of the age of administration. NGF pups gained weight at a slower rate than cytochrome-injected controls, though the difference in daily weight increment was usually rather slight (6). This effect is in agreement with previous reports (2,18), and may be attributed to the interaction of NGF with some growth regulating systems, mainly at the hypothalamic level. However, a relationship between the impairment of body weight gain and the stimulation of ChAT activity has recently been excluded in the adult rat by Williams (29). Moreover, it has to be mentioned that most of the treatments inducing a delay in body weight gain also result in a concomitant delay of neurobehavioral development. On the contrary, early NGF administration has been found to accelerate sensorimotor development and anticipate the appearance of scopolamine-induced hyperactivity (3,7), and this supports the specificity of the behavioral changes reported.

The present behavioral findings support the view that cholinergic neurons respond differentially to exogenous NGF in diverse—yet very close—developmental stages. Johnston and co-workers (13) found regional differences in the timing and magnitude of ChAT activity enhancement of hippocampus, septum, and caudate-putamen neurons to NGF administration. Thus, the differences in NGF effects on pups' behavior could reflect the involvement of distinct cholinergic brain areas at different developmental stages. Of note is that the earliest biochemical response to exogenous NGF is detected in forebrain areas traditionally involved in movement control, such as septum and caudate putamen (20). Indeed, with the exception of nipple attachment, the behavioral endpoints affected by NGF are all part of the motor repertoire of the immature animal. It can be hypothesized that NGF normally regulates the establishment of neural connections in those forebrain target areas and that its exogenous administration at critical developmental stages alters the age-specific level of behavioral activation.

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