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Postnatal Development of a Cholinergic Influence on Neuroleptic-Induced Catalepsy

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BURT, D. K., S. M. HUNGERFORD, M. L. CROWNER AND L. A. BAEZ. *Postnatal development of a cholinergic influence on neuroleptic-induced catalepsy*. PHARMAC. BIOCHEM. BEHAV. **16**(4)533–540, 1982.—The development of cholinergic influence on neuroleptic-induced catalepsy was investigated in 10-, 15- and 20-day-old rat pups. It was found that the antimuscarinic atropine was ineffective in decreasing the catalepsy produced by spiroperidol treatment at 10 and 15 days. By day 20, however, atropine attenuated cataleptic behavior in a dose-dependent manner. Atropine alone was shown paradoxically to elicit mild to moderate cataleptic responses in 10- and 15-day-olds, but not at day 20. Clozapine by itself produced the same age dependent pattern of catalepsy response as the spiroperidol and atropine combination treatment. These results suggest that cholinergic mechanisms which interact antagonistically with the dopamine systems underlying cataleptic behavior are not functionally mature until after day 15 in the rat.

Catalepsy Development Dopamine Acetylcholine Spiroperidol Atropine Clozapine

BRAIN dopamine systems provide a rich field for study of the ontogenetic development of CNS transmitter systems for a number of reasons: dopamine cell groups and projection areas have been extensively mapped; pharmacological agents with a high specificity of action for dopamine function are readily available; and behavioral measures reflecting the functional activity of dopamine pathways have been developed.

Monoamine fluorescence studies of developing rodent brain show dopamine (DA) cell bodies in the midbrain and patchy islands of dopamine axon terminals in the forebrain of newborn rats [44,50]. By the end of the second postnatal week, the adult pattern of diffuse terminal fluorescence is seen filling entire structures such as caudate-putamen, nucleus accumbens, and olfactory tubercle. During the same period, neurochemical indices of DA neuronal activity, including DA levels, [^{3}H]DA uptake, synthetic enzyme activity, and DA-sensitive adenylate cyclase activity show a steady increase, usually reaching adult levels during the third or fourth postnatal week [21, 22, 23, 24, 39]. The data uniformly suggest that DA axons have reached their target areas by the time of birth, but that a plateau of innervation and neuronal activation is not attained until after the second week of life.

Psychopharmacological experiments tend to support the neurochemical data. Dopamine agonists elicit behaviors similar to those observed in adults as early as the first week of life [40, 43, 51] implying an early maturity of postsynaptic circuitry. Furthermore, dopamine receptor blockers produce in newborn pups the same sensory-motor impairments observed in mature animals [3], suggesting that presynaptic elements are also operative in the neonate. The increase in motor activity seen in untreated rat pups between birth and day 15 [14] can be suppressed by depletion of brain catecholamines [15]. Between 10 and 15 days of age, dopamine receptor blockers show a decrease in their ability to produce catalepsy in young animals [2]. Thus, behavioral development also reflects the presence at birth of functional dopamine systems, whose levels of activity appear to increase during the early postnatal period.

The correlation between neurochemical and behavioral measures of dopamine activity is not, however, completely consistent. While biochemical markers of dopamine function continue to rise during the third postnatal week [10,39], behavioral studies indicate an attenuation of dopamine-related responses. Motor activity decreases sharply [15] and animals become more susceptible to the cataleptogenic effects of neuroleptic drugs [2]. It appears, therefore, that some factor other than a decrease in number or density of dopamine neurons or receptors is responsible for the alterations in these behaviors apparent during the third week of life.

Several lines of evidence indicate that dopamine and acetylcholine in the neostriatum have a functionally antagonistic relationship in adults. Pharmacological agents affecting dopamine transmission have a significant influence on levels and turnover rate of acetylcholine [29,59]. Cholinergic drugs, in turn, alter measures of dopamine accumulation and activity [1,5]. The two transmitters also appear to exert opposite effects on behavior [2,35].

Considerable data support the contention that maturation of forebrain cholinergic systems lags behind that of dopamine [3, 16, 25, 58]. The series of experiments reported here was designed to test the hypothesis that this late maturation of acetylcholine systems in the forebrain underlies the

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enhancement of neuroleptic-induced catalepsy found in rat pups between 15 and 20 days of age.

GENERAL METHOD

Animals

Long-Evans rat pups of 10, 15 and 20 days of age, bred in our colony, were used in all experiments. Day of birth was considered day 0. Litters were trimmed to 8 pups on day 3. All animals were housed in plastic hanging cages under a 14-hr on and 10-hr off light-dark cycle. Both males and females were tested. Just before drug treatment the mother was removed from the home cage and the litter was taken to the testing room. Pups remained with their littermates during all intervals between injection and catalepsy measurement.

Drugs

Spiroperidol (Janssen) was dissolved in a tartaric acid solution (see Baez *et al.* [2]) to concentrations of 1.0 and 0.625 mg/ml. Clozapine (Sandoz), dissolved in tartaric acid solution, was utilized in concentrations of 4.0, 8.0 and 16.0 mg/ml. Atropine sulfate was dissolved in saline in concentrations of 0.156, 0.312, 0.625, 1.25, 5.0, 10.0 and 20.0 mg/ml.

All drugs were injected intraperitoneally in volumes of 1.0 ml/kg body weight, using a 50 μl Hamilton syringe. Saline was the control solution in all experiments.

In each experiment the various dosages were distributed evenly among the pups of a given litter to control for interlitter variability. One partial exception to the practice occurred in Experiment 1, which was conducted in 2 phases. Atropine concentrations of 0.625, 0.312 and 5.0 mg/ml plus a saline control were examined first. It was subsequently decided to expand the dose response curve, so three additional atropine doses plus saline control were examined in the second phase. The saline points in Fig. 1, therefore, contained twice the usual number of animals.

Measurement of Catalepsy

Catalepsy was measured by timing bar-response latency, defined as the number of seconds elapsed between the time an animal is placed with forelimbs resting on a raised horizontal bar and the time it touches one forelimb to the testing surface. The height of the bar was adjusted to scale for each of the 3 age groups. Each pup was given three trials and the mean of the three trials served as the individual pup score. Different experimenters did injections and behavioral testing to allow for blind measurement of the catalepsy response.

Righting responses were evaluated at each observation period to insure that cataleptic behavior was not due to general CNS depression. Since righting ability improves with age, judgements of normality were made against the righting behavior of untreated animals of the same age. Pups were placed on their backs and both latency to attain an upright posture and coordination of the righting movement was observed. Overall performance was rated "normal," "slow," or "failure to right." Interrater reliability was approximately 95%.

EXPERIMENT 1: AGE-DEPENDENT EFFECTS OF ATROPINE ON SPIROPERIDOL-INDUCED CATALEPSY

It has been shown that dopamine blockade produces catalepsy in rat pups during the first two postnatal weeks, when cholinergic agonists do not [3]. In adults, cholinergic and dopaminergic agents elicit functionally antagonistic effects [35]. The purpose of Experiment 1 was to investigate the postnatal development of the dopaminergic-cholinergic interaction by testing the ability of atropine to antagonize spiroperidol-induced catalepsy in pups of varying ages.

METHOD

Pups were injected with either saline or one of the experimental doses of atropine (0.156, 0.312, 0.625, 1.25, 5.0, or 20.0 mg/kg). Fifteen minutes later, 1.0 mg/kg spiroperidol was administered.

Catalepsy measures were taken 30 and 60 minutes after neuroleptic treatment. Thirty seconds was arbitrarily set as the time at which a trial would be terminated if the animal had not yet descended from the bar.

RESULTS

Dose response curves for 10-, 15- and 20-day-old pups pretreated with atropine and tested for catalepsy 60 minutes after spiroperidol injection are shown in Fig. 1. The ability of atropine to influence neuroleptic-induced catalepsy varied dramatically with age. At 10 and 15 days, the effect of spiroperidol was not attenuated by any dose of the anticholinergic agent. In fact, at both ages, certain doses of atropine showed a tendency to increase bar latency. In 20-day-old pups, however, catalepsy was antagonised by atropine in a dose-dependent manner. Analysis of variance revealed main effects for age, $F(2)=18.91$, $p<0.001$, and dose $F(6)=2.64$, $p<0.05$, and an interaction of age with dose, $F(12)=3.48$, $p<0.001$.

Data obtained during the 30-minute catalepsy test showed the same pattern of bar latency at the three ages except that scores were generally lower, since spiroperidol had not yet produced its maximum effect.

DISCUSSION

The ability of atropine to antagonize spiroperidol-induced catalepsy is age dependent. In animals 15 days old or younger, atropine causes no diminution of the cataleptic response. By day 20, however, the drug strongly reverses this motor effect of neuroleptic treatment. Paradoxically, at 10 and 15 days atropine appears to facilitate the cataleptogenic effects of spiroperidol.

The finding that atropine does not attenuate spiroperidol-induced catalepsy until after day 15 supports the hypothesis that a relatively late maturing cholinergic system is involved in the increase in responsivity to neuroleptic drugs between day 15 and day 20 [2]. As a cholinergic input inhibitory to dopamine activity matures, drugs which block dopamine receptors are more effective in producing behavioral consequences.

EXPERIMENT 2: EFFECTS OF ATROPINE ON CATALEPSY PRODUCED BY HIGH AND LOW DOSES OF SPIROPERIDOL

The purpose of Experiment 2 was two-fold. First, to ensure that the differing effects of atropine on neuroleptic-induced catalepsy was not specific to a particular dose of spiroperidol, we selected a lower dose of the dopamine antagonist that was known to elicit a mild degree of cataleptic behavior. Secondly, we wanted to reexamine the effects of spiroperidol plus saline pretreatment because the age

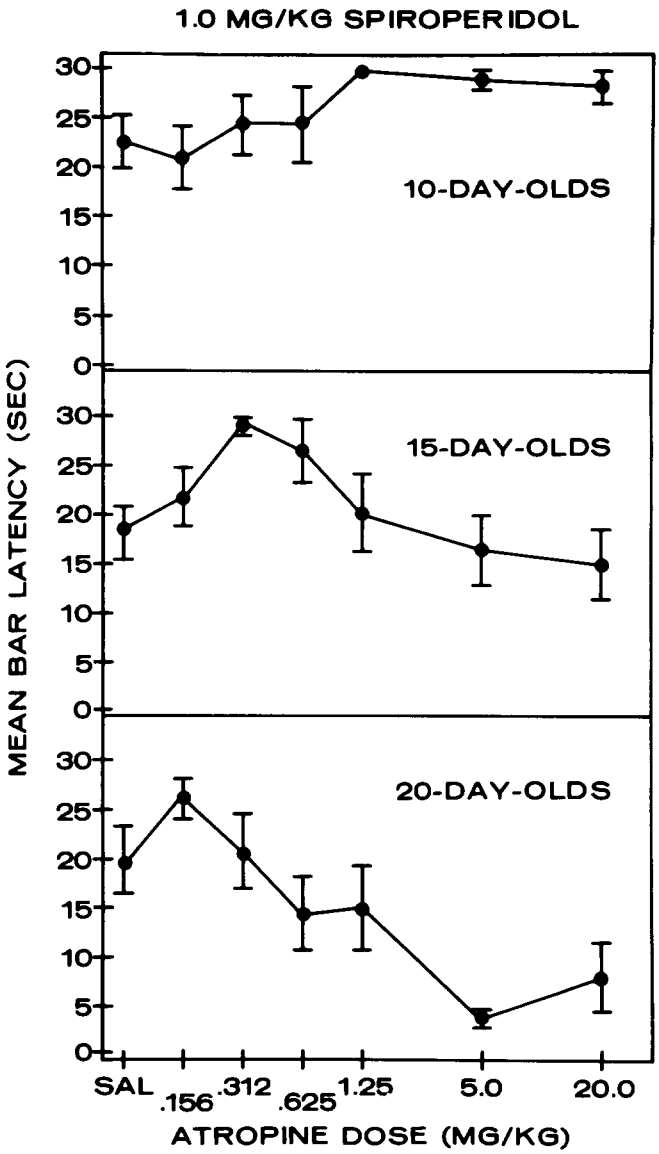

FIG. 1. The effects of atropine on spiroperidol-induced catalepsy in 10-, 15-, and 20-day-old rat pups. All animals received 1.0 mg/kg spiroperidol (IP) 15 min after IP injection of atropine. Catalepsy measures were taken 60 min after spiroperidol treatment. N's for saline groups ranged from 15 to 16. All other group N's were 8. Brackets represent standard error of the mean.

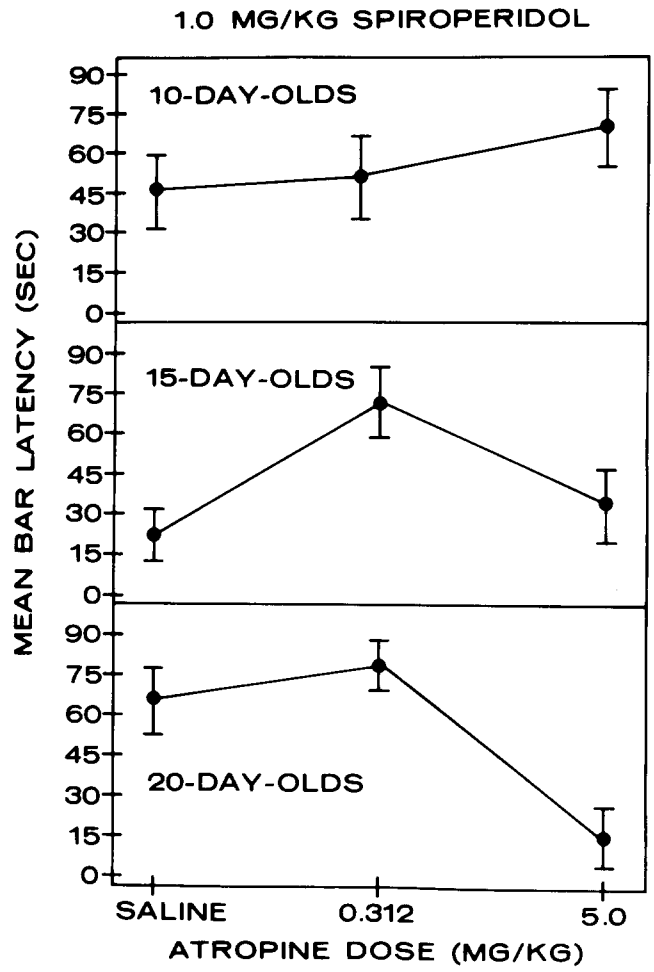

FIG. 2. The effects of high and low doses of atropine on catalepsy produced by a high dose (1.0 mg/kg) of spiroperidol in 10-, 15-, and 20-day-old rat pups. Intervals between drug treatments and testing were identical to those in Experiment 1. Group N=8. Brackets represent standard error of the mean.

difference previously observed at that dose [2] was not replicated. The difference in results was due to the fact that 10- and 20-day-old pups did not consistently attain the highest bar score possible as they had tended to do in the original dose-response study. It was reasoned that the saline pretreatment night have activated the pups, making them less susceptible to neuroleptic effects. In addition, the arbitrary test termination of 30 sec created an artificial score ceiling that would eliminate the influence on the mean of pups who might have shown a longer catalepsy response. The trial interval in Experiment 2 was, therefore, increased to 90 seconds and some pups received the same dose of spiroperidol used in Experiment 1.

METHOD

Pups received injections of either saline or one of two doses of atropine (0.312 mg/kg and 5.0 mg/kg). After 15 minutes, each animal was treated with a high (1.0 mg/kg) or low (0.625 mg/kg) dose of spiroperidol. Catalepsy testing took place 60 minutes after spiroperidol treatment.

RESULTS

The results of Experiment 2 are shown in Fig. 2 and Fig. 3. The same overall pattern of interaction between age and drug dose occurred with failure of atropine to antagonize spiroperidol catalepsy at 10 and 15 days. The age by atropine dose interaction was significant with both the high dose, $F(4)=4.18$, $p<0.01$, and low dose, $F(4)=3.92$, $p<0.01$, of

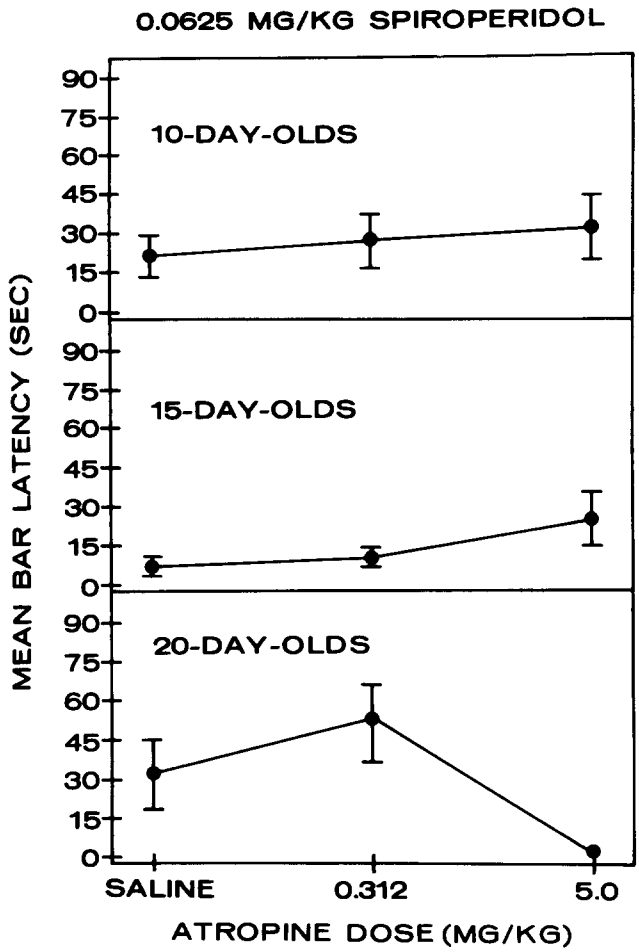

FIG. 3. The effects of high and low doses of atropine on catalepsy produced by a low dose (0.625 mg/kg) of spiroperidol in 10-, 15- and 20-day-old rat pups. Group N=8. Brackets represent standard error of the mean.

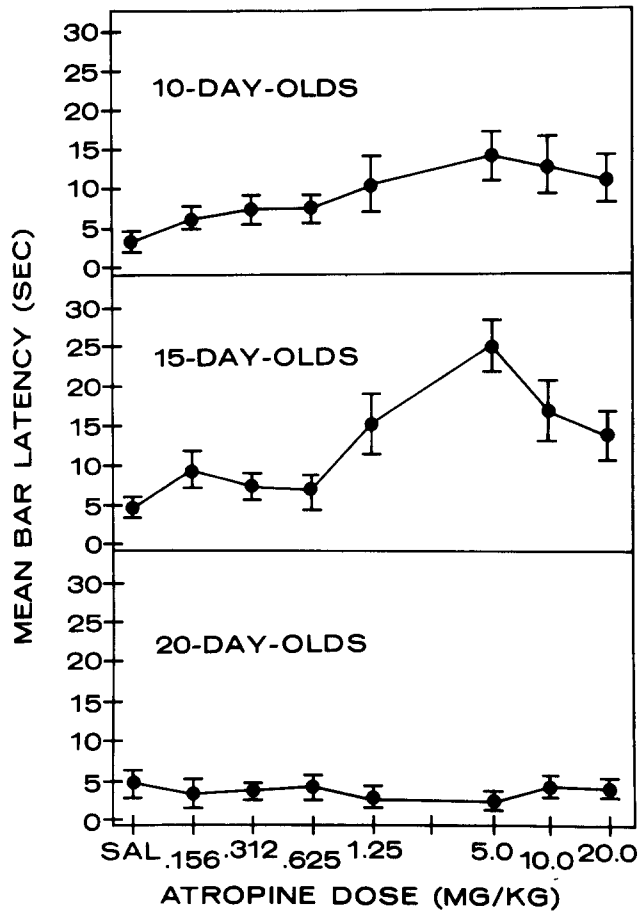

FIG. 4. The ability of atropine to produce catalepsy in 10-, 15-, and 20-day-old rat pups. Bar latencies were recorded 60 min after atropine injection (IP). Group N=8. Brackets represent standard error of the mean.

spiroperidol. Main effects for age and dose were not statistically significant in Experiment 2. With the higher trial cut-off point, the substantial decrease in cataleptic behavior at day 15 previously observed at all doses of spiroperidol [2] was again apparent in the spiroperidol plus saline groups. Planned comparisons analysis showed 15-day-olds to be less cataleptic than other age groups at both doses of spiroperidol with $p<0.05$.

DISCUSSION

The inability of atropine to antagonize neuroleptic catalepsy until after day 15 is replicable and not dependent on the dose of spiroperidol used in treatment.

EXPERIMENT 3: ATROPINE-INDUCED CATALEPSY

In experiments 1 and 2, atropine had exhibited a consistent tendency to enhance neuroleptic-induced catalepsy in 10-day-old pups and, at low doses, in 15-day-olds. It seemed advisable, therefore, to examine the effects of atropine alone on bar latency in the three age groups.

METHOD

Pups were treated with either saline or one of several doses of atropine (0.156, 0.312, 0.625, 1.25, 5.0, 10.0, or 20.0 mg/kg). Sixty minutes later bar latencies were recorded. Since atropine was not expected to produce long latencies, the original trial termination point of 30 seconds was used once more.

RESULTS

Figure 4 shows the effects of varying doses of atropine on bar latency in 10-, 15- and 20-day-old pups. At ten days there was a gradual increase in catalepsy which reached plateau at a dose of 5 mg/kg with mean bar scores between 10 and 15 seconds. Fifteen-day-olds showed little or no increase in bar latency at the three lowest atropine doses, followed by a steep increase peaking at 5.0 mg/kg to a mean of 25 sec, and then diminishing somewhat between 5.0 and 20.0 mg/kg.

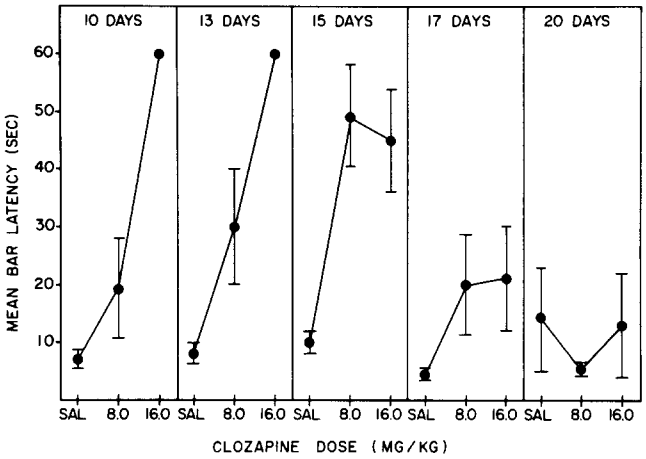

FIG. 5. The ability of clozapine to produce catalepsy in 10-, 13-, 15-, 17-, and 20-day-old rat pups. Bar latencies were recorded 60 min. after clozapine injection (IP). Group N=6. Brackets represent standard error of the mean.

In contrast to the younger groups, the dose-response curve for 20-day-olds is completely flat, demonstrating the absence of a cataleptic effect of atropine at any of the administered doses by 20-days of age.

Analysis of variance of data in Experiment 3 revealed main effects of age, $F(2)=21.98$, $p<0.001$, and dose, $F(7)=4.31$, $p<0.001$. The age by dose interaction was not significant.

DISCUSSION

Atropine alone elicits a marginal cataleptic response at 10 days and substantial bar latency scores at day 15, but is without effect on day 20. These results are consistent with the unexpected influence of atropine in the enhancement of catalepsy in 10- and 15-day-olds observed in Experiment 1.

The catalepsy produced by atropine in 10- and 15-day-old pups is not as profound as that seen with neuroleptic treatment. Bar latencies do not reach the same ceilings as those found after spiroperidol injection. The behavior, however, does appear to be true catalepsy in that impairment of righting responses was not seen except in a few instances in 10-day-olds receiving the 20.0 mg/kg dose.

EXPERIMENT 4: CLOZAPINE-INDUCED CATALEPSY

This experiment was conducted to examine in developing animals the catalepsy-producing capability of clozapine, a neuroleptic reported to possess both anticholinergic and antidopaminergic properties [52]. In adults, clozapine is devoid of extrapyramidal effects. It was hypothesized that if clozapine did indeed block both dopamine and acetylcholine receptors, then its effect on catalepsy in developing pups should mimic the results obtained in our atropine plus spiroperidol experiments.

To expand data points on the age variable, two additional groups of 13-day-olds and 17-day-olds were added to the design. The trial cut-off was set at 60 seconds to allow for a broad range of scores without necessitating an overlap of injection and testing periods required by the 90-second time.

METHOD

Pups were injected with either saline or 8.0 mg/kg or 16.0 mg/kg clozapine. Behavioral testing was conducted 60 minutes later.

RESULTS

The results of Experiment 4 are shown in Fig. 5. Clozapine produced cataleptic behavior in a dose-dependent manner at 10, 13, and 15 days. However, by 17 days the catalepsy was greatly decreased, and twenty day-olds were totally unresponsive to the drug. Analysis of variance results included main effects of age, $F(4)=7.17$, $p<0.001$, and dose $F(2)=27.01$, $p<0.001$, and an age by dose interaction, $F(8)=4.32$, $p<0.001$.

DISCUSSION

The mechanism of action of clozapine and the explanation of why, unlike the classical neuroleptics, it is devoid of extrapyramidal side effects at clinical dosages, are questions that have stirred controversy ever since the drug was introduced [9, 32, 38, 55, 61]. There is evidence that the drug is both antidopaminergic [27, 52, 57] and anticholinergic [42,52]. If such were the case. clozapine should produce the same effects on catalepsy as a combination of spiroperidol and atropine, and it should duplicate the developmental pattern found in the first experiments. Indeed, clozapine does produce catalepsy in animals 15 days or younger, and thereafter loses its potency as a cataleptic agent. In fact, the age-related effects are remarkably similar. Fifteen-day-olds, who are even more cataleptic than 10-day-olds when given atropine plus spiroperidol, are also more sensitive to the extrapyramidal effects of clozapine than the younger animals. The parallels between the two types of drug treatment suggest that the same neurochemical/neuroanatonical substrates underly the behavior in both cases.

It has been proposed that the atypical neuroleptics lack extrapyramidal side effects because they act preferentially on mesolimbic rather than nigrostriatal structures [4,36]. If catalepsy is mediated by pharmacological activity in the neostriatum as is commonly accepted [18, 19, 31, 48], then our data argue that, at least in young animals, clozapine is having a significant influence on extra-pyramidal structures.

GENERAL DISCUSSION

It has been repeatedly demonstrated that dopamine circuits are present and functioning in the rodent brain at birth [3, 10, 20, 40, 43, 44, 51] and even prenatally [22, 23, 24, 49]. Central cholinergic systems, by contrast, appear according to most reports to be immature and inaccessible to pharmacological manipulation during the early postnatal period [3, 11, 13, 16, 25]. Our data suggest the onset of an interaction between the two systems during the third week of life. This suggestion is consistent with other data on the relationship between dopaminergic and cholinergic neurons in developing brain at morphological [11], biochemical [23,47], pharmacological [23,33], and behavioral [30] levels of analysis.

Not all studies, however, are in agreement with the assertion that central cholinergic neurons are not functional during the first prenatal weeks. Intracerebral injections of anticholinergic and cholinomimetic agents into the neostriatum produce behavioral effects in 2-day-old and 10-

day-old rat pups (Hungerford and Van Hartesveldt, in preparation, See also [60]. It may be that a relatively small population of early maturing cholinergic cells are present, whose effects on behavior can only be modified under conditions associated with intracerebral administration (e.g., a rapid saturation of many receptors in a circumscribed region). In regard to cholinergic interactions with striatal dopamine neurons, the possibility remains that synaptic relationships between the two populations of neurons have not yet been established by day 2. Further intracerebral infusion experiments using combinations of cholinergic and dopaminergic drugs are needed to explore the question of early transmitter interactions.

Paradoxical Effects of Antimuscarinics in Neonates

The discrepancy between experiments utilizing different routes of drug administration suggests that the notion of a single, early maturing dopamine pathway later inhibited by a newly functioning cholinergic system, is overly simplistic. Our own data demonstrate that the developmental picutre is not so straightforward, since in very young pups atropine has the opposite effect from that seen later in ontogeny. While atropine counteracts spiroperidol-induced catalepsy in animals 20 days or older, it enhances bar scores at 10 and 15 days. Furthermore, atropine itself is capable of producing catalepsy in the younger pups.

Paradoxical behavioral effects of cholinergic agents in the young rodent appear in several experimental reports [7, 8, 30], but only recently has the phenomenon been explicitly discussed [56]. Other evidence indicates that 2 or 3 different subpopulations of muscarinic receptors exist in brain [6,37]. If such subgroups possess different functional properties and show differential rates of maturation, their existence might help explain the puzzling age related alterations found with administeration of cholinergic drugs.

Another possibility is that two or more distinct populations of dopamine neurons are contributing to these changes in behavioral development. Olson *et al.* [50] extended such a proposal years ago when they speculated on the significance of the early developing islands of fluorescence in the forebrain. It has also been suggested that the dopamine receptor may possess 2 conformational states [26] and that 2 different receptor types, one excitatory and the other inhibitory, exist in the forebrain [17,54].

Implications for Model of Neurotransmitter Interactions in Development

As research accumulates on the complexities of receptor types and transmitter physiology in the neostriatum, it becomes increasingly difficult to base detailed models of synaptic connectivity during development on psychopharmacological data. Behavioral responses to disruption of normal transmitter activity can, however, provide a valuable index of overall systems interactions of neurotransmitters. Our data conform to a general model which casts dopaminergic and cholinergic systems in complementary roles in sensorimotor integration. The model proposes that the nigrostriatal dopaminergic input to the basal ganglia is involved in crucial "survival" functions which are already essential at the time of birth. At this point the dopamine input may be directly on efferent cells of the striatum, providing rapid, relatively reflexive motor responses to relevant cues. Thus, direct interference with dopamine activity produces significant behavioral effects, but cholinergic manipulations fail to alter dopamine function.

As the organism ages, environmental demands require more complex integrative functions. One mechanism for such change is the maturation of interneurons which can serve as modulators of afferent activity on the output of the striatum. During the third week of life synaptogenesis in rat striatum reaches a peak [34] and a rapid differentiation of cholinesterase-containing cells occurs [11]. Acetylcholine is present primarily in local circuit neurons of the striatum [12, 45, 47]. By week 3, therefore, proliferating dopamine terminals may form synapses on cholinergic interneurons, probably inhibitory in nature [46]. The cholinergic interneuron provides a link between the nigrostriatal axons and other afferents from neocortex and thalamus, as well as an influence on output which may feed back to the nigral cell bodies. Now functional antagonism between the two systems can be observed at both biochemical and behavioral levels.

What happens to the orginial dopamine input? One possibility is that a "pruning" effect occurs wherein those neurons projecting directly onto striatal efferents are eliminated. There is an apparent decrease in number of nigral dopamine cells after day 15 [44]. An alternative is that the original connections remain, but are quantitatively diluted by the new dopamine-to-acetylcholine contacts. Fluoresecent dopamine islands reappear in the striatum after transmitter synthesis inhibition [50]. These early dopamine synapses may be the ones associated with excitatory responses to dopamine that are found in the striatum with electrophysiological recording [41].

Systemically injected atropine is clearly unable to reverse neuroleptic-induced catalepsy at day 15, yet exerts a dramatic attenuation of cataleptic behavior by day 20. An interesting effect of dopamine cell destruction has recently been shown to alter suddenly at about the same point in time [28]. 6-Hydroxydopamine given before day 14 prevents the depression of locomotor activity normally seen after day 15. The effect is no longer attained if the neurotoxin is given after day 14. The intriguing possibility is raised that early postnatal dopamine may serve a second role as a neurohumoral agent, influencing development of neuronal systems which normally inhibit dopamine transmitter activity. In light of our data, the relevant inhibitory system may be acetylcholine.

In summary, systemically injected anticholinergic drugs are ineffective in antagonizing spiroperidol-induced catalepsy until after 15 days of age in the rat. This suggests that the complementary relationship between cholinergic and dopaminergic systems in the neostriatum is not functionally established until the third postnatal week.

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