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Central Cholinergic Involvement in Behavioral Hyper-Reactivity[1]

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RUSSELL, R. W. AND J. MACRI. *Central cholinergic involvement in behavioral hyper-reactivity*. PHARMAC. BIOCHEM. BEHAV. **10**(1) 43–48, 1979.—The present experiments were designed to test the hypothesis that behavioral reactivity to changes of environmental stimulation varies concomitantly with changes induced acutely by pharmacological and chronically by morphological manipulations of acetylcholine in the central cholinergic system. Acute reduction of acetylcholine levels was achieved by cerebroventricular injections of hemicholinium-3 at five dosages and chronic reduction by electrolytic lesions in the septum. Significant and quantitatively equivalent hyper-reactivity occurred when animals were exposed to a variety of stimulus changes at peak effect times for the two experimental treatments. Such concordance of behavioral effects was not observed during non-contingent shock stimulation, when septal animals were significantly hyper-reactive and hemicholinium-3 animals were not. These findings may be interpreted in terms of results of recent multivariate analyses showing that several independent behavioral factors constitute the septal "syndrome": a particular factor, e.g., reactivity, may be related to a neurochemical substrate which is not shared by other factors. The present experiments revealed a highly precise relation between acute changes in acetylcholine levels and dose effect trends in hyper-reactivity, i.e., hyper-reactivity followed hemicholinium-3 injections (IVt) which reduced acetylcholine and returned to control levels when acetylcholine recovered. Under conditions of chronic acetylcholine reduction (septal lesion) initial hyper-reactivity disappeared with time, an observation consistent with the "imbalance" hypothesis about interactions between transmitter systems which assumes that changes in one system may be followed by compensatory changes in a related system.

Hemicholinium-3 Septal lesions Hyper-reactivity Cholinergic system

AN EXTENSIVE body of knowledge has been accumulated which implicates central cholinergic mechanisms in various behaviors of both human and infrahuman animals [14, 18, 28, 34]. Among the behaviors observed hyper-reactivity appeared as a response to changes in the environment of an organism. Results of research indicate that these responses consist of reactions to stimulus change and are not merely general or random activity [10,35]. Work in our laboratory has shown that they constitute one feature of the behavior of animals whose endogenous brain acetylcholine (ACh) levels have been reduced by interference with high affinity choline (Ch) transport following cerebroventricular (IVt) injection of hemicholinium-3 (HC-3) and the consequent decrease in ACh levels [9, 17, 30].

Hyper-reactivity is also one of the features of behavior following morphological lesions at the site in which ACh is synthesized in the septal-hippocampal pathway of the CNS. First noted by Brady and Nauta [4], subsequent empirical observations have reported ". . . very characteristic hyper-reactivity which appears as an exaggerated reaction to normally nonoxious stimuli [26]." This behavioral effect is most pronounced shortly after surgery and disappears within 10–15 days [24], a process which can be accelerated by handling and in animals living in groups rather than singly. It is only one of the behavioral consequences which, collectively, constitute the "septal syndrome," a syndrome for which more than one explanatory concept is required [41].

Superficially, both these pharmacological and morphological manipulations of the CNS appear to be associated with certain similar behavioral consequences. Studies of effects of septal lesions on cholinergic events in the hippocampus suggest what may be a common factor underlying the similarities. Chronic experiments have shown that most of the cholinergic input to the hippocampus is eliminated by lesions in the medial septal area [20,38]. Destruction of the input reduces endogenous hippocampal ACh levels by more than 80%, Ch levels by 20–23% and choline acetyltransferase (ChAT) activity by 85–90%. Maximum effects occur by four days postoperative and persist when assayed two months later. As with septal lesions, the effect of HC-3 IVt is to

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reduce ACh levels, an effect which is dose-dependent. Studies using ^{14}C-labelled HC-3 IVt have shown that HC-3 reaches the walls of the ventricles and accumulates in the caudate nucleus and the hippocampus [8]. The intracellular distribution of ^{14}C-HC-3 is associated with nerve endings in both these neuroanatomical regions [37]. These facts suggest the possibility that the common factor in both types of CNS manipulation may be the decrease of ACh levels in the hippocampus.

The present experiments were designed to test the hypothesis that behavioral reactivity varies concomitantly with changes induced in the cholinergic system acutely by the pharmacological and chronically by the morphological manipulations described above.

GENERAL METHOD

Animals

Forty-five Sprague-Dawley (Simonson, Gilroy, CA) male rats served as subjects. All were housed in individual cages in temperature and humidity controlled rooms with continuous light. They were maintained on ad lib laboratory chow and a constant water supply. There were no significant differences in body weights of the several groups at the start of each experiment, the means varying around 225 g.

Each experiment involved a minimum of five replications of the research design. Animals were assigned randomly to the treatment cells. All behavioral testing was carried out at the same time of day in order to eliminate the possible confounding effects of diurnal changes in ACh or other neurochemical variables [12].

Measures of Behavior

Multiple stimulus rating scale. Measures of behavior were selected after study of the extensive literature on behavioral changes following septal lesions in the rat. The general procedure has consisted of a standardized rating scale to evaluate the relative reactivity of animals during exposure to a variety of test conditions [4, 15, 19, 39]. The exact procedure used in the present experiments was adapted from that described by King [19] and had been employed in earlier experiments in our laboratory [9,17]. In brief, during a test session an animal's reaction to visual and tactile stimuli were observed; in addition its resistance to capture and handling, its vocalization, and its muscular tension after capture were rated. A 6-point scale was used for each of the five test conditions during which the above reactions were observed. Thus, the total score for an animal could vary from 5 in the most hypo-reactive state to 30 in the most hyper-reactive. Animals were rated by a trained experimenter who was not aware of the pretreatment schedules to which the animals had been exposed. Checks were made of the consistency with which the rating scales were applied. The high level of consistency achieved is reflected, for example, in a comparison of scores for septally lesioned animals in experiments conducted in our laboratory two years apart; the means were 23.8 and 24.2, with no significant difference between groups (Mann-Whitney $U=20.5$).

Non-contingent aversive stimulation. Measures were also made of reactivity in a situation involving noncontingent aversive stimulation, i.e., unconditioned reactions (UR) to inescapable electric shock. Low shock intensities produce a flinching response, followed at higher intensities by skeletal activity which is intensity dependent [3,25]. All shock intensities in the present experiments were above the threshold for the flinching reaction. Experimental observations were made with the animal in a test chamber 30.5×30.5×30.5 cm. The top and sides of the box were made of transparent plastic. Shocks at the various intensities required were delivered by a Grason-Stadler shock generator to the stainless steel rods which constituted the floor of the chamber. The polarity of the electrified grid was scrambled automatically. The duration of each shock pulse was 0.5 sec and shocks were delivered at a frequency of 20 per min. Shocks of different intensities were given in groups of 10 shock pulses in the following counterbalanced order: 1.3, 2.5, 0.5, 1.3, 0.5, 2.5, 1.3, 2.5 and 0.5 mA. Thus each test session involved reactions to 90 shock pulses, 30 at each of the 3 intensities. Records were kept as to whether or not each shock pulse stimulated a UR, i.e., jumping, prancing or running [25]. All trials were carried out with the experimental room in darkness except for a 60-W electric bulb directly over the test chamber. Extraneous sounds were masked by a continuous white noise background.

Analysis of Results

Statistical analyses were carried out using nonparametric methods and following the accepted procedure of testing first by analysis of variance, then by two-way comparisons when such testing indicated significance at or better than the 0.05 level of confidence. The Kruskal-Wallis (H) statistic was used in analyzing independent samples and the Friedman (χ_r^2) related samples. These were supplemented by Mann-Whitney (U) and Wilcoxon (T) tests where appropriate. Whenever statistical analysis involved predictions about the direction of a difference, one-tailed tests of significance were used; otherwise the tests were two-tailed and are so noted in the text.

EXPERIMENT 1

ACUTE SUPPRESSION OF CHOLINE TRANSPORT

Experiment 1 was planned to investigate effects on the behavior patterns of interfering acutely with the high affinity transport of Ch in brain, thereby causing varying endogenous levels of ACh.

Method

Pharmacological manipulation was carried out by IVt injections of HC-3 (Eastman 9702). ACh levels were varied by administration at five dose levels, including a saline control: saline, 0.01, 0.1, 1.0 and 10 μg. Previous research in our laboratory had provided data on ACh levels in whole brain following microwave fixation which established that these doses result, at peak effect times between 2 and 4 hr, in decreases in levels ranging from 0% (saline) to 81% (10 μg) [9]. The HC-3 used had been recrystallized from absolute ethanol/methanol (1:1) in order to remove possible contaminants [17]. All doses were injected in a volume of 2 μl.

The research design required 25 animals. Multiple stimulus rating scales were applied four times relative to HC-3 injections: 24 hr pretreatment to establish baseline reactions and 2, 24 and 168 hr post-treatment. Tests for reactions to noncontingent shock stimulation occurred 24 hr pretreatment and once only thereafter, i.e., 2.5 hr post-treatment.

Experiment 1 involved injections directly into the cere-

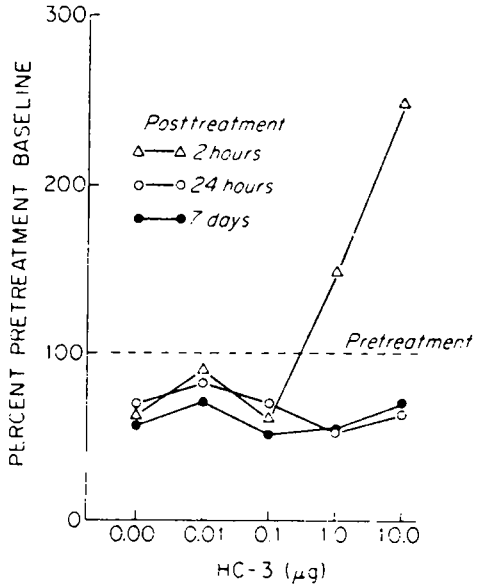

FIG. 1. Dose-response effects of HC-3 on reactivity: triangles show effects 2 hr after cerebroventricular injection; open circles, after 24 hr; and filled circles, after 7 days.

broventricular system of the brain. Cannulation was carried out using the basic technique described by Balbion Vester *et al.* [2]. A polyethylene cannula was inserted freehand into the right lateral ventricle through a hole placed 2 mm lateral to the saggital suture and 1 mm caudal to the coronal suture. Stainless steel, self tapping screws were placed in two holes nearby as a means of anchoring the cannula, which was bonded to the skull with dental acrylic resin. Injections were made with a 10 μl syringe (Hamilton 701-SN, 26 ga). Checks for placement of cannulae were made on a random sample of the animals. Gentian violet (5 μl) was injected via the cannula, the animals sacrificed with pentobarbital anesthesia, their brains removed and sectioned along the line of the cannula. In all cases staining of the ventricle was clearly visible.

Results

Figure 1 summarizes effects of HC-3 on reactivity as evidenced in the total scores for the various behavioral ratings. There were no significant differences among the five dosage groups on tests 24 hr prior to HC-3 treatment ($H=5.3536$, 4 *df*, $p>0.05$). Two hours after treatment significant effects did occur ($H=17.1677$, 4 *df*, $p<0.005$). Although there were no significant differences among controls and experimental animals at the two lower doses, animals with the two highest doses showed a very marked hyper-reactivity. The hyper-reactivity began to occur when ACh levels reached approximately 50% of normal, as determined by Freeman *et al.* [9], and increased exponentially thereafter. The effect disappeared by the 24 hr post-treatment test session and there were no significant differences among groups when retested at 168 hr, i.e., 7 days post-injection.

Friedman two-way analysis of variance by ranks confirmed the prediction [25] that the URs recorded in the noncontingent shock situation would be dependent upon stimulus intensity: hyper-reactivity increased very significantly as shock intensity increased ($\chi^2_r=27.02$, 2 *df*, $p<0.001$). However, there was no evidence of dose dependency during tests 2 hr after injection when HC-3 was having its peak effect. Kruskal-Wallis analyses of variance provided "H" statistics of 1.8941, 3.0948, and 3.8292 for the 0.5, 1.3 and 2.5 mA shock intensity trials, respectively, none of these being significant at $p<0.05$ with 4 *df*.

EXPERIMENT 2

CHRONIC SUPPRESSION OF HIPPOCAMPAL ACh AND Ch CONTENT AND OF ChAT ACTIVITY

In Experiment 2 morphological lesions in the septal area of the brain were used as a means of producing chronic suppression of ACh and Ch levels and of ChAT activity in the hippocampus [20,38].

Method

Twenty rats served in the experiment. Each was assigned randomly to one of three groups: septal lesioned (n=10), sham operated control (n=5) and unoperated control (n=5).

Electrolytic lesions and sham operations were performed under sodium pentobarbital anesthesia. The coordinates for the lesioning electrode were, from Bregma: 2 mm anterior, 0 mm horizontal, and 6 mm vertical. The second electrode was placed in the rectum. A 2.0 mA anodal dc current was passed for 20 sec through the stainless steel electrode (0.46 mm dia.) and insulated except for 1 mm at the tip. The same surgical procedure was followed for both groups except that in sham operated animals the electrode was lowered vertically by only 3 mm and no current was applied.

After the final behavior test all lesions were verified histologically. Animals were perfused intracardially with 0.9% saline followed by 10% Formalin. Brains were removed and kept in Formalin for 7 days before being sectioned and stained. The stained sections were then examined microscopically. Although lesions were not all completely uniform they damaged the precommissural septum, including the medial and lateral septal nuclei.

Responses of all animals were recorded on the multiple-stimulus rating scales at three different postoperative times: 3, 7 and 28 days. Repeated tests of the same animal always occurred at the same time each day. Reactions in the standardized noncontingent shock situation were measured on the seventh postoperative day in order to be clearly within the peak effect period of the septal lesions [38].

Results

Although all animals included in the present report had damage which included the medial and lateral septal nuclei, there were differences in the areas involved. It would be expected that inadequate lesions, i.e., too small, too large or misplaced, would be reflected in behavioral effects which would differ from those induced by proper lesioning [11]. In analysis of results, omission of behavioral ratings for animals with the three smallest lesions had no significant effect on the group means: 23.0 vs 23.4 for the full sample. Tests for outliers [6,40] established that the extreme values at both ends of the sample actually belong to the normal distribution of the remaining scores ($E_k=0.783$, $p>0.05$). Of the extreme outliers only one was among those animals with the smallest lesions. Thus, the empirical evidence justifies treatment of the behavioral measures involved in the present analysis as belonging to the same distribution.

TABLE 1

EFFECTS OF SEPTAL LESIONS ON BEHAVIORAL VARIABLES

Multiple-Stimulus Rating			Aversive Stimulaton*		
Days Post lesion	Lesion	Control	Shock (mA)	Lesion	Control
3	23.80 ± 2.78†	9.40 ± 3.66	0.5	5.90 ± 3.91‡	2.40 ± 1.15
7	23.40 ± 4.12†	8.50 ± 2.51	1.3	15.30 ± 4.31‡	9.10 ± 2.02
28	9.00 ± 3.37	8.70 ± 4.27	2.5	15.13 ± 3.57‡	9.20 ± 2.22

N = 10; Mean ± SD.

*The lowest shock intensity was above the threshold for the flinching response [25]. Only skeletal activity is included in the anaylsis.

†More hyper-reactive than controls: $p<0.001$.

‡More hyper-reactive than controls: $p<0.01$.

Comparisons were made of performances of the sham operated and the unoperated control groups during tests on the seventh day following surgery to the former. There was no significant difference between the groups ($U=8$, $p>0.05$) and, therefore, the two were combined to form the control group for later analysis.

Effects of septal lesions and their neurochemical sequelae on the multiple-stimulus behavioral ratings are summarized in Table 1. It is clear that for a time after surgery, the treated animals were very significantly more hyper-reactive to the test stimuli than were the controls ($p<0.001$). However, this condition had disappeared by the time of testing on the 28th day post-lesion.

Tests for reactions to noncontingent aversive (shock) stimulation were conducted on the seventh day after lesioning. The results are also summarized in Table 1. At all levels of shock intensity the lesioned group was significantly more hyper-reactive than the controls ($p<0.01$). Indeed, at each level there was an overlap of only one animal between the two samples. The Wilcoxon matched pairs statistic indicates that differences within groups between the 1.3 and 2.5 mA conditions are not significant ($T=27$, $p>0.05$) suggesting that the grading of skeletal activity according to stimulus intensity [25] approaches an asymptote within this range of values. Friedman two-way analyses of variance using the data from all shock levels shows that, despite this trend, there exist significant positive relations between stimulus intensity and hyper-reactivity in both septal ($\chi^2_r=15.8$, 2 *df*, $p<0.001$) and control ($\chi^2_r=7.60$, 2 *df*, $p<0.02$) groups.

DISCUSSION

Effects on Behavior

Multiple-stimulus situation. Both experimental treatments, administration of HC-3 IVt and septal lesioning, were consistent in their effects on responses to the multiple-stimulus test situations. In all instances treated subjects were hyper-reactive at peak effect times, a state which disappeared as duration of the post-treatment period increased. It is important to emphasize that the responses were reactions oriented to experimentally induced changes in stimulation and not merely increased general activity. Effects of the two experimental treatments in producing hyper-reactivity in identical test situations were remarkably similar quantitatively. The mean for HC-3 animals is 23.00 ± 4.207 as compared with means for septally lesioned subjects tested at 3 and 10 days postoperatively of 23.80 ± 2.181 and 23.40 ± 4.115, respectively (there are no significant differences among the controls for these groups). This similarity cannot be accounted for in terms of limitations of the measuring instruments, which provided a range extending well beyond these levels. Although hyper-reactivity occurred at peak effect times for the two treatments, it must be remembered that these times had different dimensions, i.e., hours in the case of HC-3 injection and days for septal lesioning. Further consideration of the implications of this is given below.

Non-contingent shock situation. The two experimental treatments did not produce a concordance of behavioral effects in the noncontingent shock situation. Septal animals showed very significant hyper-reactivity at their peak effect times, but the HC-3 injected groups showed no dose-dependent effects and were no more hyper-reactive than control animals.

Such differences between effects of the same experimental treatments in the two behavioral test situations is not surprising. Multivariate analyses of behavioral effects of septal lesions have shown that several independent factors constitute the septal syndrome [42]. There are no rational grounds for assuming that all these factors share the same neurochemical substrate. That behaviors in the two test situations studied in the present experiments may, indeed, be associated with "pharmacologically distinct components of the septum" is consistent with results reported by Miczek *et al.* [24]: when hyper-reactivity of the kind observed in our Experiment 1 was eliminated by pre- and postoperative handling, reactivity to shock continued unaffected. It has been suggested that reactions to electric shock may be related to decreased levels of serotonin [21] or catecholamines [26], rather than to changes in the cholinergic system.

Critical Level

Earlier studies involving pharmacological manipulation of the cholinergic system using anti-ChE agents have provided results requiring introduction of a concept of "critical level" [32]. Dose-response functions showed that relations between brain AChE activity and behavioral changes were not linear: there was a critical level between 40 and 50% of normal

enzyme activity below which the magnitudes of changes in behavior became significant and, therefore, were inversely related to AChE activity. This critical level has more general significance in that it is, for example, in the range where Aprison [1] reported that the enzyme lost control of its substrate, where a crossover from potentiation to decline occurs in a respiratory reflex [23], where a sharp drop in nerve conductance begins to be clearly observable [44].

Results of the present experiments indicate that the concept of critical level is also applicable to them and suggest that the levels are within the same range as those already reported from other behavioral patterns. Hyper-reactivity followed HC-3 injections IVt only at the two highest doses when ACh was reduced to 27% and 23%, respectively, of its normal level. It was also observed that hyper-reactivity had disappeared 24 hr after HC-3 injection when the ACh concentration for the animals affected earlier had recovered to 76% of normal.

Reduction in AChE activity is related to increased ACh levels; the opposite effect, i.e., reduced levels, follows administration of HC-3. Observations of behavioral effects when deviations in the cholinergic system occur beyond critical points in either direction from normal suggests that limits to behavioral plasticity may be set in terms of the state of its neurochemical substrate(s) [28].

Relations to Changes in the Cholinergic System

Acute changes. Results of Experiment 1 demonstrated that the significant effect of acute manipulation of the cholinergic system with HC-3 IVt was to induce hyper-reactivity in the behavioral rating situations during testing 2 hr following treatment, a change which disappeared at tests 24 hr after administration. Research in our laboratory using the same doses, stock of animals and procedures for injecting HC-3 has shown that ACh concentrations in brain follow a similar time course [9], ACh levels decreasing to a low of 19% at 4 hr and recovering to 76% by 24 hr. Ch levels were not affected significantly during this period. The association between the dose-effect trends for hyper-reactivity and ACh levels as measured by the Spearman rank order correlation coefficient is −1.00.

Chronic changes. The immediate effect of septal lesions was also to induce changes in behavior: septal animals were significantly more hyper-reactive than control subjects during tests both three and seven days after surgery. Reports by Kuhar *et al.* [20] and by Sethy *et al.* [38] discussed above, have established that septal lesions reduce endogenous ACh levels and cause decreases in events involved in ACh synthesis, these effects reaching a maximum 4 days post-operative and persisting chronically. The concomitant changes in the cholinergic system and in behavior during the initial period after lesioning was analogous to the acute effects described above, i.e., decreased availability of ACh was associated with hyper-reactivity.

Behavioral recovery. This relationship changed, however, as chronicity of reduced ACh levels increased. Although it has been shown that these levels are not changed when assayed 2 months post-lesion [20], hyper-reactivity of septal animals in the present experiment had disappeared by the time reactions in the multiple-stimulus situations were measured 28 days after surgery. A dissociation had occurred between the neurochemical and behavioral variables, which, in acute studies, had appeared to be highly related, i.e., changed from pretreatment base lines and recovered concomitantly.

This discussion suggests that another process (or processes) is involved, which is activated by and compensates for the acute effects of manipulating the cholinergic system. The circumstances are reminiscent in general terms of the development of behavioral tolerance to anti-ChE agents, where return to pretreatment base line occurs even when AChE activity is maintained at a constant percent of normal [31]. Recovery can only be accounted for by postulating a change(s) in some other feature of the cholinergic system or the involvement of another system(s). In the case of tolerance development to chronic administration of anti-ChE agents research has focussed attention on postsynaptic events, i.e., changes of sensitivity of postsynaptic muscarinic receptors [29,33].

Many research findings suggest an alternative view, i.e., the general hypothesis that there exists a central cholinergic system which exerts an "inhibitory" effect on behavior by modulating sensory input, suppressing response output, or a combination of the two [5, 27, 43]. It is plausible that, in the normal organism, such a cholinergic system would function collaboratively with other transmitter systems. The interactions could be based upon dynamic "balances" between systems or upon "redundancies" by which one system is capable of taking over the roles of another when the latter malfunctions [31].

Recent clinical, as well as experimental, investigations have led to special interest in the "interaction" model [7, 16, 21, 22, 26, 36]. Evidence that increased central cholinergic activity can ameleorate symptoms in mania has suggested that a relative cholinergic underactivity is involved where hyper-reactivity is a behavioral feature. Such observations are consistent with those of our present experiments in which hyper-reactivity followed decreases in ACh levels in the central cholinergic system. The observations have led to the proposal of an "imbalance" hypothesis of affective disorders: mania is viewed as a condition of decreased cholinergic activity relative to catecholaminergic or indoleaminergic activity and depression, as an imbalance in the opposite direction. To test the interaction model fully requires two basic research strategies: (a) analyses in tissue from the *same* animal of the neurochemical systems whose balance is hypothesized to be involved in the behavior under study, and (b) pharmacological manipulation of the *balance* between such systems while measuring consequent changes in the behavior.

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