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Fourth Ventricle Effects of Nicotine, 2-Methylpiperidine and Cytisine in Dogs

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MARTIN, W. R., J. W. SLOAN, R. HOOK, E. KAPLAN AND C. WASH. *Fourth ventricle effects of nicotine, 2-methylpiperidine and cytisine in dogs.* PHARMACOL BIOCHEM BEHAV **25**(4) 843–848, 1986.—Four distinguishable nicotinic binding sites have been identified as well as four nicotinic ligands with different specificities: (±)-2-methylpiperidine which binds to a very high affinity site (Site 1) and produces up-regulation of the high affinity site (Site 2); (−)-nicotine which binds to Site 1 and Site 2 as well as to a low affinity site (Site 4); (+)-nicotine which binds to Site 1, Site 4 and Site 3 which is also a high affinity site; and (−)-cytisine which binds to Sites 1 and 2. These drugs were injected into the 4th ventricle of 5 dogs in graded concentrations (12.5 to 400 μg) and their effects on the EEG, skin twitch reflex latency, heart rate, rectal temperature, pupillary diameter, blood pressure and the amplitude of the flexor reflex were measured. Drugs which act predominantly on Site 1 [(±)-2-methylpiperidine and (+)-nicotine] produced EEG synchronization and hyperalgesia while drugs which interact with Sites 2 and 4 produce EEG desynchronization, analgesia and tachycardia. These data indicate that nicotinic ligands which have different binding specificities have different actions in medullary function and support the hypothesis that the different binding sites have different pharmacologic significance.

(−)- and (+)-Nicotine Up-regulatory site High affinity site Low affinity site
Multiple modes of nicotine's actions (−)-Cytisine (±)-2-Methylpiperidine Conscious dog
Intracerebroventricular EEG Analgesia Pupillary diameter Body temperature Behavior

IN a series of studies of the binding of (+)- and (−)-nicotine as well as other related drugs four nicotine binding sites and ligands which have some degree of specificity for these sites have been identified [22]. Four of these ligands which have been employed in the present study and their binding sites are summarized in Table 1. Site 1 enhances the binding of (+)- and (−)-nicotine probably at a very high affinity site and is thought to produce up-regulation. Site 2 is the (−)-nicotine high affinity site. Site 3 is the (+)-nicotine high affinity site which may be a sub-population of Site 2. This latter issue is unresolved. Site 4 is a low affinity site and it has been postulated to be a nicotinic cholinergic site.

It was first suggested by Abood [2] that nicotine may exert some of its actions through a non-cholinergic mechanism. The present study investigates graded amounts of nicotinic ligands with different patterns of binding specificities [(+)- and (−)-nicotine, (−)-cytisine and (±)-2-methylpiperidine] administered into the 4th ventricle of intact or low spinal dogs to determine if they exhibit different pharmacologic effects. The 4th ventricle was chosen as a site for study because of previous findings [12] which indicated that an infusion of (−)-nicotine into the 4th ventricle altered several physiologic parameters.

METHOD

All experiments were conducted in five dogs; one intact and 4 chronic spinal preparations. The chronic spinal dogs were prepared under pentobarbital anesthesia in an approved facility under sterile conditions. The spinal cord was transected at approximately the T-10 level and polyethylene cannulae (PE 10) were inserted beneath the dura such that their tips were approximately at the C-7 and L-7 levels of the spinal cord. These intrathecal cannulae were led through a special holder which was screwed onto the T-11 vertebral spine through a modified Clay Adams plastic tubing adaptor with a stainless steel guide. All polyethylene cannulae were cemented to a guide with a Super Glue. The polyethylene cannulae were cut nearly flush with the guide and plugged with a 30 gauge stainless steel wire. A screw-on cap was placed on this receptacle. Further, a polyethylene cannula was inserted into the fouth ventricle through a guide cannula with a sharpened stylet. The cannula was oriented stereotaxically and penetrated the atlanto-occipital membrane and the posterior medullary velum. When the sharpened stylus was removed from the guide cannula the free flow of spinal fluid indicated the presence of the tip in the fourth ventricle. The

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TABLE 1

A SUMMARY OF THE BINDING SITES FOR (−)- AND (+)-NICOTINE, (±)-2-METHYLPIPERIDINE AND (−)-CYTISINE AND THEIR PHARMACOLOGIC ACTIONS WHEN ADMINISTERED INTO THE 4TH VENTRICLE OF INTACT OR CHRONIC LOW SPINAL DOGS

	Site	K_D*	EEG	Skin Twitch Reflex Latency	Blood Pressure	Pulse Rate	Miosis	Rectal Temperature
	1						+	
(−)-Nicotine	2	2.5×10^{-8}						
	4	1.5×10^{-5}	Desyn	↑				
	1		Syn	↓			+	
(+)-Nicotine	3	6.7×10^{-7}			↑	↑		↑
	4	1.5×10^{-4}	Desyn	↑		↓		
(±)-2-methyl-piperidine	1	1.8×10^{-8}	Syn	↓			+	
(−)-Cytisine	1						+	
	2	3.7×10^{-9}			↓	↑		↓

Syn=synchrony; Desyn=desynchrony; ↑ =increase; ↓ =decrease; and + =present.
*The K_Ds for (−)- and (+)-nicotine and for (−)-cytisine are from [22].
*The K_D for (±)-2-methylpiperidine is an unpublished observation of Sloan and Martin obtained using [^{3}H]-(±)-2-methylpiperidine.

polyethylene cannula was inserted through the guide cannula to a predetermined distance calculated to be at Horsely-Clark posterior −7. The cannula was further advanced in the fourth ventricle until its movement was obstructed by the anterior end of the fourth ventricle and then withdrawn to the predetermined distance to further confirm the AP location of the tip.

Stainless steel bolts were placed head-down on the parietal cortex bilaterally in a key slot and EEG leads and a tower form (see below) were bolted on. Further, an indifferent screw electrode was placed on the occipital ridge. These leads were attached to a Cannon plug (MIKO-1-7SH14).

The polyethylene fourth ventricle cannula was inserted into a length of stainless steel tubing (18 gauge-14 mm long). This tube and cannula were led into a tower form made of a cut-off top of an appropriately sized plastic container. The form with the Cannon plug and fourth ventricle cannula was filled with dental acrylate. A cap was placed on the tower form. After recovery from surgery, animals were trained to lie on an observation table. Several physiologic measures were made. The bipolar parietal lobe ECoG was recorded on a Grass polygraph. Fifteen minute segments of the ECoG were digitized using an IQS series 401 FFT spectrum analyzer (Model 4000) and were stored on a magnetic disk. The digitized data were subsequently analyzed, again using the IQS series 401 FFT spectrum analyzer Model 4000 in 10 second epics and the power of frequencies from 0.5 to 30 Hz was obtained. Further, the ECoG was integrated using a Grass Polygraph Integrator (Model 7P10E) and the ramps (electrogenesis) displayed on a Grass polygraph. These data were digitized for 15 seconds each minute. Further, the EKG was also digitized for 15 seconds out of each minute. The flexor reflex was evoked with a pneumatic toe squeezer in which the toe was squeezed with 15 lb of pressure [17] once every minute. The amplitude of the flexor reflex was recorded using a specially designed stain gauge transducer and a low-level DC amplifier (Model 7P1). The electrical output of this strain gauge was also digitized for 24 seconds of each minute. The sequence of the digitizing process was controlled using an Isaac Computerized Data Acquisition and Control System and was initiated by the toe squeezer. The digitized data were recorded on magnetic disks for subsequent analysis. The sequence of digitizing the data was (1) flexor reflex, (2) EKG and (3) the electrogenesis. These data were then expressed as output/minute using an Apple IIE computer. Each of these variables were printed out and time action curves obtained for each dog. Further, the area under appropriate segments of the time action curve was calculated. All data were transformed into a percent of the mean of 30 minutes of predrug control values. These data were also stored for subsequent analysis. Pupils were photographed using a Polaroid Close-Up Camera [15]. Body temperature was measured with a needle thermister probe placed in the spinal muscles below the level of transection. The skin twitch latency was also determined every 15 minutes using a previously described method [10]. Estimates of systolic blood pressure were obtained from the tail of the dog using a Narco Biosystems-Programmed Electrosphygnomanometer (PE 300) from tracings recorded on one channel of the polygraph. In the studies of (+)- and (−)-nicotine the data acquisition system was not in place and the analyses were made from the polygraph record. In these studies EEG and EKG data were taken for one minute every 15 minutes. The mean amplitude of the flexor reflex was measured for each 5 minute interval. To make all data equivalent since different areas under the time action curves were analyzed, the difference between the saline control and the drug condition was divided by the number of minutes comprising the area.

Experimental Design

Five dogs were employed in these studies. Four concentrations of all drugs were administered into the fourth ventricle in a 150 μl volume. The four doses were randomized

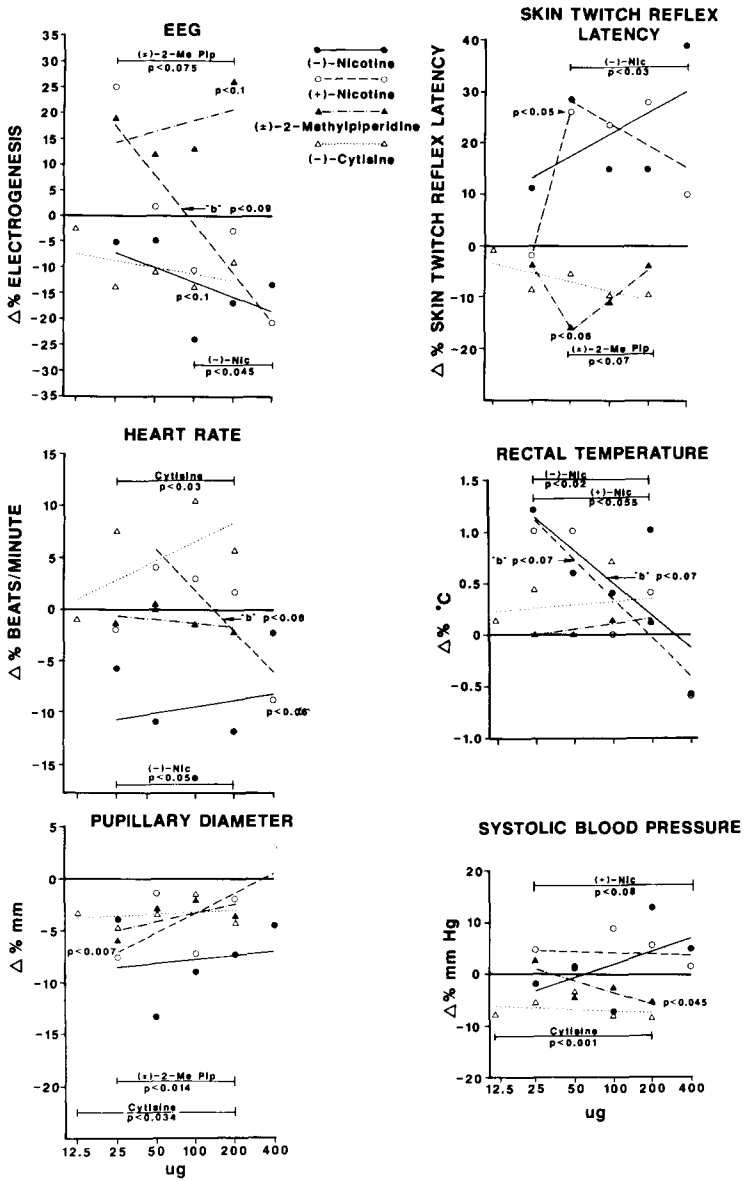

FIG. 1. Effect of (+)- and (−)-nicotine, (±)-2-methylpiperidine and (−)-cytisine on the EEG, skin twitch reflex latency, heart rate, rectal temperature, pupillary diameter and systolic blood pressure in the dog. All points are the mean of observations made in 4 chronic spinal and 1 intact dog. p-Values for points and segments of slopes (b) of the dose-response lines are presented.

and administered in a Latin square design to four spinal dogs. Studies in one intact dog were subsequently conducted. If additional doses seemed approprite they were incorporated at the end of the Latin Square Block. Following a 30 minute control period, drugs were administered and observations were made for an additional 90 minutes. A saline control study was done periodically. The dose levels employed were administered in geometric increments ranging from 12.5 to 400 μg. The data for all experiments were transformed into percent of mean predrug control observations. The area under the appropriate portions of the time action curve was determined for each animal under each treatment condition. The data were analyzed by a two-way analysis of variance in which between dogs, between weeks and between doses variance was determined. Dogs participated in experiments at approximately weekly intervals. The data were further analyzed by partitioning the between doses variance into a linear and quadratic component. If the quadratic component accounted for most of the variance the curvilinear dose-response curve was resolved into two linear components. Dose-response curves, or segments of dose-response curves, were also compared using a bioassay procedure [7]. These analyses of variance were done using the Statistical Analysis Systems General Linear Models (GLM) procedure for multiple regressions and an unbalanced ANOVA.

Four of the 5 dogs were sacrificed and the sites of the cannulae were verified by identifying a slight identation on the floor of the 4th ventricle which was the cannula bed. The cannula bed was in the mid-line and the rostral end of the beds were at the intended locus. Bromophenol (150 μl) was injected into the 4th ventricle of one dog. The medulla was stained approximately 1 mm on either side of the cannula bed which extended from the middle of the 4th ventricle to its caudal end.

RESULTS

Figure 1 presents the mean values for all parameters for the four drugs. Two two-way analyses of variance revealed that there was no significant between weeks variance for any of the drugs or measures presented in Fig. 1. There were only several instances when the between dogs variance was significant. Further analysis was directed at determining the configuration of the dose-response curves. All doses of (−)-cytisine desynchronized the EEG, however the degree of desynchronization was not statistically significant. Further, (−)-cytisine produced no dose-related EEG changes. The desynchronization was due to a modest decrease in delta, theta and alpha activity. In contrast, all doses of (±)-2-methylpiperidine increased EEG electrogenesis which was manifest as an increase in delta, theta and alpha activity. There was not a significant linear or quadratic dose-response relationship. The three largest doses of (−)-nicotine produced a significant degree of desynchronization. However, the linear and the quadratic components of the regression analysis were not statistically significant. It is important to note that the lowest dose of (+)-nicotine produced synchronization and that 100 and 400 μg produced desynchronization which was comparable to that produced by the highest dose of (−)-nicotine. Further, the slope (b) of the (+)-nicotine dose-response line approached statistical significance ($p<0.1$).

The effects of nicotine on the skin twitch reflex latency are also complicated. Both (+)- and (−)-nicotine produced a significant prolongation of the skin twitch reflex, however simple dose-response relationships were not obtained. There was not a statistically significant linear or quadratic component for the (−)-nicotine dose-response line, however doses from 50 to 400 μg produced a statistically significant degree of analgesia which was frequently observed within 5 minutes following injection and persisted for over 50 minutes. The (+)-nicotine dose-response line had a significant quadratic but not a linear component and the degree of analgesia produced by the 50 μg dose was not statistically significant. The hyperalgesia produced by the 50 μg dose of (±)-2-methylpiperidine was almost statistically significant ($p<0.06$) and the dose-response curve had a significant quadratic but not a linear dose-response line. The hyperalgesia produced by (−)-cytisine over the 50 to 200 μg range also was almost statistically significant.

(−)-Nicotine produced a significant bradycardia over a dose range of 25 to 200 μg however the dose-response line had neither a statistically significant linear or quadratic component. The 25 μg dose of (+)-nicotine had no effect on pulse rate whereas the 50 μg dose produced tachycardia and this effect was less with higher doses. A bradycardia was observed with the 400 μg dose. The dose-response line had a negative slope (b) and the linear regression component over the dose range of 50 to 400 μg approached statistical significance ($p<0.076$). (−)-Cytisine (25–200 μg) produced a statistically significant tachycardia while (±)-2-methylpiperidine had no statistically significant effect on heart rate.

(+)-Nicotine (25 to 400 μg) produced a modest increase in blood pressure ($p<0.08$) while (−)-cytisine (12.5 to 200 μg) produced a decrease in blood pressure ($p<0.001$). Niether (−)-nicotine or (±)-2-methylpiperidine altered blood pressure. None of the drugs administered into the fourth ventricle altered the amplitude of the flexor reflex.

DISCUSSION

Many investigators have shown that nicotine alters medullary functioning. Previous studies in our laboratory have shown that (−)-nicotine administered into the 4th ventricle of the conscious dog has several important effects: enhanced respiratory rate, prolonged latency of the skin twitch reflex, decreased EEG electrogenesis and depression of body temperature [12]. In these studies nicotine was infused into the 4th ventricle in a large volume and histologic studies using a dye marker indicated that dye was not only found in the entire 4th ventricle but on the ventral surface of the medulla, the upper cervical spinal cord, and to a slight degree in the 3rd ventricle. The drugs were probably at their highest concentration along the 4th ventricle cannulae bed in the present study. The use of the chronic indwelling 4th ventricle cannula allowed drugs to be injected into the 4th ventricle over a nine month period and there was no statistically significant change in responsivity to drugs across time.

(−)-Nicotine can produce both EEG desynchronization associated with arousal and EEG synchronization and sedation when administered intravenously and into the 4th ventricle of the cat [23,24] and when administered into the vertebral artery or into the lateral cerebral ventricle of the dog [8,9]. Intravenously administered (−)-nicotine produces an initial desynchronization and arousal followed by EEG synchronization and sleep in the cat [26]. (+)-Nicotine had a similar action except that it was less potent. In extensive studies conducted to localize the site of action of (−)-nicotine responsible for EEG arousal in the dog it was found that nicotine did not produce EEG desynchronization in dogs whose brainstems were transected anterior to the pons but produced desynchronization when the brainstem was transected at the mid-pontine level [13]. These investigators concluded that the desynchronizing action of (−)-nicotine was at the ponto-mesencephalic level of the brainstem. Since the alerting and desynchronizing effects were antagonized by mecamylamine it has been also concluded that a nicotinic-cholinergic mechanism was involved. It has been found that (−)-nicotine administered intraventricularly to the rat produces prostration and minor seizure [1]. Similar signs have been reported in the rabbit following large doses of nicotine IV. Because d-tubocurarine did not antagonize these effects while they were antagonized by N-benzylnicotine and N-benzylpiperidine, it was concluded that (−)-nicotine was exerting its effects through a non-cholinergic mechanism [1]. The data presented in this paper also suggest that (+)-nicotine has two different actions on the EEG; one a desynchronizing action and the other a synchronizing effect. Synchronization of the EEG may be produced by the drugs interacting with the up-regulatory site and desynchronization by agonists interacting with a low affinity site. The data with (+)- and (−)-nicotine are also consistent with this formulation. (+)-Nicotine is more potent (10^3–10^4 times) in producing up-regulation than (−)-nicotine

whereas (−)-nicotine has a higher affinity for the low affinity site than does (+)-nicotine [21,22]. Low doses of (+)-nicotine produced EEG synchronization. The present studies also demonstrate that (+)- and (−)-nicotine's EEG synchronizing and desynchronizing systems are present in the caudal 4th ventricle probably near the dorsal surface. It is not known whether the (+)-nicotine induced synchronization is related to the cholinergic sleep system which ascends in the ventral tegumentum to the medial forebrain bundle [11]. Further, a caudal medullary nicotinic desynchronizing system has been identified which may be part of the pontine nicotinic activating system [13].

The effects of (−)-nicotine administered into the 4th ventricle on pupillary diameter and rectal temperature have not been previously reported. All drugs studied tended to produce miosis. Both (+)- and (−)-nicotine tended to increase temperature in lower doses. The fact that none of the drugs studied significantly altered the amplitude of the flexor reflex of the chronic spinal dog suggests that the drugs were not producing their effects by being absorbed systemically.

Nicotine has been shown to produce analgesia in mice [18], rats [20,25] and dogs [12]. Although nicotine produces analgesia when administered intravenously it failed to do so when administered into the third ventricle [12]. The fact that nicotine produced analgesia over a wide range of doses when injected into the 4th ventricle is consistent with previous observations [12]. The present studies in which nicotine was restricted to the 4th ventricle establishes the 4th ventricle as a site of action. Others have shown that (−)-nicotine injected into the common carotid, vertebral artery or intrathecally in the lumbar cord produces a short latency, short duration analgesia in the rat as measured by the tail flick [16] and concluded that nicotine acted at spinal and supraspinal sites. In the intact dog, IV (−)-nicotine prolonged the latency of the skin twitch reflex during a 20 minute infusion and through a twenty minute recovery period [12]. (+)-Nicotine also produces analgesia of short duration in the mouse [3]. In the dog studies herein reported the analgesia produced by (+)-nicotine also persisted for over 50 minutes. The disparity of the time action of both (−)- and (+)-nicotine in the mouse and dog are probably not a consequence of the route of administration since (−)-nicotine had a relatively long duration of analgesic action when it was administered into the 4th ventricle and intravenously in the dog. Species may be of importance. It has been shown that (−)-nicotine is more potent as an analgesic in the rat than in the mouse [25]. Further, the nociceptive reflexes (hotplate reaction vs. skin twitch) and the type of data generated (quantal vs. prolongation of latency) were also different in the two studies. In contrast to (+)- and (−)-nicotine, (±)-2-methylpiperidine produced hyperalgesia which approached statistical significance at the $p<0.06$ levels. (−)-Cytisine which interacts only with the high (−)-nicotine affinity site (Site 2) and the up-regulatory site (Site 1) tended to produce hyperalgesia but the intensity of this action did not attain statistical significance.

The actions of nicotine and related drugs on medullary vasomotor regulation in the dog have not been extensively studied but there are reasons to believe that this site plays an important role in nicotine action. It was observed that intravenously administered (−)-nicotine (10 μg/kg) produced a depressor followed by a pressor effect in the dog [5]. These effects were markedly reduced by pentobarbital anesthesia and spinal cord transection (C-1) and somewhat reduced by a midcollicular transection. In the cat, intravertebral artery administration of (−)-nicotine produces a fall in blood pressure and heart rate [19]. It was also found that (−)-nicotine superfusion of the medulla produced a fall in blood pressure [4,6]. In the urethane anesthetized rat, nicotine microinjected into the vicinity of the area postrema produced a biphasic effect consisting of an initial vasopressor effect followed by a depressor effect and bradycardia [14]. (−)-Nicotine produced a significant slowing of heart rate but no consistent effect on blood pressure in the present studies. Higher concentrations of (+)-nicotine tended to slow heart rate while lower concentrations tended to increase heart rate and increase blood pressure. In contrast (−)-cytisine increased heart rate and lowered blood pressure. (±)-2-Methylpiperidine did not alter heart rate but the 200 μg dose produced a significant decrease in blood pressure.

These studies have not definitely identified the mode of action of these drugs. Their sites of action must be near the midline of the caudal 4th ventricle. Table 1 summarizes the results and relates them to the postulated receptors based on binding studies in the rat. The data is consistent with the hypothesis that Site 1 (up-regulatory) is associated with EEG synchrony, hyperalgesia and miosis. These effects are polarly opposites to effects associated with Site 4 (low affinity) which include EEG desynchronization, analgesia and bradycardia. (−)-Cytisine, which appears to interact predominantly with Site 2 and (−)-nicotine, had different profiles of effects. The role of (−)- and (+)-nicotine and Sites 2 and 3 in altering brain stem function have not been clearly delineated in these studies.

In conclusion, the complexities of (+)- and (−)-nicotine binding appear to be associated with complex changes in brain stem function. To further elaborate modes and sites of action of (+)- and (−)-nicotine, more specific agonists and antagonists will have to be developed and their pharmacology elaborated at various brain stem sites.

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