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Hippocampal Electrical Activity in Relation to Behavior Following Ethylcholine Aziridinium Ion (AF64A) Treatment

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STEWART, D. J., S. M. LEVENTER, I. HANIN AND C. H. VANDERWOLF. *Hippocampal electrical activity in relation to behavior following ethylcholine aziridinium ion (AF64A) treatment.* PHARMACOL BIOCHEM BEHAV **26**(2) 357–364, 1987.—The effects of intracerebroventricular (ICV) injections of ethylcholine aziridinium ion (AF64A; 3 nmol/3 μl/side) on the pattern of hippocampal electrical activity were studied in freely moving and urethane anesthetized rats. AF64A treated rats showed a significantly smaller increase in 6–12 Hz hippocampal rhythmical slow activity (RSA) with struggling in the no drug condition in comparison to the vehicle injected rats. However, neither AF64A treatment nor a control injection abolished the presumed cholinergic form of RSA that is present during urethane anesthesia. Systemic injection of atropine in waking rats did not significantly alter RSA in either the AF64A or vehicle injected rats. Analysis of histological brain sections revealed extensive damage to the fimbria-fornix, CA3 of the hippocampus, corpus callosum, neocortex and striatum. Acetylcholinesterase staining of the remaining hippocampus appeared normal in the AF64A treated rats. The data indicate that the depletion of cholinergic markers in the hippocampus following ICV administration of AF64A is not sufficient to disrupt the cholinergic form of RSA. Further, the question is discussed as to whether AF64A produces its cholinoselective effects via a specific pharmacological action or through a nonspecific destruction of the fimbria-fornix.

AF64A Ethylcholine aziridium ion Hippocampus Behavior EEG Fimbria-fornix system
Atropine Urethane RSA Septohippocampal system

RECENTLY, it has been suggested that certain neurotoxic analogs of choline (Ch) might be useful in producing selective long-term deficits in central cholinergic function. One such analog is ethylcholine aziridinium ion (AF64A; [9–11, 29]). AF64A is structurally similar to Ch but also contains a cytotoxic aziridinium moiety which produces an irreversible inhibition of high affinity choline transport (HAChT; [9,29]) the rate limiting step in the synthesis of acetycholine (ACh; [5]).

A number of studies have indicated that AF64A produces depletions of a variety of presynaptic cholinergic markers such as HAChT, choline acetyltransferase (ChAT) and ACh, with little or no effect on postsynaptic cholinergic markers in the neocortex, striatum and hippocampus, following intracerebroventricular (ICV) injection [11, 40, 41]. Presumably the changes are due to damage to cholinergic projections from the basal forebrain to the neocortex and hippocampal formation [20, 22, 31, 32]. No significant long-term alterations in biochemical markers associated with other neurotransmitters such as GABA, noradrenaline, dopamine or serotonin have been reported after AF64A treatment [23, 30, 41].

There is evidence that the occurrence of rhythmical slow activity (RSA) in the hippocampus in urethane anesthetized rats may be a good indicator of the function of cholinergic septohippocampal inputs. In rats anesthetized with urethane, RSA can be elicited in the hippocampus by sensory stimulation, electrical stimulation of the reticular formation or by the administration of physostigmine. The RSA produced in these ways can be completely abolished by systemic injections of anticholinergic drugs such as atropine, scopolamine or quinuclidinyl benzilate in urethane anesthetized rats [4, 16, 34]. Dudar, Whishaw and Szerb [8] have shown that ACh release in the hippocampus is increased during behaviors associated with RSA. Cells in the medial septal nucleus (in which many cells contain cholinergic markers) fire in rhythmic bursts that are phase-locked with RSA [26,36]. Further, ICV injections of hemicholinium, which deplete brain ACh, also abolish RSA in urethane anesthetized rats [27]. Microinfusions of cholinergic agonists directly into the hippocampus have been reported to produce RSA that can be antagonized by local microinfusion of atropine [28]. All this evidence indicates strongly that RSA in urethane anesthetized rats is due to activity in cholinergic

septohippocampal fibers. However, in waking rats the situation is complicated by the existence of a second RSA-producing input which is not sensitive to anticholinergic drugs and may be dependent on ascending serotonergic fibers [39]. Activity in this pathway is suppressed by urethane and other anesthetics.

Previous work on the effects of AF64A has relied largely on biochemical measures to demonstrate the selective destruction of cholinergic septohippocampal neurons. The recording of hippocampal RSA could supplement this work by providing an electrophysiological measure of central cholinergic function. This was attempted in the present experiment which investigated the effects of ICV injection of AF64A in rats on spontaneous hippocampal electrical activity and hippocampal activity evoked by medial septal stimulation. Hippocampal electrical activity was studied during normal behavior, after systemic administration of atropine and during urethane anesthesia.

METHOD

Subjects and Surgery

Animals. Male Sprague-Dawley rats (Zivic Miller laboratories, Allison Park, PA) were used in the present experiment. Food and water were available ad lib.

AF64A preparation. AF64A was prepared essentially as described previously [11]. Briefly, a solution of acetylethylcholine mustard HCl (Research Biochemicals Inc., Wayland, MA) was brought to pH 11.5 with NaOH. After stirring for 20 min at room temperature, the pH was lowered to 5.0–6.0 with 6 N HCl, then adjusted to pH 7.4 with dilute NaOH. For injection, the final concentration was 1.0 mM.

AF64A administration. Animals were anesthetized with chloral hydrate (325 mg/kg) and positioned in a Kopf small animal stereotaxic frame. AF64A (3 nmol/3 μl/side), or vehicle solution was infused bilaterally (ICV) with a 30 gauge needle inserted through a burr hole drilled in the skull. The needle was positioned according to the atlas of Pellegrino, Pellegrino and Cushman [25]. The accuracy of the injection was confirmed with dye in several animals. Stereotaxic coordinates were (from bregma): posterior 0.8 mm, lateral +2.5 mm, ventral (from dura) 3.0 mm. The rate of infusion was 0.5 μl/min and the needle was left in place for 2 min at the end of the infusion. Vehicle solution consisted of double distilled water, with the pH adjusted in an identical manner to that used in AF64A preparation. Animals were treated with AF64A or vehicle in Pittsburgh and then were shipped by air to London, Canada for subsequent studies.

Electrode implantation. At least two weeks following treatment with AF64A or vehicle solutions, the rats were anesthetized (Somnotol, 65 mg/kg, IP) and placed in a stereotaxic apparatus with the skull leveled such that lambda and bregma were in the same horizontal plane. Bipolar recording electrodes (125 μm in diameter Teflon coated, stainless steel wires) were placed in the dorsal hippocampus bilaterally and in the anterior neocortex unilaterally, according to standard techniques [6]. One member of each bipolar pair was approximately 1 mm shorter than the other. A single wire was also placed such that the tip was located in the medial septal region. A screw fixed in the skull over the cerebellum served as an indifferent for monopolar recording and for stimulation while a separate screw placed in the frontal bone served as a ground connection.

Drugs

Drugs (Sigma Chemical Co.) dissolved in saline and injected intraperitoneally included atropine sulfate (50 mg/kg) and urethane (ethyl carbamate). Drugs were given to the rats not less than two weeks after the electrode implantation and successive drug administrations were separated by at least 3 days. Urethane was used only in a terminal procedure and was given to five of the AF64A treated rats within 60 days of the AF64A injection. The remaining 6 rats in the AF64A group received urethane within 40 days of the AF64A treatment. Control rats received urethane at the same time as the AF64A treated rats. Rats were anesthetized with an initial injection of 1 g/kg of urethane with supplements of 0.25–0.5 g/kg added as necessary to abolish all head and limb movements in response to a strong tail pinch.

Electrical Recording

Electrical recording was begun not less than one week after the electrodes were implanted. Spontaneous slow wave activity and behavior were recorded using an inkwriting polygraph and a magnet and coil type of movement sensor [38]. Hippocampal electrical activity between 6–12 Hz in the undrugged and atropine conditions and 4–10 Hz in the urethane condition was quantified by passing the hippocampal signal through a band-pass filter (attenuation of 48 dB/octave), followed by full wave integration over successive 10 second periods. The amplitude of the integrator output served as a measure of 6–12 or 4–10 Hz activity under the various drug and behavioral conditions. Comparisons of the amount of 6–12 or 4–10 Hz activity within an individual rat or group of rats was made on the basis of the absolute value of the integrated hippocampal signal. However, the amplitude of the hippocampal signal varies across rats as a function of the precise location of the recording electrodes [3]. Therefore comparisons between the AF64A and vehicle injected groups were made in terms of the percent increase in 6–12 Hz activity during elicited walking as compared to waking immobility in the no drug and atropine conditions. The percent increase in 4–10 Hz activity during a tail pinch as compared to undisturbed periods after urethane administration was also determined in the two groups.

Evoked potentials were collected by delivering stimuli of various intensities using a Grass stimulator (model S48) and a stimulus isolation unit. Cathodal stimuli of 0.5 msec duration were delivered to the medial septal area, using a screw in the skull over the cerebellum as a diffuse anode. Hippocampal evoked responses were recorded bipolarly (surface to depth) from the best hippocampal site. Averaged evoked potentials (AEP's) were plotted by a Digitimer Neurolog NL 750 averager and plotted with an X-Y plotter (Hewlett-Packard model 7034A). AEP's were constructed under the no drug and atropine conditions during immobility and induced struggling and under urethane anesthesia during tail pinching and undisturbed periods, using 32 stimuli per condition.

Behavior

Behavior was categorized primarily as: (1) waking immobility, head held up against gravity, eyes open and motionless except for respiration and various tremors; (2) walking, stepping with all four limbs resulting in a horizontal displacement of the body; (3) elicited walking, induced by picking up and pushing the rat forward; and (4) tail pinching following the administration of urethane.

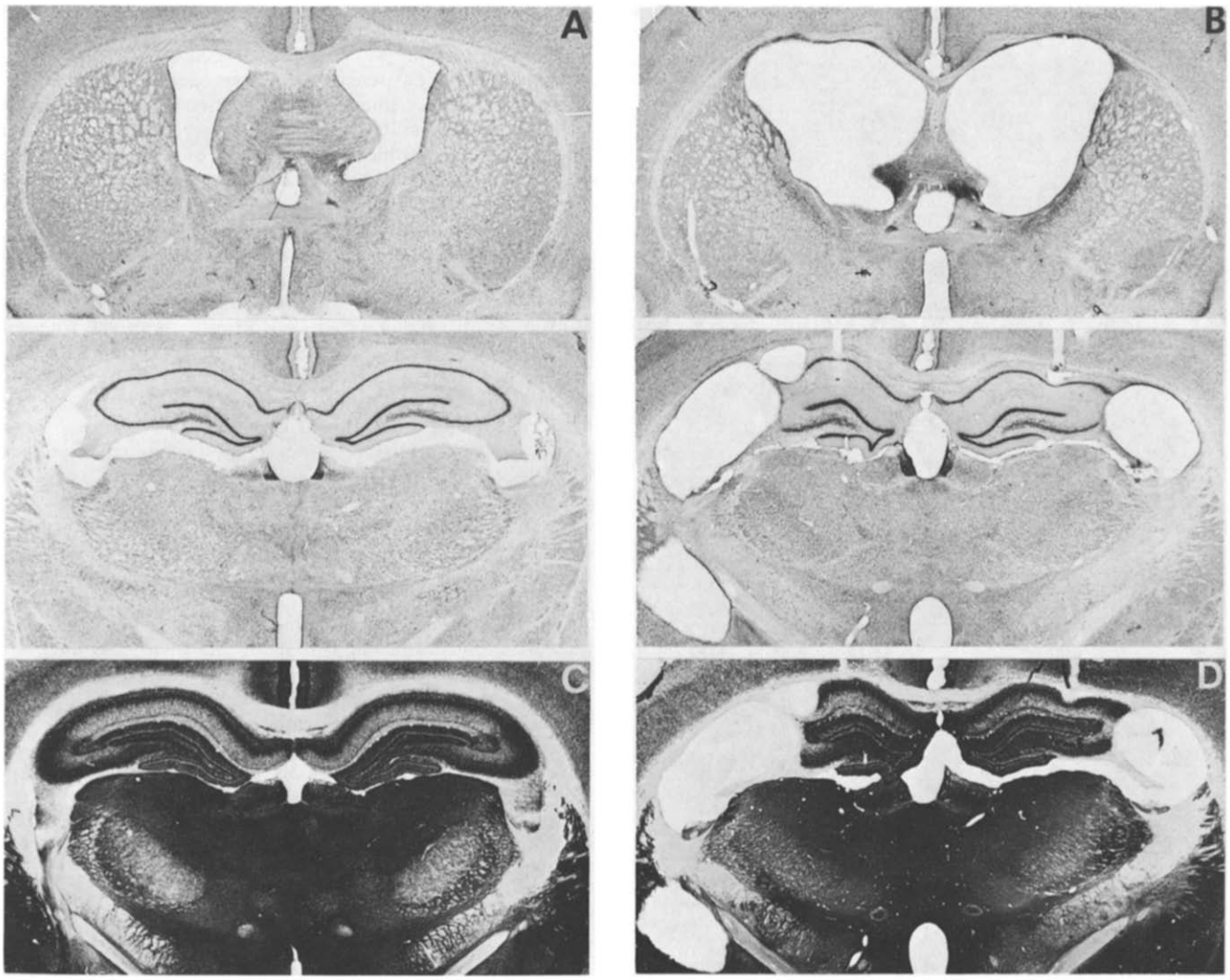

FIG. 1. Anatomical changes associated with intracerebroventricular (ICV) injections of AF64A. A. Nissl stained coronal sections in a vehicle injected rat. B. Nissl stained coronal sections at comparable levels from a rat receiving ICV injections of AF64A. Damage is present in the striatum, neocortex, hippocampus, fimbria and corpus callosum of the AF64A treated brain. A gliotic margin surrounds the enlarged lateral ventricles. The CA3 region of the hippocampus is severely damaged especially at the outer edge of the hippocampus and the fimbria are markedly shrunken in comparison to the vehicle injected rat. The third ventricle also appears somewhat enlarged in the AF64A treated rat. C. Acetylcholinesterase (AChE) stained section through the hippocampus of the same vehicle injected rat as shown in A above. Note the area of enhanced AChE staining at the outer edge of the hippocampus in the CA3 region. D. AChE stained section through the hippocampus of the same AF64A treated rat as shown in B above. Apart from the tissue missing at CA3, the AChE staining appears normal.

Histological Procedure

At the conclusion of the experiments the brains of the rats were prepared for acetylcholinesterase (AChE) staining using a modification of the Koelle copper thiocholine method [34]. Briefly, under deep urethane anesthesia, the rats were perfused with cold physiological saline followed by a cold formal-sucrose ammonia solution. The brains were removed and stored overnight in the formal-sucrose solution at 4°C then cut on a freezing microtome (40 μm) and immediately floated onto chrom-alum coated slides. Alternate sections were kept for AChE, Nissl or modified Luxol fast blue staining. All slides were examined microscopically with reference to the atlas of Paxinos and Watson [24]. The degree of damage to various anatomical structures was rated on a scale of 0–3 with a score of 0 corresponding to no visible damage.

Statistical Analysis

The data were analyzed using various nonparametric tests [33].

RESULTS

Examination of the histological material revealed areas of damage to tissue surrounding the site of AF64A injection in

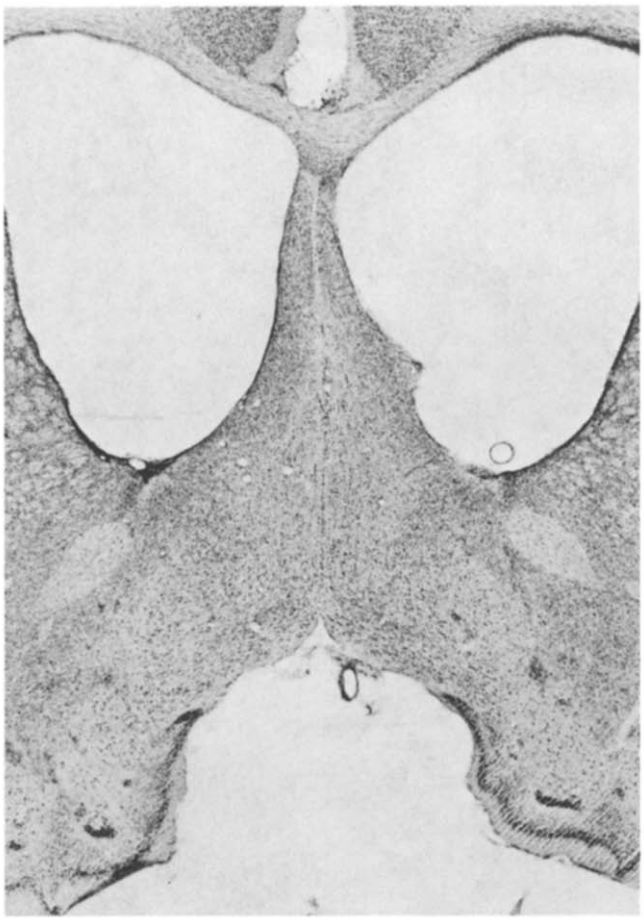

FIG. 2. Higher power photomicrograph of the septal region in a rat receiving ICV injections of AF64A. Despite the large amount of cell and fiber damage evident in the septal region and in other tissue surrounding the injection area, an abundance of neurons in the medial septal area and diagonal band have survived in the remaining septal tissue.

the lateral ventricles. The lateral and third ventricles were enlarged and damage could be seen in the septum, striatum, fimbria, fornix, hippocampus, neocortex and corpus callosum. Both cellular regions and fiber pathways were susceptible to destruction by AF64A.

Figure 1 shows coronal sections at comparable levels in a vehicle injected rat and in a rat that received AF64A and sustained damage to the fornix, fimbria, corpus callosum, neocortex and hippocampus. In the hippocampus the CA3 region was particularly affected by AF64A injection, although some cell loss also occurred in other areas of the hippocampus. AChE stained sections through the hippocampus appeared normal, apart from the missing tissue at CA3 (Fig. 1). It should be noted that the primary area of cell loss and tissue damage in the hippocampus occurs in CA3 where the level of AChE staining is highest in normal rats (Fig. 1). Damage to the fimbria and fornix was severe in these rats. Despite, in some cases, severe cell and fiber damage to the septal region, the remaining septal tissue contained many neurons which appeared intact (Fig. 2). Similarly, fiber stained sections revealed the presence of intact fibers passing through the septal area as well as in the fimbria and fornix.

Records of spontaneous slow wave activity from the hippocampus of AF64A treated rats were not obviously different from those seen in the vehicle injected rats (Fig. 3). In the no drug condition, both AF64A and vehicle injected rats displayed a pattern of large amplitude irregular activity (LIA) during periods of immobility. When the AF64A treated rats were induced to walk, a pattern of 6–12 Hz rhythmical slow activity (RSA) was observed that was similar to that seen in the vehicle injected rats (Fig. 3A). Similarly there was little consistent difference in hippocampal activity between the two groups of rats following the administration of atropine (Fig. 3B). Both groups of rats showed LIA with large sharp waves during immobility and RSA during elicited walking or spontaneous head movement and locomotion. Under urethane anesthesia, both groups of rats displayed a pattern of mixed irregular slow and fast wave activity during undisturbed periods. A tail pinch produced large increases in 4–10 Hz activity in both groups in this condition (Fig. 4A). Analysis of the data from each rat showed that 7 of the 10 rats in the AF64A group showed an increase of 4–10 Hz RSA during the tail pinch under urethane anesthesia. In the vehicle injected group, 9 of 11 rats showed such an increase. Quantitative comparisons of the amount of RSA present in the hippocampus of AF64A and vehicle injected rats indicated that, although there was a slight trend for the AF64A treated rats to show less RSA under all conditions, this difference was significant only in the no drug condition (Table 1). No significant correlations could be found between the amount of hippocampal RSA present and the ratings of anatomical damage to the septum, fornix, fimbria, hippocampus, neocortex or corpus callosum.

Figure 4B shows AEP's from an AF64A and a vehicle injected rat during immobility in the no drug condition. No consistent difference in the evoked potentials could be seen. The waveforms and latencies of the various components of the AEP's did not appear to be altered in any consistent way as a result of the AF64A treatment.

DISCUSSION

There is abundant evidence from a variety of experiments (see the Introduction section) that one of the two forms of RSA depends on a cholinergic input to the hippocampus. This input likely arises from the cholinergic cells in the medial septum and diagonal band region and appears to be responsible for any RSA seen in the urethane anesthetized preparation.

AF64A has been reported to produce a selective depletion of hippocampal cholinergic markers following ICV administration (see the Introduction section). Therefore, AF64A treatment might be expected to disrupt selectively the cholinergically mediated form of RSA. The fact that the AF64A treated rats showed significantly less increase in RSA in correlation with elicited walking than the vehicle injected rats in the no drug condition suggests that the AF64A treatment did affect the production of RSA. Since there was no difference between the two groups in the atropine condition it appears that atropine-resistant (presumably serotonergic) RSA was intact. Since RSA occurring during elicited walking in normal rats appears to be a result of both cholinergic and serotonergic inputs to the hippocampus, this finding offers some support for the hy-

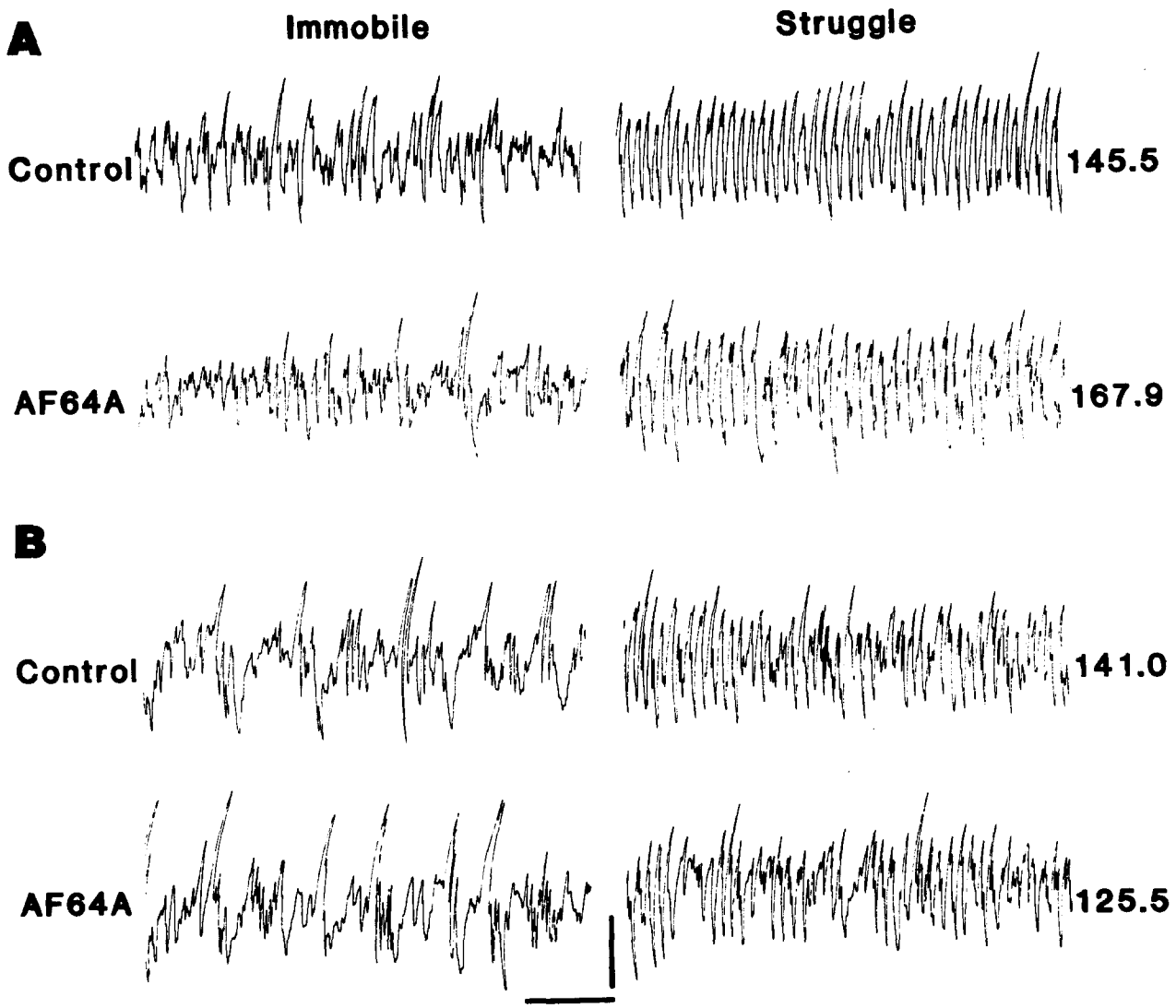

FIG. 3. Hippocampal electrical activity in a vehicle injected (Control) rat (No. 1736) and a rat receiving an ICV injection of AF64A (No. 1796). A. Hippocampal activity in the no drug condition during immobility and elicited walking. B. Hippocampal activity following an intraperitoneal injection of atropine (50 mg/kg). In both conditions the hippocampal activity in the vehicle and AF64A treated rats appears indistinguishable. Large amplitude irregular activity is present during immobility and is replaced by rhythmical slow activity when the rats are induced to walk. The numbers to the right refer to a mean percent increase in integrated 6–12 Hz activity in the elicited walking (struggling) condition as compared to immobility. Data are based on five 10 sec integration periods per condition. Calibration: 0.5 mV, 1 sec.

pothesis that AF64A produced a selective loss of such cholinergic inputs. However, the AF64A and vehicle injected rats did not differ in the amount of 4–10 Hz RSA produced under urethane anesthesia. After urethane only 4 of the AF64A treated rats failed to show a normal increase in RSA. Two of the rats in the vehicle injected group likewise failed to show an increase in RSA in the urethane condition. Although these data suggest a slight trend toward a reduction in cholinergic RSA in the AF64A treated rats, the effect is not significant and was not evident in most of the rats.

The lack of a clear-cut disruption of cholinergic RSA in the present study may be related to the amount of cholinergic depletion produced by the AF64A treatment. Depletions of hippocampal markers after ICV administration of AF64A are typically about 40–80% depending on the dose [11, 15, 41]. In the present study, the dose of AF64A used has been shown to produce depletions of approximately 60% [41]. Depletions larger than this may be necessary in order to obtain significant effects on hippocampal activity. There is evidence that extremely large depletions of other neurotransmitters must be produced before brain function is noticeably impaired [35,39].

The anatomical damage in the area surrounding the injection site in the present experiment was similar to that reported by others after ICV administration of AF64A [12,14]. In particular, there was a marked loss of fibers in the fimbria-fornix system as well as cell loss in the CA3 area of the hippocampus. It is unlikely that the cell loss in CA3 was a

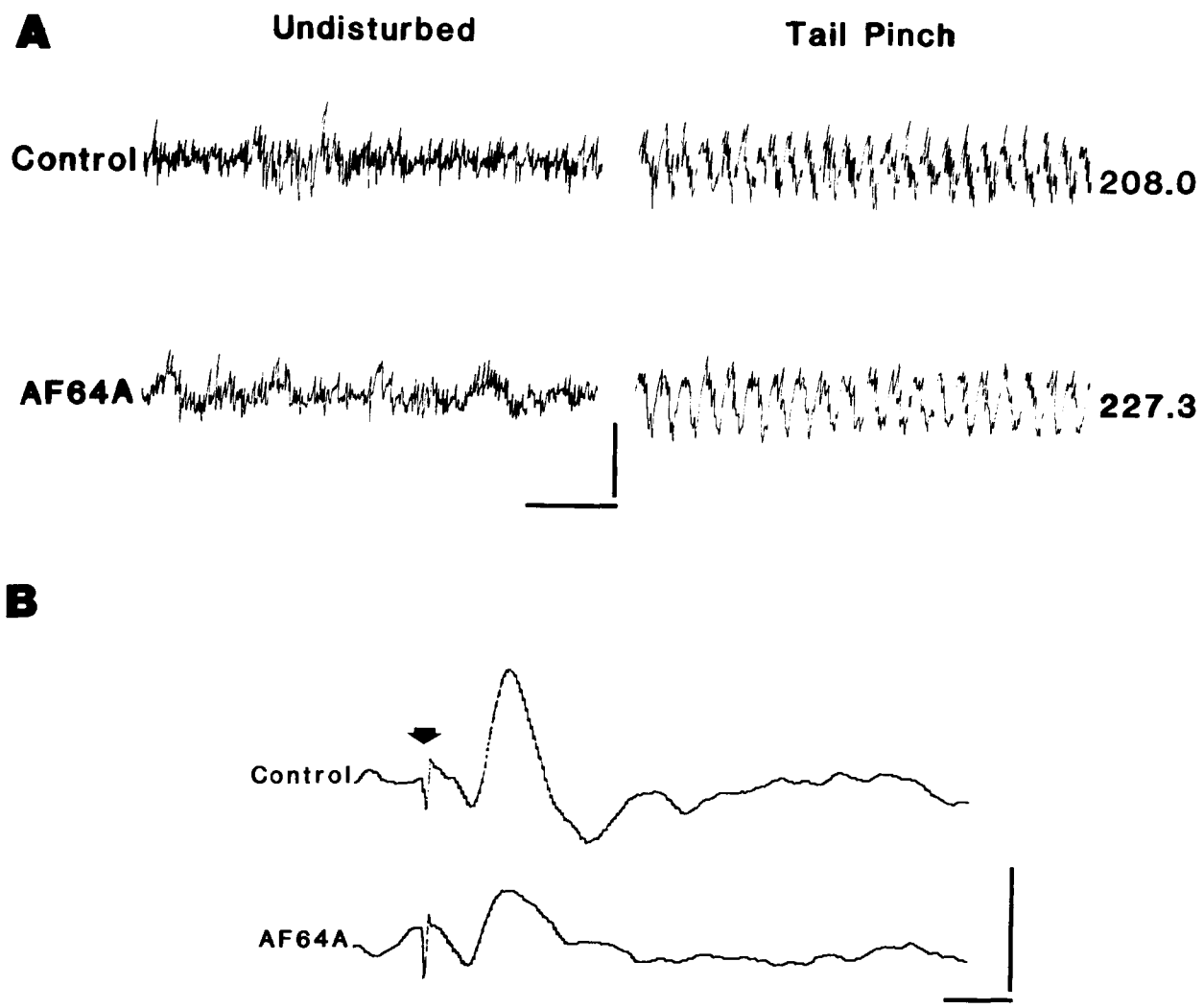

FIG. 4. Spontaneous and evoked hippocampal electrical activity in rats receiving ICV injections of either vehicle (Control; No. 1736) or AF64A (No. 1796). A. Spontaneous hippocampal activity under urethane anesthesia during undisturbed and tail pinch conditions. Both rats show a pattern of mixed irregular slow and fast wave activity during the undisturbed periods. This pattern changes to rhythmical slow activity during a tail pinch. The numbers to the right refer to a mean percent increase in integrated 4–10 Hz hippocampal activity during tail pinching as compared to the undisturbed condition. Calibration: 0.5 mV, 1 sec. B. Hippocampal activity evoked by electrical stimulation of the medial septal region. The waveforms and latencies of the various components of the evoked potential do not appear to differ across the two groups but the amplitude is reduced in the AF64A treated rat. Calibration: 0.5 mV, 50 msec. Stimulus artefact at arrow.

consequence of a loss of the cholinergic input produced by AF64A. Complete surgical transections of the fimbria and fornix, which would presumably have a similar deafferenting effect, are not accompanied by a comparable pattern of cell loss in CA3 [7]. Further, the pattern of tissue damage observed in the fimbria, fornix, corpus callosum, septum, hippocampus and, in many cases, the neocortex would suggest that AF64A in high enough concentrations may be highly toxic to any tissue that it comes into contact with. Similar nonspecific tissue damage has been reported following intrastriatal [21] or intranigral administration of AF64A [18]. The extent of this type of damage likely depends on factors such as the rate and site of injection, the volume and concentration injected as well as a number of other factors since there are other reports suggesting that nonspecific damage can be limited [30,41]. In order to limit the non-cholinergic effects of AF64A it may be necessary to use lower doses than those used here [13]. Further study is required to determine whether lower doses of AF64A will be capable of producing specific morphological and neurochemical cholinotoxicity of significant magnitude.

While it is clear that AF64A can produce relatively selective depletions of cholinergic markers in the hippocampus after ICV administration it is not yet clear whether or not this effect is due to a selective cholinotoxicity. AF64A may damage cholinergic projections to the hippocampus by a selective pharmacological action, producing selective depletions of cholinergic markers. Evidence for this is provided by a number of neurochemical studies. The other neurotransmitter systems may be less susceptible to the neurotoxic effects of AF64A and would therefore not be expected to show similar depletions. Alternatively, it is possible that AF64A

TABLE 1

HIPPOCAMPAL RHYTHMICAL SLOW ACTIVITY IN AF64A TREATED RATS IN VARIOUS DRUG AND BEHAVIORAL CONDITIONS

	Group		
	No Drug	Atropine	Urethane
Vehicle	164.5 ±12.7	105.6 ±13.5	257.1 ±43.2
N =	11	11	11
AF64A	124.6 ±12.8	89.1 ±9.8	162.8 ±40.2
N =	11	11	10

Under the no drug and atropine conditions, the numbers shown are the mean percent increase (plus or minus the standard error of the mean) in integrated 6–12 Hz activity during elicited walking as compared to waking immobility. Under urethane anesthesia, the percent increase in 4–10 Hz activity during a tail pinch as compared to undisturbed periods is shown. The data are based on five 10 sec integration periods per condition per rat. There was a slight trend for the AF64A treated rats to show less RSA than the vehicle injected rats during elicited walking or during the tail pinch, but this trend was significant only in the no drug condition (Mann-Whitney U-test, $p=0.05$).

produces its cholinergic effects in the hippocampus primarily as a result of its ability to damage (much as a surgical lesion would do) the fimbria and fornix, the major route by which the cholinergic cells in the medial septal region reach the hippocampus. Lewis, Shute and Silver [20] have shown similar depletions of cholinergic markers in the hippocampus after surgical transection of the fimbria. Evidence in favor of this hypothesis is the damage to non-cholinergic structures such as the corpus callosum that is seen in the present experiment, and in other work [14, 18, 21]. The relative lack of any long-term effect on other neurotransmitter systems such as noradrenaline and serotonin may be due to the fact that they reach the hippocampus primarily by a supracallosal route [1, 2, 37] that may not be affected by ICV AF64A injections.

It is unclear why the rats in the present study had a normal pattern of AChE staining in the hippocampus following AF64A. Gaal *et al.* [12] reported a profound decrease in AChE staining intensity in the hippocampus after AF64A treatment. It is possible that this difference may be due to the fact that in the present experiment the survival times were 30–60 days, whereas Gaal *et al.* [12] reported decreases in AChE staining intensity in the first few days after AF64A administration. The intensity of this decrease was much less pronounced with longer survival times. However, the long survival times used in the present experiment cannot explain the relative lack of an effect on cholinergic RSA since cholinergic markers may remain depleted for up to 12 months following AF64A treatment [17].

In conclusion, irrespective of the mechanism of action, it appears that AF64A injection into the ventricles does not produce a sufficiently large depletion of cholinergic markers in the hippocampus to disrupt the cholinergic form of RSA.

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