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POTENTIAL ANIMAL MODELS FOR SENILE DEMENTIA OF ALZHEIMER'S TYPE, WITH EMPHASIS ON AF64A-INDUCED CHOLINOTOXICITY

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INTRODUCTION

A number of excellent reviews have been published over the past decade, dealing with neurotoxic substances specific for neurotransmitter systems (1–12). Application of a few such agents has provided new animal models for various neuropsychiatric disease states, including Parkinson's disease, Huntington's disease, Senile Dementia of Alzheimer's Type (SDAT), epilepsy, and some ataxias.

These all serve as background for the subject of this review, which focuses on literature pertaining to ethylcholine aziridinium ion (AF64A) (e.g. 13–45). We have recently proposed this agent as a potential tool in developing an animal model for SDAT (13, 20, 26), a disease in which a central cholinergic hypofunction has been implicated (46–55).

In this review, the clinical, neuropathological, and behavioral features of SDAT will first be described briefly, to enable us to evaluate the relevance of AF64A-induced cholinotoxicity in vivo as a potential animal model for SDAT. We then compare the AF64A model with other experimental models of SDAT.

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We provide an overview of research conducted with the AF64A-treated animal, based on published reports in the literature. Finally, we discuss the potential of the AF64A-treated animal as a model of SDAT, in light of AF64A's biological effects in vivo.

THE CHOLINERGIC HYPOFUNCTION IN SDAT

The neurological disorder SDAT is highly prevalent today. Yet, to date there is no effective therapy for this disease state (46, 47). The term SDAT is now generally applied to cases of presenile dementia in which onset occurs in the fifth decade, as well as to cases of senile dementia in which patients are 60–65 years or older. SDAT is characterized by a progressive, chronic cognitive dysfunction, with severe impairment of memory of recent events, while memory for the more distant past remains relatively intact (46).

Histopathological studies on brains taken at autopsy from patients with SDAT show characteristic abnormalities such as plaques, consisting of abnormal neurites and having a central core of amyloid surrounded by argentophilic granules and filaments; neurofibrillary tangles, which are composed of bundles of paired helical filaments that accumulate within the cell bodies of neurons; and granulovacuolar degeneration. These abnormalities are most prominent in the cerebral cortex and hippocampal formation (46, 47).

A loss of specific populations of nerve cells from SDAT patients' brains taken at autopsy has been reported in the frontal and temporal cortexes, the medial septum, diagonal band of Broca (dbB), and the nucleus basalis of Meynert (nbM), a basal forebrain cholinergic network that projects directly to the hippocampus and neocortex (reviewed in 46, 47). It is therefore important to note that, over the past few years, neurochemical studies have also demonstrated that presynaptic cholinergic markers[1] are significantly reduced in the cerebral cortices and hippocampi of affected individuals (55), as well as in their medial septa, dbBs, and nbMs (46–69).

These changes are paralleled by increasing morphological alterations, probably due to nerve terminal degeneration, which in turn correlate with increasing dementia scores (46, 49). Such observations, coupled with pharmacological investigations in humans and animals indicating a major role of the cholinergic system in learning and memory (52–56), are consistent with a hypothesis of a cholinergic dysfunction in SDAT. In fact, SDAT appears to be caused primarily by a cholinergic hypofunction in selected brain areas, while other neurotransmitter systems appear to be affected less or later in the course of the disease (46, 47, 51, 53–57, 65).

[1]Presynaptic cholinergic markers include choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities; high-affinity transport of choline (HAChT); acetylcholine (ACh) synthesis; and muscarinic receptor ($mACh_R$) binding.

Clinical trials have been conducted in which lecithin or physostigmine have been administered to SDAT patients, because of the presumed ability of these agents to elevate and thus restore cholinergic activity in the central nervous system (66, 67, 69). Unfortunately, results to date have not been conclusive, and clinicians do not agree about the efficacy of treating SDAT with such drugs. Recently, however, a marked improvement in SDAT patients was reported following the intracerebroventricular infusion of bethanechol, lending further credence to the cholinergic hypothesis of SDAT (68).

RELEVANT ANIMAL MODELS OF SDAT

A major problem in the basic research and drug development for SDAT is the lack of adequate animal models that can mimic all aspects of this disease. Generally, an animal model should allow for a more detailed evaluation of the neurochemical, neuropathological, and behavioral sequelae of the primary cholinergic hypofunction suggested in this disorder. Such a model would be instrumental in testing a wide variety of drugs, to determine whether they correct the cholinergic hypofunction and restore cognitive functions to normal. An animal model would obviously provide information in studies that cannot readily be performed in humans (e.g. analysis of neurotransmitter levels and metabolism in brain areas in vivo). Finally, an animal model that reproduces the specific neuronal deficits and cognitive dysfunction of SDAT may offer some clues about the underlying deficits of this disease state.

The ideal model would be one that exhibits the same biochemical, behavioral, and histopathologic abnormalities as the human disease state. However, there is at present no homologous animal for SDAT, since the etiology of this disorder is still unknown. Partial success could therefore be achieved at this stage only with *isomorphic models;* that is, despite parallelism between the model and the human conditions, the cause of the condition in the animal may be quite different from the cause in man.

A number of experimental approaches or paradigms have been used that mimic different aspects of this progressive neurological disorder. These include:

1. aged rodents and aged monkeys;
2. anoxic/hypoxic rodents;
3. scopolamine- or hemicholinium-treated experimental animals;
4. aluminum-treated experimental animals;
5. excitotoxin-lesioned rats or monkeys; and
6. AF64A-lesioned rodents.

The following is an overview and critique of each of these specific experimental models. Available space precludes an extensive evaluation of each

of these models since the primary emphasis of the review is on the AF64A-treated animal. The reader is therefore urged to refer to the pertinent publications listed in the discussion of each of the other individual models for further specific information.

Aged Rodents and Aged Monkeys

Learning and memory deficits are recognized as severe and consistent behavioral impairments in the elderly. Since SDAT is a neurological disorder associated with aging, aged animals have been evaluated in many studies as possible animal models for age-related memory deficits and for SDAT (56).

Aged rodents (mice and rats) were shown, for example, to suffer deficits of retention of a single-trial passive-avoidance task. This deficit is conceptually similar to the severe memory loss reported in aged monkeys and humans (56). Control studies have indicated that a major source of this impairment is loss of memory for the learned event.

Drugs known to produce subtle improvements in SDAT patients also improved the performance of aged rodents in a task sensitive to age-related loss of memory (56). Thus, such experimental animals can be useful in evaluating drugs for the treatment of SDAT. Similarly, old monkeys showed reduced memory for recent events, increased perseveration in a reversal learning task, and hypersensitivity to interference. Drug effects on memory in the aged monkey also appeared to resemble those in humans (56).

Neuritic plaques occur in aging human brain and are more numerous in SDAT. Furthermore, in SDAT they correlate with the severity of dementia and magnitude of cortical cholinergic deficits (49). Neuritic plaques have also been found in aged (23–31 years) rhesus monkeys (70). In this regard, therefore, these aged nonhuman primates are an important model for testing hypotheses about the evolution and significance of these plaques.

Thus, the aged rodent or monkey does have value as an experimental animal in modelling SDAT. This model also has a few disadvantages:

1. Since SDAT is a neurological progressive disease distinct from normal aging (46, 48, 49, 71–73), aged rodents or monkeys are not ideal animal models for this disorder (74). Moreover, aged rodents mimic to a certain degree the neurochemical changes associated with normal aging, but not those presynaptic cholinergic dysfunctions (e.g. decreased ChAT activity) reported in SDAT (54).

2. The high cost of old monkeys and even old rats in drug screening also severely restricts the practical use of these animals for research purposes.

3. Poor health of aging animals and individual pharmacokinetic variabilities in drug absorption, metabolism, and distribution may sometimes yield statistically inconclusive data.

Anoxic or Hypoxic Rodents

The brains of many aged mammals differ from those of young mammals in energy-dependent biochemical functions (56). By depriving young rodents of oxygen, an energy-deficient state is induced that resembles the functional characteristics of the aging brain. Anoxia may be analogous to aging because both aging and anoxia impair oxidative metabolism and lead to similar behavioral deficits.

The anoxic model is produced by training animals in a single-trial passive-avoidance task and then exposing them immediately to an oxygen-deficient environment. The anoxic situation causes a retrograde retention deficit for the training trial (56). Hypoxia as a model for aging is analogous in many aspects to the anoxia-induced experimental model (74).

The anoxia/hypoxia experimental model generally resembles the aged rodent both neurochemically and behaviorally, thus allowing evaluation of drugs to be used for experiments in and treatment of geriatric memory and learning deficits. In fact, drugs shown to have some marginal beneficial effects in SDAT are effective in this test as well (56). This simple and inexpensive model can therefore be used for preliminary screening in developing drugs for memory disorders.

On the other hand, anoxic or hypoxic animals do not selectively exhibit those brain-area specific cholinergic deficits generally reported in this disorder. In fact, the key disadvantage in this model is that the anoxic/hypoxic animals mimic a generalized neuropathology associated with various neurotransmitters, including the cholinergic system. Another disadvantage of this model is its lack of histopathological characteristics similar to those of SDAT brains.

Scopolamine- or Hemicholinium-Treated Experimental Animals

The primary pharmacological support for the theory that the cholinergic system plays an important role in cognitive function is the finding that blockade of central $mACh_R$ binding induces a cognitive deficit in young volunteers. The deficit is qualitatively similar to that occurring naturally in aged subjects. Scopolamine administration induced cognitive deficits similar to those found naturally in aged subjects tested on the same clinical battery (reviewed in 54). One such deficit was loss of memory in young subjects for recent (but not immediate) events. Similarly, scopolamine injected into young monkeys caused memory deficits similar to those occurring naturally in aged monkeys (54).

Although the scopolamine-treated animal provides a good model for evaluating the cognitive effects of pharmacological disruption of cholinergic function, it lacks certain features necessary for studying the pathophysiology of SDAT: (*a*) SDAT is a progressive, irreversible, neurological disease whereas the effects induced by scopolamine in animals (or humans) are reversible. (*b*) In

SDAT the cholinergic system is irreversibly compromised as a result of presynaptic degeneration of cholinergic neurons, without a significant decrease in postsynaptic $mACh_R$ (54, 60, 62–64; but see also 61); therefore scopolamine, which causes mainly reversible blockade of postsynaptic $mACh_R$, mimics only some features of SDAT (75).

Hemicholinium-3 injected intracerebroventricularly (i.c.v.) in mice, rats, or marmosets has also been shown to impair certain cognitive functions (75, 76). Hemicholinium-3 induces a state of presynaptic cholinergic hypofunction by blocking HAChT. However, although this model may mimic some aspects of the memory disorder in SDAT it, again, is not the ideal model for this disease state since it only partially mimics the central cholinergic hypofunction. Moreover, the effect is reversible, whereas SDAT is a chronic and progressive disorder.

Aluminum-Treated Experimental Animals

Aluminum has been implicated as being involved with SDAT. Perl & Brody (77) have reported the presence of aluminum in the nuclear region of neurofibrillary tangles containing neuronal cells from Alzheimer's patients. Aluminum injected into rabbit brains also causes formation of neurofibrillary tangles (78). Additionally, following aluminum administration into cat brains, some memory loss and abnormal behavior have been observed (79). Also, recently, intracranial administration of aluminum was shown to produce avoidance-learning deficits in immature rabbits (80).

The morphology of the neurofibrillary tangles induced by aluminum, however, differs significantly from that of human SDAT-type tangles (46, 79, 80). Central cholinergic activity (which is markedly reduced in SDAT) is, moreover, normal in rabbits with aluminum-induced neurofibrillary changes, and the direct activity of aluminum on cholinergic markers such as ChAT is minimal (81). Also, the brain areas affected by aluminum in animals are significantly different from those areas affected in SDAT (81). In addition, McDermott et al (82) showed that there was no difference in aluminum accumulation between SDAT and age-matched controls. Furthermore, they found an increase in brain aluminum content associated with aging.

Based on the above-mentioned data it appears that the induction by aluminum of neurofibrillary tangles in experimental animals, while an intriguing phenomenon definitely worth further exploration, is not a perfectly matching animal model for SDAT. Moreover, its relevance to the etiology of this disorder is still an unsettled issue.

Excitotoxin-Lesioned Rats and Monkeys

The magnocellular system of the basal forebrain consists of clusters of large neurons located in the septum, dbB, and nbM. This system has been shown to

be cholinergic (48, 83). In SDAT the cholinergic deficiency in the cortex and hippocampus appears to be due to a loss of neurons from this magnocellular cholinergic system. In concordance with this observation, electrolytic or excitotoxin-induced lesions of the ventromedial corner of the rat globus pallidus (equivalent to the nbM in humans and nonhuman primates) reduce ChAT activity, ACh levels, and Ch uptake in different parts of the cortex (48, 72, 83).

Electrolytic lesions are nonspecific, however, and result in axonal destruction and anterograde and retrograde neuronal degeneration. Intracerebrally injected excitotoxins, including kainic acid, ibotenic acid, *N*-methyl aspartate, and quinolinic acid do, on the other hand, produce a selective pattern of neuronal degeneration. This effect is focussed at neuronal perikarya near the injection site; axons of passage or of termination are essentially spared from destruction (72, 84–86).

Some of the excitotoxins listed above have been used for lesioning the nbM, in order to induce in experimental animals (rats or monkeys) cortical cholinergic lesions similar to those reported in SDAT. A wealth of information regarding these types of lesions in the nbM has been accumulated in the last few years, including neurochemical, histochemical, behavioral, and pharmacological observations. Some representative examples follow.

Neurochemical studies with kainic acid–induced lesions in the rat nbM have demonstrated a 45–50% loss of cortical ChAT (72, 87). This decrease in cortical ChAT has been paralleled by a decrease in ACh levels and in HAChT and AChE activity, but with no significant change in cortical GABA-ergic, noradrenergic, serotonergic, or histaminergic presynaptic markers (72). Behaviorally, this kind of lesion with kainic acid induces a marked retention deficit, 24 hr after the initial training trial, in a step-through passive-avoidance task (87). Thus, this lesion with kainic acid may be capable of producing a potential animal model for SDAT (72, 87).

However, kainic acid, as a potent convulsant, can produce distant damage (88, 89). Therefore ibotenic acid is presently preferred in a large number of laboratories (72, 84, 90–100).

Ibotenic acid–induced lesion of the nbM reduces ChAT activity in different parts of the cortex; the decrease is generally smaller than that reported with kainic acid (but see also 72, 84). Behaviorally, rats with ibotenic acid–nbM lesions show deficits in the retention of a step-through passive-avoidance response; an impairment of spatial-reference memory in a 16-arm radial maze; and recent-memory deficits (in an 8-arm radial maze), analogous to those seen in SDAT patients (84, 99). On the other hand, working memory is not disrupted by such lesions (84). Lesions of the nbM and medial septal areas of rats with ibotenic acid also impair spatial "working" memory in a T-maze task (98).

A few pharmacologically oriented studies have already been initiated in which attempts have been made to reverse learning and memory deficits caused

by nbM lesions induced by ibotenic acid. Such deficits can be reversed by treatment with the cholinesterase inhibitor physostigmine (84, 95), but not with the direct muscarinic agonist oxotremorine (99). Monkeys injected with ibotenic acid into the nbM can still perform normally in a recognition memory task, but their performance is more easily disrupted than that of normal animals when challenged with the muscarinic antagonist scopolamine (93).

All these results, when combined, lend support to the view that excitotoxin-induced lesions can be useful in production of animal models of the cortical cholinergic deficiency in SDAT. However, several deficiencies in these models should be pointed out. Specifically, these are the following.

1. The excitotoxin-induced lesions do not produce the histopathological features characteristic of SDAT such as neuritic plaques and neurofibrillary tangles. Nor do they cause deficits in somatostatin, which is also affected in SDAT (46, 51, 96).
2. Since these excitotoxins generally affect all neuronal perikarya regardless of neurotransmitter used, these lesions provide only limited information on the specific role of the cholinergic system in memory and learning deficits.
3. Another deficiency with these excitotoxin lesions is that they damage contiguous noncholinergic neurons at the injection site (72). Thus, rats with ibotenic lesions in the dorsolateral globus pallidus exhibited passive-avoidance deficits even though their cortical ChAT activity was not decreased (90). This limitation might be avoided through the use of AF64A (see below).
4. Finally, excitotoxin-induced lesion of the nbM mimics only the cortical presynaptic cholinergic deficit in SDAT; ChAT activity in the hippocampus is not affected in these excitotoxin-lesioned animals.

AF64A-Lesioned Rodents

Progress in the amelioration of SDAT could be hastened considerably if an animal model could be found that *directly* mimics the cholinergic abnormality reported in SDAT (13). Steps in this direction have been taken, based on our current understanding of factors regulating cholinergic function in vivo.

Although cholinergic neurons do not take up their neurotransmitter ACh, they possess an avid, high-affinity transport mechanism for its precursor choline, the HAChT, which appears to be restricted to, or at least highly concentrated on, cholinergic neurons, and tightly linked to ACh synthesis. It can be distinguished from a nonspecific transport process for choline, found in most cells that have a much lower affinity for choline transport (LAChT) (13, 101). A chemical analog of choline was therefore sought, which would be selectively targeted toward the HAChT system because of its structural similarity to choline, but which, at the same time, would be cytotoxic at the site of its accumulation in vivo.

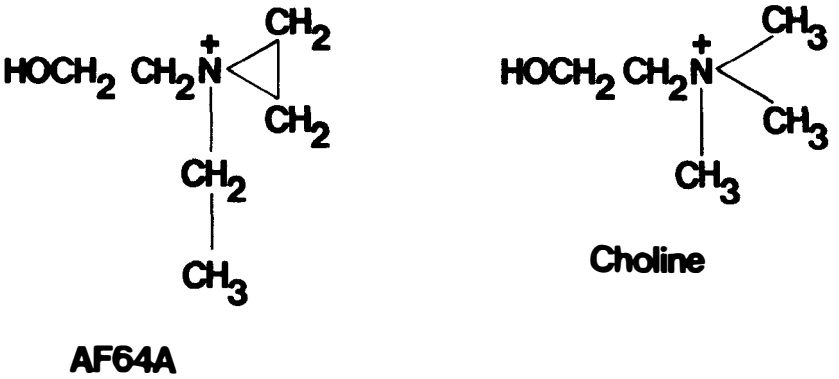

Figure 1 Structural representation of AF64A and choline. Note the close similarity in chemical structure of these two molecules.

Such a cholinotoxin, ethylcholine aziridinium, (AF64A) (Figure 1) fulfills that requirement. It induces in vivo a persistent central cholinergic hypofunction of presynaptic origin. To date, we and others have conducted extensive neurochemical, electrophysiological, behavioral, and histochemical studies to evaluate the AF64A-treated animal as a potential animal model for SDAT.

The following is an overview of some key reports on the biological effects of AF64A in vivo. Because the data are pertinent vis-à-vis animal models of SDAT, and selectivity of action of the neurotoxin, we focus in this section on results from studies conducted with rats, with particular emphasis on neurochemical findings as well as on behavioral consequences of AF64A administration.

INTRAHIPPOCAMPAL ADMINISTRATION OF AF64A In early studies performed in vitro on rat hippocampal tissue, we were able to show that AF64A is a very specific inhibitor of HAChT (IC_{50} = 3.4 μM). Its effect on HAChT was 17, 1209, 1494, 1735, and at least 10,000 times more potent than on the LAChT, $mACh_R$, ChAT, or AChE activity, or on serotonin uptake, respectively. Thus, at low concentrations in vitro, AF64A interacts only with the HAChT system (25). These findings are in concordance with the in vitro studies of Rylett & Colhoun (19), in which they demonstrated irreversible inhibition of HAChT in rat forebrain synaptosomes, using ethylcholine aziridinium.

We have expanded these data in rats, in vivo. When 2 nmol (in 2μl) AF64A were injected intracerebrally (i.c.) into the dorsal hippocampal area of rats, ACh levels in this tissue were significantly lowered (−57%) within 5 days. This was paralleled by a significant decrease in the activity of HAChT (−77%), and ChAT (−58%), while choline levels and $mACh_R$ were unchanged in the same brain area. Moreover the LAChT (found on many cell types), serotonin uptake, and norepinephrine levels were also unchanged (25).

In a longitudinal study 5 days, 3 weeks, and 6 weeks following i.c. injection

of AF64A (6 nmol/side into the dorsal hippocampus), HAChT and AChE activity were greatly reduced (−60–70%), and AChE staining was markedly decreased (23).

INTRASTRIATAL ADMINISTRATION OF AF64A Striatal lesions induced in rats by AF64A (8 nmol bilaterally) resulted in significant impairments in the acquisition and retention of a step-down passive-avoidance task. No significant differences between the same control and AF64A-injected rats were found in sensitivity to electric shock or in various measures of spontaneous locomotor activity. Striatal ChAT activity was significantly decreased (−25%) in these AF64A-treated rats when compared with controls, whereas glutamic acid decarboxylase activity was not affected in the same brain area. Furthermore, there were no significant differences between the two groups in activities of ChAT and glutamic acid decarboxylase, in either the cortex or the hippocampus, thus supporting the specificity of the lesion to the striatum (33).

The above study concurs with a previous series of experiments employing similar conditions, where it was shown that AF64A acted specifically on striatal cholinergic neurons, inducing a long-lasting cholinergic underactivity (lasting at least up to 3 months), while sparing neurons containing norepinephrine, dopamine, serotonin, and GABA (31). Interestingly, under similar experimental conditions AF64A increased spontaneous nocturnal locomotor activity in rats (35). The hyperactivity found in these rats after intrastriatal injection of AF64A was interpreted as evidence that the striatal cholinergic system plays a role in locomotor behavior.

INJECTION OF AF64A INTO THE nbM Very low concentrations of AF64A (0.02 nmol/μl or 10 μl), unilaterally administered directly into the rat nbM, decreased AChE staining in the nbM when the brain region was analyzed 7 or 14 days after injection. This reduction was paralleled by a significant decrease in cortical activity of ChAT (−17%); cortical levels of dopamine and serotonin were not affected in the same tissue (41).

On the other hand, in another study doses above 0.5 nmol of the cytotoxin, when administered into the striatum and nbM, produced appreciable nonselective damage in both the caudate putamen complex and nucleus basalis. More selective effects were seen, however, in the range of 0.1–0.5 nmol, and these were accompanied by minimal effects on cholinergic neurons (42).

The consequences of AF64A administration into the nbM thus remain equivocal, and are subject to establishing the optimal dose of the substance that should be used in future studies.

INTRACEREBROVENTRICULAR ADMINISTRATION OF AF64A A considerable amount of information has been generated recently as a result of the

i.c.v. administration of AF64A in rats. A variety of neurochemical parameters, as well as behavioral correlates of administration of various concentrations of this substance, have been measured in different brain areas. The following summarizes some of the findings obtained so far.

In the studies of Walsh and coworkers (38), rats were infused i.c.v., bilaterally, with either 7.5 or 15 nmol of AF64A (i.e. 15 and 30 nmol per brain). The results obtained in this set of experiments were as follows:

Retention of step-through passive-avoidance task, assessed 35 days after dosing, was impaired in both the 7.5- and 15-nmol groups.

Radial-arm maze performance, measured 60–80 days following treatment, was markedly impaired in the treated groups. Animals treated with AF64A made fewer correct responses in their first eight choices, required more total selections to complete the task, and had an altered pattern of spatial responding in the maze. It appears that the "working memory" rather than the "reference memory" was impaired in this test.

In these same rats neurochemical changes were evaluated 120 days later (following an extensive behavioral evaluation). Significant decreases in ACh levels in both the hippocampus (−44 to −62% in the 7.5- and 15-nmol groups) and the frontal cortex (−63% in the 15-nmol group) were observed, whereas ACh levels in the striatum were unchanged when compared with the control group. The concentrations of catecholamines, indoleamines, their metabolites, and of choline in these brain regions were not affected by AF64A treatment (38). Thus, AF64A caused a persistent decrease in ACh levels in selected brain areas reminiscent of the data obtained in mice (17).

In a similar behavioral study, smaller doses of AF64A (3 and 6 nmol) were injected i.c.v. into rats, and performance in an 8-arm radial maze was evaluated 21 days following treatment (30). "Working memory" function in the radial maze was impaired on both "place" and "cue" tasks, while "reference memory" performance was disrupted only on the "place" task. Treatment of rats i.c.v. with 3 nmol AF64A induced, after one week, a large reduction of ACh levels in the hippocampus and striatum without affecting norepinephrine or dopamine levels in the hippocampus and striatum, respectively. The authors claimed that this behavioral pattern is similar to the behavior exhibited following lesions of the fimbria-fornix. However, they also indicated that, under their experimental conditions, it was not clear whether the behavioral and neurochemical effects of AF64A were due to cholinergic specificity of this agent, or whether they resulted from a nonspecific lesion of the fimbria-fornix.

Comparable behavioral observations were also made with AF64A (3 and 5 nmol/side, icv) (44), using the one-trial passive-avoidance test. Interestingly, this AF64A-induced memory impairment could be reversed by intraperitoneal (i.p.) pretreatment of animals with physostigmine (0.06 mg/kg), thus emphasizing the potential use of this animal model for SDAT in evaluating new drugs for the disorder (44).

When rats were injected bilaterally with AF64A (3 nmol/3 μl/side) and analyzed for various cholinergic parameters 7 and 21 days after injection (45), hippocampal ChAT was found to be reduced to 42% of control level both 7 and 21 days after AF64A treatment; HAChT was reduced to 33% and 48% of control respectively; AChE was reduced to 40% and 30% of control, respectively; and K^+-stimulated ACh release was reduced to 24% or 35% of control, respectively. Meanwhile $mACh_R$ binding ([^{3}H]QNB binding) was either unchanged (at 7 days), or slightly decreased (−11%, by 21 days). Neither HAChT nor [^{3}H]QNB binding in striatum and cortex were altered by AF64A treatment. Interestingly, under the specific conditions of this study, the effect of AF64A appeared to be selective for the hippocampus. These data, using a low dose of i.c.v. AF64A, further emphasize the value of using AF64A as a selective tool for inducing a persistent cholinergic hypofunction in vivo.

In yet another study (36) AF64A was administered i.c.v. (20 nmol/5μl) to 3–4-month-old rats, and a variety of tests were performed three months later with the following results:

Locomotor activity was markedly increased (which is also seen with anticholinergics). Habituation was not influenced by AF64A treatment. REM sleep was reduced, and REM latency increased. The latter was also found to be the case in old rats (>30 months) that were compared with the AF64A-treated rats. Finally, postsynaptic excitability of hippocampal pyramidal cells was not changed, while a presynaptic cholinotoxic site of action of AF64A was indicated. In some respects the changes observed were also found in old animals.

Thus, AF64A mimics, at least qualitatively, the profound reduction of presynaptic cholinergic markers observed in most regions of the forebrain, and particularly the hippocampus and cortex, of SDAT patients (46, 48).

In addition, the persistent cholinergic deficiencies in hippocampus and cortex induced by AF64A (administered to rats) are paralleled by a long-term impairment of cognitive function in the affected animals. In SDAT a decrease of presynaptic cholinergic markers is paralleled by chronic cognitive dysfunction (46, 49, 51).

The cognitive deficit in SDAT is severe for memory of recent events (short-term memory) whereas memory for the past remains relatively intact. A similar behavior pattern was observed in the 8-arm radial maze, as a result of AF64A-induced cholinotoxicity following i.c.v. injection in rats (38, 44).

Finally, AF64A administration is superior to the excitotoxin-lesioning approach of developing animal models for SDAT since the excitotoxins, because of their postulated cytotoxic mechanism, must be injected very close to the cell bodies rather than in the terminal field, where they are inactive.

However, the AF64A-animal model also has its deficiencies:

1. We have not shown yet that it can mimic the histological characteristic found in SDAT (that is, plaques and tangles). This limitation is inherent in the excitotoxin-induced lesions as well (72).

2. This animal model mimics the cholinergic hypofunction reported in SDAT. However, we do not yet know if it can reproduce deficits in somatostatin that have been reported to be reduced in SDAT (46, 50).
3. Still to be established, using conventional cholinergic drugs such as arecoline or oxotremorine, is whether such agents are capable of alleviating memory disorders induced by AF64A. In this regard the beneficial effect of physostigmine in this animal model (44) holds great promise for the future.
4. It still is important to evaluate the effect of nootropic drugs (54) on this animal model.

POSSIBLE MECHANISMS OF AF64A-INDUCED CHOLINOTOXICITY

From the literature to date we can deduce that AF64A, when used at appropriate concentrations, is a unique, selective, and specific presynaptic cholinotoxin capable of inducing a long-term or even persistent cholinergic hypofunction in vivo. How AF64A actually induces such a state of diminished cholinergic activity, while sparing other neurotransmitter systems such as the catecholaminergic, serotonergic, or GABA-ergic systems, remains to be answered.

At this stage, we have some clues regarding the chain of events leading to a long-term presynaptic cholinotoxicity and eventual cytotoxicity of cholinergic neurons following AF64A administration:

The specificity of AF64A to cholinergic neurons originates from its very close structural similarity to choline; the volume of the cationic head in AF64A is slightly larger or almost equal to the cationic head of choline (13). Therefore, AF64A is "recognized" by the HAChT system, since the aziridinium moiety in AF64A is rather stable at physiological pH (19, 27; A. Fisher and I. Hanin, unpublished).

AF64A is most probably targeted toward the HAChT and is not "used up" on its way toward the cholinergic neurons on other neuronal systems. In addition, AF64A could conceivably be transported via the HAChT system into cholinergic neurons (34). Only part of the HAChT system might be alkylated on nucleophilic sites on the choline carrier, since alkylation (i.e. a covalent bond formation) is probably a much slower reaction than the transport process itself. [That the transport of choline via the HAChT is an extremely fast process is evident from a variety of studies (101).] This would allow for an accumulation of the neurotoxin into the cholinergic neurons (mainly terminals) (22, 23, 37).

Once inside the cholinergic neurons, the compound could disrupt fundamental metabolic processes required for viability. This possible mechanism of cholinotoxicity and cytotoxicity of AF64A is evident from studies in which cholinergic and noncholinergic neuroblastoma cell lines, and various partially purified enzymes, were reacted with AF64A in vitro, revealing selective

cytotoxicity of this drug against cholinergic substrates only (29, 40). The results indicated that AF64A enters the cell via HAChT and inhibits enzymes involved in choline metabolism but spares enzymes that do not use choline as a substrate or inhibitor.

Eventually, this effect could conceivably cause a decrease in the cellular concentration of phosphatidylcholine that may, in turn, cause disruption of plasma membranes. Such a mechanism may explain, in part, the selective cytotoxic effect of AF64A toward cholinergic neurons. In this regard, we can speculate that AF64A is even more specific for cholinergic neurons than the well-known specific neurotoxin, 6-hydroxydopamine (6OHDA), is for catecholaminergic neurons. The latter neurotoxin can decompose extraneuronally and intraneuronally to a variety of free radicals and H_2O_2 (1–3), which are certainly not as specific for catecholaminergic neurons as AF64A is for cholinergic neurons.

Another related intraneuronal possibility, not contradictory but in fact complementary to the above-mentioned mechanism, is the transformation of AF64A to its acetylated analog, acetylethylcholine aziridinium ion, via ChAT. This compound potentially formed, in situ, could exert toxicity, when accumulated in the presynaptic cytoplasm, by alkylating enzymes involved in vesicular ACh transport, as well as by alkylating other cytoplasmic enzymes (22).

In conclusion, while we have some excellent leads from a variety of studies, the exact mechanism of neurotoxicity and of cytotoxicity induced by AF64A is only partially understood at this time. Further studies are clearly necessary to answer a number of questions.

It is important in this context to point out that AF64A belongs to the class of neurotoxic alkylating agents: AF64A, DSP-4, and xylamine (4). These three alkylating neurotoxins for the presynaptic cholinergic and noradrenergic neurons, respectively, are excellent proof for the notion that alkylating agents of this type are not merely indiscriminately cytotoxic. A careful design of the molecule induces marked specificity, especially if the molecule resembles a substrate molecule such as norepinephrine (for DSP-4 and xylamine) (4), or a precursor such as choline (for AF64A).

COMMENTS REGARDING THE SELECTIVE NEUROTOXICITY OF AF64A

AF64A is a very potent neurotoxin, and on a molar basis is at least as toxic as kainic acid. Therefore, it is clear that certain precautions should be taken when one wants to obtain selective cholinotoxic effects versus nonspecific effects.

Nonspecific effects at the injection site can occur and have been reported in a few studies (28, 30–32, 42). Differences seen among these studies include variations in the size of the necrosis [large (28, 30, 32) or smaller (31, 42)];

different interpretation of the data (31 versus 30); use of different batches of AF64A[2] (17, 18, 27, 28, 43); and different experimental conditions.

No pathological changes in the hippocampus, septum, fimbria-fornix, amygdala, or caudate nucleus were found in our studies using conventional histology following AF64A administration (i.c.v.) at doses that induced selective persistent cholinergic hypofunction and memory disorders in rats (46). In addition we did not detect gross nonspecific histological changes with 1–2 nmol of AF64A, administered i.c. into the dorsal hippocampus, a dose that induced a selective, persistent, presynaptic, cholinotoxic effect in this brain area. However, as the dose was increased a proportionally higher incidence of edema, focal hemorrhage, and in extreme cases, liquefactive necrosis was found (35, 37).

It is therefore still not entirely clear to us whether or not AF64A actually causes a destruction of cholinergic neurons, or whether this agent modifies their functional status. The accumulated histochemical studies are not conclusive at this stage, and much work has yet to be done in this particular area of investigation.

A few general comments should be made in this context regarding the specificity of neurotoxicity of AF64A, as well as of other neurotoxins:

Cavitary lesions along the cannula tract bordered by a narrow zone of gliosis is a *common* feature rather than an exception in the case of use of all the neurotoxins. This is seen with 6OHDA (102–109), kainic acid (110–113), and with 5,6- or 5,7-dihydroxytryptamine [5,6- or 5,7-DHT (114)]. Sometimes these lesions are evident, sometimes they are undetected, depending on the experimental techniques used. Selectivity toward a certain neurotransmitter neuronal system should therefore be defined in such a way that at areas remote from these so-called cavitations only this one neuronal system is impaired while other neurotransmitter systems (in the same area) are spared. Such a condition can be fulfilled by AF64A and to a certain degree with 6OHDA, and with 5,6- or 5,7-DHT, but not with an electrolytic or mechanical lesion. Interestingly, with 6OHDA for instance, nonspecific effects were reported at areas remote from the site of injection (103).

Following any lesion, even if it is selective for a particular neuronal population, secondary changes in tissue morphology will also occur. Death of this neuronal population could lead to extensive gliosis, tissue shrinkage, and if the brain region is adjacent to the ventricles, ventricular enlargement that sometimes might cause deformation of the entire structure in this region. This effect sometimes can be misinterpreted as being a nonspecific lesion, reminiscent of electrolytic lesions, for example.

[2]Batches of AF64A used in our studies are identical to the compound sold by Research Biochemicals Inc. (RBI), USA, and are free of potential cytotoxic impurities such as $EtN(CH_2CH_2Cl)_2$ that could be a source of nonspecific-induced damage in the brain. We do not know whether other batches of AF64A, prepared by others, are free of such impurities.

Nonspecific destruction of tissue may also occur due to artifacts of tissue preparation. If we assume that AF64A would specifically alter the membrane morphology in cholinergic neurons (by impairing processes in which choline is either a substrate or inhibitor), and decrease its phosphatidylcholine content, for example (29, 40), then it is possible that the membrane fluidity and fragility might change. This could reveal macroscopically liquefactive tissues in the affected brain area. Once the chemical consistency of these membranes is changed, infusion of chemicals usually used for fixation (formaldehyde and/or glutaraldehyde) could osmotically disrupt these membranes, eventually leading to marked cavitations. Thus, in fact, an experimental method used in histological evaluations could even cause an artifact in interpretation of the data.

For all of the above reasons, in each case that AF64A is employed in a new application, it is recommended that comprehensive histological as well as neurochemical evaluations be conducted to allow reliable interpretation of the data. If by both these methods nonspecific effects are revealed above a certain dose, this dose should be adjusted in order to obtain specific cholinotoxic effects.

SUMMARY

In this review we have described and critiqued several commonly used proposed animal models for SDAT. In particular, we have focussed on the AF64A-treated animal. Major pertinent neurochemical and behavioral data obtained so far with AF64A have been presented, and these effects have been compared with neurochemical and behavioral changes in the SDAT patient. We have commented on the possible mechanism(s) of action of AF64A in vivo, and have also presented some observations and speculations concerning the selectivity of action of AF64A as a specific presynaptic cholinotoxin.

Much work has yet to be done with all the available animal models, including the AF64A-treated animal, before one could definitively state which one is the ideal model for SDAT. Data obtained to date with the AF64A-treated animal are nevertheless most encouraging. Despite some caveats, AF64A is a valuable neurochemical tool with which one can induce a persistent cholinergic deficiency of presynaptic origin.

As with all new tools, however, one must always exercise due care to use it properly, and interpret the results obtained following its administration with caution.

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