THE MUSCARINIC RECEPTOR BLOCKER QUINUCLIDINYL BENZILATE INDUCES ACETYLCHOLINESTERASE POSITIVE FIBER INCREASES WITHIN UNDAMAGED ADULT RAT HIPPOCAMPUS

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BY

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Abstract

Prolonged blockade of nicotinic receptors induces sprouting of axon collaterals at the neuromuscular junction (Holland, R.L. and Brown, M.C., 1980). The present study examined the role of muscarinic receptors in the control of collateral sprouting in the adult hippocampus. It was hypothesised that the state of muscarinic receptor activation could promote plastic change at hippocampal cholinergic synapses. In experiment one, quinuclidinyl benzilate (QNB; 20 μg/2 μl), an irreversible muscarinic antagonist was injected unilaterally into the hippocampus of female Sprague Dawley rats. Beta hydroxypropylcyclodextrin (36%) served as a vehicle which was injected into the opposite hemisphere. Subjects were sacrificed 1, 4, 14 or 21 days after treatment. Brain sections (40 μm) were reacted for acetylcholinesterase (AChE). Increased AChE staining in the area of the injection site was visible at the 4 day interval in the QNB treated hippocampi. No increase in AChE staining was seen at the 4 day interval in vehicle treated hippocampi. Brains examined after a 1 day interval showed increased staining limited to the needle track in both vehicle and drug injected hemispheres which appeared to be a function of blood accumulation. At the 14 day interval staining increases were still present at the QNB injection site but were reduced compared to the 4 day interval. An increase in AChE staining was no longer evident at the QNB injection site at the 21 day interval. These data suggest that irreversible blockade of muscarinic receptors can induce collateral sprouting of AChE reactive fibers in the hippocampus. It is hypothesised that sprouting is rapidly induced and retraction occurs following renewed receptor activation.

Experiment two assessed the effect of hemisphere, sex and drug dosage on QNB-induced collateral sprouting in Sprague Dawley rats. Quinuclidinyl benzilate (20 μg/2 μl or 40 μg/2 μl) was intrahippocampally injected into either right or left hemispheres of male and female rats. Rats were sacrificed four days following treatment and brains were reacted for AChE. Acetylcholinesterase positive fiber outgrowth was demonstrated using both QNB doses, in right and left hemispheres and in both male and female rats. Neither variable of experiment two effected the occurrence of AChE positive collateral sprouting in the hippocampus.

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The present study investigates a form of hippocampal plasticity that involves the potential of this region to physically restructure its cholinergic inputs under a situation of prolonged muscarinic antagonism. Prior to introducing the hypothesis and background information that support the predictions of the present study, a section will be dedicated to discussing hippocampal anatomy and the septohippocampal cholinergic tract. This will serve to familiarize the reader with the area of the brain under investigation in this study.

Hippocampal anatomy and the septohippocampal track

The hippocampus can be differentiated into distinct anatomical regions (see figure 1). The hippocampus proper or cornu ammonis (CA) is composed of three distinct areas referred to as CA1, CA2 and CA3 (Swanson, Kohler and Bjorklund, 1987). These areas are comprised primarily of pyramidal cells. Cornu ammonis can further be subdivided in to strata oriens, pyramidale, radiatum and lacunosum-moleculare (Swanson et al., 1987). The second major region of the hippocampus is the dentate gyrus or fascia dentata. This "U"-shaped structure can be divided into medial and lateral blades also known as infrapyramidal and suprapyramidal blades respectively (Swanson et al., 1987 ; Amaral and Witter, 1989). The dentate gyrus can be divided into three layers known as molecular, granular and polymorph zones. The medial and lateral blades are composed of a molecular layer (outer, middle, inner sublayers) containing granule cell dendrites that receive terminal connections from entorhinal, septal and commissural

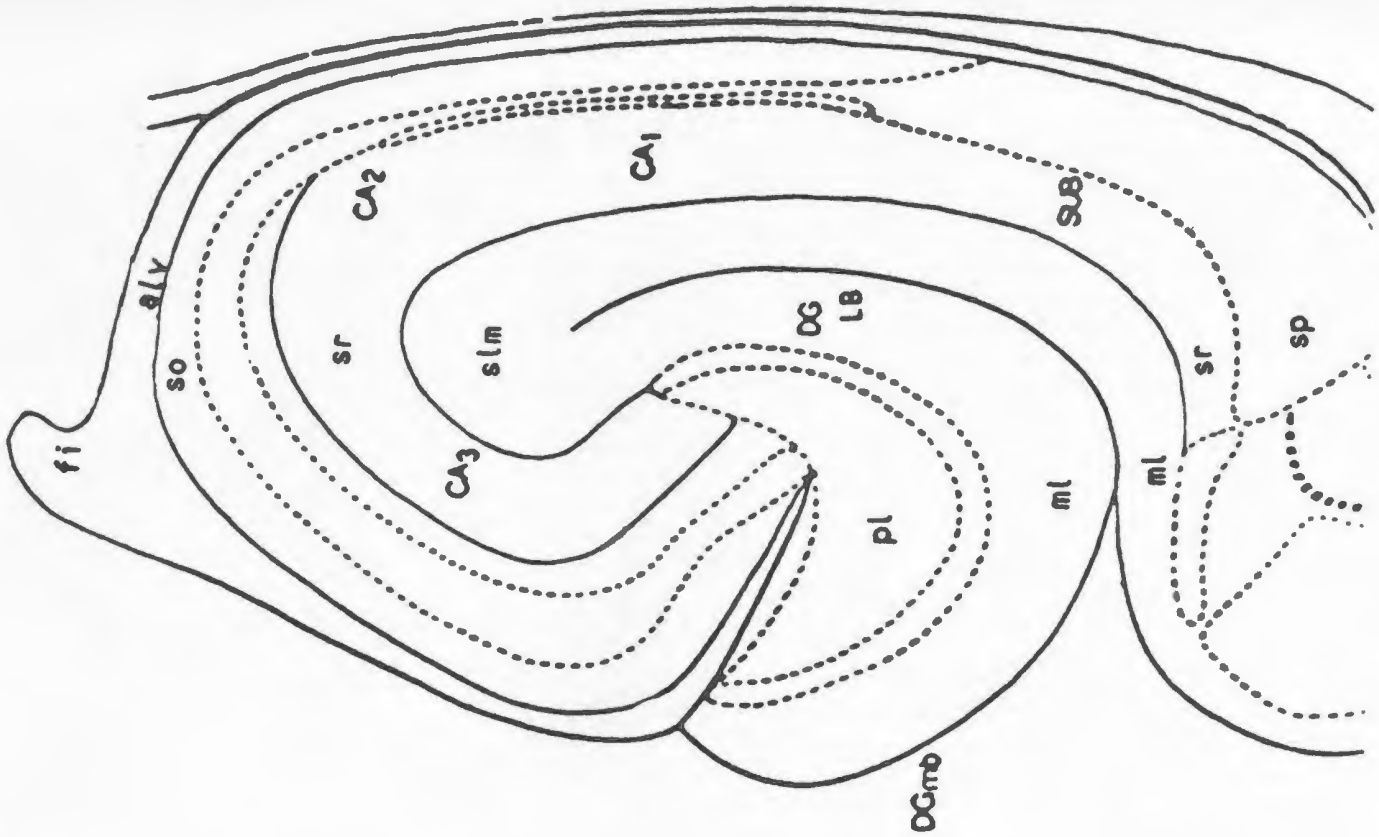


Figure 1. Horizontal section through the hippocampus of the rat.

Adapted from Swanson, Kohler and Bjorklund (1987) In Handbook of Chemical Neuroanatomy. (With permission from Elsevier Science Publishers).

Abbreviations: CA1 = field CA1 of Ammon's horn ; CA2 = field CA2 of Ammon's horn ; CA3 = field CA3 of Ammon's horn ; DG (lb,mb) = dentate gyrus (lateral and medial blades) ; SUB = subiculum ; alv = alveus ; fi = fimbria ; gl = granule layer ; hf = hippocampal fissure ; ml = molecular layer ; pl = polymorph or hilar layer ; sl = stratum lucidum ; slm = stratum lacunosum-moleculare ; so = stratum oriens ; sp = pyramidal layer ; sr = stratum radiatum.

association fibers (Swanson et al., 1987). Sensory information travels to the molecular layer via perforant path fibers originating in the entorhinal cortex (Amaral et al., 1989). A granule cell layer is surrounded by the molecular layer in the dentate gyrus. The area between the lateral and medial blades and extending toward the boundary of CA3, is referred to as the hilar region or polymorph layer and is composed of a variety of neuronal types (Swanson et al., 1987 ; Amaral et al., 1989).

The cholinergic system of the brain is widespread in that it supplies acetylcholine (Ach) fibers to numerous regions from two major sources. Cholinergic neurons of the basal forebrain project to cortical regions while mesopontine tegmentum cholinergic fibers project mainly to the thalamus (Semba, 1990). The medial septal nucleus and the nucleus of the vertical limb of the diagonal band are the main sources of an extensive cholinergic pathway to the hippocampus that travels in the fimbria-fornix fiber bundle (Wainer, 1990 ; Semba, 1989 ; Amaral and Kurz, 1985 ; Lynch, Rose and Gall, 1977). This pathway provides 90 percent of the cholinergic innervation to areas of the hippocampus (Gage. Bjorklund and Stenevi, 1983). The other ten percent of cholinergic fibers arise from a path that innervates the ventral hippocampus after traversing the piriform lobe (Gage et al., 1983 ; Wainer, 1990).

A prominent transmitter of the septohippocampal pathway is Ach (Swanson, 1977 ; Wainer, 1990). Within the regions of the hippocampus there are distinct patterns of Ach innervation (Swanson, Kohler and Bjorklund, 1987). Cholinergic inputs can be assessed by observing the number of acetylcholine binding sites in a given region using radio-

ligand binding techniques or reacting tissue for biochemical markers of cholinergic activity (Sokolovsky, 1984).

There are two types of cholinergic receptors - muscarinic and nicotinic (Zilles, 1988). Of the muscarinic class, there are M1 and M2 receptor subclasses differentiated primarily on ligand affinities and tissue localization (Zilles, 1988). The M1 receptors are thought to be postsynaptic while M2 receptors are believed to be presynaptic in nature (Cooper, Bloom and Roth, 1986 ; North, Slack and Surprenant, 1985). Postsynaptic muscarinic receptors are thought to activate physiological changes at the postsynaptic membrane consequent to Ach input to the hippocampus while presynaptic receptors act to regulate the release of Ach from cholinergic axons (Sokolovsky, 1984). The absolute density of M1 binding sites exceeds that of the M2 receptor sites in the hippocampus (Cortes and Palacios, 1986).

The absolute density of nicotinic receptors is much lower than the values of muscarinic receptors in the hippocampus (Zilles, 1988). While both pre and postsynaptic muscarinic receptors have been reported, the nicotinic receptor is believed to be presynaptic only with respect to the brain (Cheney, 1991). Conversely, nicotinic receptors are found postsynaptically in the peripheral nervous system (PNS) at the neuromuscular junction. The present study focused on antagonism of muscarinic receptors in the hippocampus in an attempt to elucidate information regarding their involvement in plastic responsiveness of cholinergic fiber inputs. Given the numerical dominance of postsynaptic muscarinic receptors in the hippocampus over presynaptic

receptor types, any manipulation of the muscarinic receptor system in the present study, is presumed to exert its major physiological effect at the postsynaptic region. While muscarinic manipulations do affect presynaptic receptors, this effect is primarily with respect to transmitter regulation and will have little consequence for postsynaptic receptor activation when the postsynaptic receptor population is antagonized as in the treatment of the present study.

Swanson et al. (1987) discuss the relative densities of muscarinic receptors in the hippocampus. Receptor densities were determined using the labelled antagonist tritiated N-methyl scopolamine (NMS), a general marker of both types of muscarinic receptors (Swanson et al., 1987). Table 1 illustrates the relative distributions of muscarinic receptors in the hippocampus and compares this to acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) densities in the same region. The highest densities of muscarinic receptors are found in molecular and hilar regions of the DG, strata oriens and radiatum of CA1 and stratum oriens of CA3. Moderate levels of muscarinic receptors are observed in stratum pyramidale of CA1 and strata radiatum and moleculare of CA3. The lowest relative densities of muscarinic receptors are found in the granule cell layer of the DG, strata lacunosum and moleculare of CA1 and strata pyramidale and lucidum of CA3.

The distribution of specific muscarinic receptor binding sites within the hippocampus varies across its anatomical regions. Research is still necessary to fully differentiate binding distributions of M1 and M2 muscarinic receptor subtypes (Zilles,

Table 1. The relative density of muscarinic receptors in different layers of hippocampal fields and their relationship to the relative density of known cholinergic innervation

Anatomical region	Muscarinic ([3H]NMS)	Cholinergic innervation	
		AchE	ChAT
DENTATE GYRUS			
molecular layer	+++	++	++
granular layer	+	0	0
hilar region	+++	+	++
AMMON'S HORN, FIELD CA1			
stratum oriens	+++	++	++
stratum pyramidale	++	+++	+++
stratum radiatum	+++	+	+
stratum lacunosum	+	+	++
stratum moleculare			+
AMMON'S HORN, FIELD CA3			
stratum oriens	+++	++	+++
stratum pyramidale	+	+++	+++
stratum ludicum	+	+	+
stratum radiatum	++	+	+
stratum moleculare	++	++	++
SUBICULUM			
stratum pyramidale	+++	++	+++
stratum radiatum	++	+++	++
stratum moleculare	+	+	+

Table adapted from Swanson, Kohler and Bjorklund (1987) In. Handbook of Chemical Neuroanatomy. (With permission from Elsevier Science Publishers).

Abbreviations: AChE = acetylcholinesterase ; ChAT = choline acetyltransferase ; NMS = N-methylscopolamine

1988). To date however, preliminary muscarinic receptor levels for M1 and M2 binding sites have been mapped out for the hippocampus (Zilles, 1988). The highest relative densities of M1 binding sites are found in the strata pyramidale and radiatum of CA1 and in stratum moleculare of the fascia dentata (Zilles, 1988). The lowest M1 binding site densities are found in all layers of CA2 and CA3 and in hilar fascia dentata (Zilles, 1988). The highest density of M2 binding sites is found in stratum lucidum of CA2 and in stratum pyramidale of CA1 and CA2 (Zilles, 1988). The lowest binding densities of the M2 receptor are found in stratum lacunosum-moleculare of CA1 and in stratum moleculare of the fascia dentata (Zilles, 1988).

Table 1 illustrates the distributions of AChE and ChAT reactivity in the hippocampus. Acetylcholinesterase is an indirect marker of cholinergic innervation as it is an enzyme associated with Ach degradation (Cooper et al., 1986 ; Sokolovsky, 1984). Choline acetyltransferase, on the other hand is a direct marker of cholinergic neurons and axons and is the enzyme that catalyses the synthesis of Ach from choline (Cooper et.al. (1986) ; Sokolovsky (1984)). Acetylcholinesterase distributions correlate relatively well with those of ChAT in the hippocampus. There is moderate AChE staining in the molecular layer of the DG and a similar level of ChAT activity. Choline acetyltransferase activity is moderate in the hilus while AChE is low in the same region. In the CA1, distributions of both AChE and ChAT are similar in strata oriens, pyramidale and radiatum. There is a similar consistency between the two markers in CA3 with the exception of stratum oriens which has moderate AChE staining and high ChAT reactivity.

This comparison suggests that AChE and ChAT are similarly distributed throughout the hippocampus with only a few exceptions, and either marker is adequate when assessing cholinergic activity in that region.

Embryonic septal cell transplants

Recently, considerable information has been generated regarding the potential of the hippocampal cholinergic system to regenerate connections following their removal by surgical means (Dunnett, 1991 ; Bjorklund, Gage, Stenevi and Dunnett, 1983). Through embryonic septal cell transplant procedures neuroscientists have been able to better understand the plastic properties of the hippocampus. Embryonic cholinergic grafts placed in the hippocampus of the fimbria-fornix lesioned rat have produced both anatomical reinnervation and functional recovery from behavioral deficits (Bjorklund and Gage, 1984 ; Dunnett et al., 1991). These findings suggest that perhaps the hippocampus can recover from states of cholinergic depletion with the aid of tissue implants that effectively restore cholinergic input to the damaged hippocampus.

Research is attempting to disclose the factors that can influence graft innervation of host tissue and subsequent viability of this input (Dunnett, Whishaw, Bunch and Fine, 1986 ; Gage, Buzsaki and Armstrong, 1990). One focus is neurally produced nerve growth factor (NGF) (Gage et al., 1990). Nerve growth factor (NGF) exists in highest central nervous system (CNS) concentrations in the hippocampus and its activity is increased following fimbria-fornix lesions (Collins and Crutcher, 1985 ; Gage et al.,

1990). It is thought that NGF is synthesised in the hippocampus and transported retrogradely to cholinergic neurons of the basal forebrain where it prevents neuronal degeneration following fimbria-fornix lesions (Seiler and Schwab, 1984).

Buzsaki, Bickford, Varon, Armstrong and Gage (1987b) investigated the effects of NGF on host septal cells following a fimbria-fornix lesion in rats. Buzsaki et al. (1987b) placed fetal hippocampal grafts into the fimbria-fornix lesion cavity. These grafts were assumed to act as a bridge for isolated host septal cells as they extended their axons toward the denervated hippocampus. This technique, without NGF administration, consistently yields small increases in ChAT and AChE in the hippocampus. Growth of the host septal cells through the fetal cell bridge into the host hippocampus is limited presumably due to the death of host septal cells in the absence of retrogradely transported NGF (Buzsaki et al., 1987b). It was hypothesized that delivery of NGF to the lateral ventricle could promote survival and the extension of cholinergic axons to the hippocampus from the host septum. Buzsaki et al. (1987b) combined the transplant technique with infusions of NGF and measured subsequent ChAT and AChE staining in the hippocampus. They found that NGF administered after graft implantation lead to increased choline acetyltransferase immunoreactivity (Chat-IR) in host septal cells and increased reinnervation of the hippocampus compared to a graft plus fimbria-fornix condition alone (no NGF treatment). It appears as if NGF enhances reinnervation and survival of embryonic septal cell transplants.

Neurotransmitters too can influence graft outgrowth and survival. Noradrenergic

transmitter availability within a system receiving a cholinergic septal graft influences the reinnervation of host tissue by that graft. Dunnett, Whishaw, Bunch and Fine (1986) assessed the survival of embryo cholinergic cell transplants to the adult rat forebrain with intrinsic cholinergic innervation removed through nucleus basalis lesions. They measured the extent of AChE innervation of the host brain by a cholinergic graft under control and noradrenaline depleted conditions. Nucleus basalis lesioned subjects were depleted of forebrain noradrenaline by 6-hydroxydopamine (6-OHDA) followed by cortically placed cholinergic grafts. This manipulation resulted in a significant enhancement of AChE positive fiber outgrowth relative to an intact noradrenergic system control condition. This suggests that neurotransmitter influences also play a role in regulating plastic change in the CNS.

Sprouting

When discussing forms of hippocampal plasticity and the factors that promote regeneration of cholinergic system communication within the hippocampus, sprouting research can provide some valuable insight. Injury of brain regions can induce a trophic signal that results in a level of recovery that can be physically measured. Terminal sprouting or reactive synaptogenesis, in response to a lesion has been reported in the CNS (Cotman and Nieto-Sampedro, 1984). This sort of central sprouting differs from the widely studied PNS sprouting in that growth distances are usually shorter, less than 50 to 100 micrometers (μm) (Gage et al., 1983). The onset of CNS terminal sprouting

responses are quick. The initiation of terminal sprouting in the CNS requires one to five days in the adult (Cotman et al., 1984 ; Gage et al., 1983). During reactive synaptogenesis, reinnervated areas are restricted to regions of sprouting terminals. It is this form of sprouting that is believed to immediately follow the infliction of injury upon a system. Two to four days after lesioning the perforant pathway, reactive synaptogenesis can be observed in the molecular layer of the dentate gyrus (Cotman et al., 1984). Levels of the synaptic growth associated phosphoprotein (GAP-43), a marker of developing axons, increase within the molecular layer after such a lesion and are indicative of axon terminal outgrowth (Benowitz, Rodriguez and Neve, 1990).

Other forms of sprouting require more time before reinnervation is seen and thus do not fall into the same class as reactive synaptogenesis (Gage et al., 1983). An example is compensatory collateral sprouting which requires one to three months before regeneration of fibers is noted. This time difference is primarily due to the distance across which fibers must travel to effect reinnervation. Compensatory collateral sprouting is not simply terminal in nature as with reactive synaptogenesis but involves a rerouting of fibers of a given intact system to reinnervate a denervated system. For example, Gage et al., (1983) demonstrated that cholinergic fibers innervating ventral hippocampus can sprout to dorsal sites where they replace denervated dorsal cholinergic inputs. The response was elicited by lesioning the fimbria-fornix tract of rats. One to two weeks following the lesion ChAT levels were reduced by 95 percent in dorsal hippocampus. One to three months after the lesion there was up to 40 percent recovery of ChAT levels.

The lengthened time course relative to reactive synaptogenesis can be understood given that fibers from ventral hippocampus had to traverse a distance of three to four millimeters (mm) to reach dorsal hippocampus.

Acetylcholinesterase as a marker of cholinergic fiber outgrowth

Acetylcholinesterase activity is widely used to assess sprouting within the hippocampus (Gage et al., 1983 ; Lynch, Matthews, Mosko, Parks and Cotman, 1972 ; Storm-Mathisen, 1974 ; Nadler, Cotman and Lynch, 1977 ; Nadler, Cotman, Paoletti and Lynch, 1977 ; Steward and Messenheimer, 1978 ; Amaral, Avendano and Cowan, 1980 ; Scheff, Benardo and Cotman, 1980 ; Stanfield and Cowan, 1982 ; Woodruff and Baisen, 1990 ; McKeon, Vietje and Wells, 1989). For example, the hippocampus receives afferents from the septum, subicular and entorhinal cortices. If the entorhinal cortex is lesioned electrolytically, an intense band of AChE reactivity appears in the outer part of the molecular layer of the dentate gyrus. If the septum is lesioned at the time of an entorhinal lesion, no stain increases are seen (Lynch et al., 1972). The study of Lynch et al., (1972) suggests that septal neurons sprout into the outer molecular layer of rats following entorhinal lesions and that this response can be measured by increases in AChE staining (Lynch et al., 1972).

Stanfield and Cowan (1982) also investigated the effects of unilateral entorhinal cortex lesions on AChE staining in the molecular layer of the DG. Entorhinal lesions

produced an intensification of AChE stain in the molecular layer of rats when conducted four to six weeks prior to sacrificing the subjects. Septal fibers were believed to be the source of the AChE positive intensification seen in that study (Stanfield and Cowan, 1982).

Woodruff et al. (1990) employed the neurotoxin trimethyltin chloride (TMT) and produced massive cell loss in the entorhinal cortex. Orally administered TMT effectively lesions the entorhinal cortex input to the hippocampus (Woodruff et al., 1990). Administration of TMT resulted in significantly denser AChE staining in the dentate molecular layer relative to control subjects. Woodruff et al. (1990) concluded that the increased AChE staining is the result of sprouting septal axon terminals into the zone denervated by the entorhinal cortex lesion.

Acetylcholinesterase activity is also a widely used marker of cholinergic transplant growth (Dunnett, Low, Stenevi and Bjorklund, 1982 ; Dunnett et al., 1986 ; Bjorklund et al., 1983). Embryo septal cells implanted into a damaged septohippocampal system reinnervate host tissue over approximately one to six months. The amount of reinnervation has been assessed by the levels of AChE staining in the hippocampus. As the donor cholinergic fibers grow into host tissue, AChE staining increases indicating restoration of cholinergic innervation of the hippocampus (Dunnett, 1991 ; Dunnett et al., 1982 ; Bjorklund et al., 1983).

In the present study, AChE staining will be used to assess the level of cholinergic fiber terminal sprouting following prolonged muscarinic receptor antagonism.

Acetylcholinesterase staining has served as a reliable marker of cholinergic fiber outgrowth in both sprouting and transplant paradigms and is therefore a logical and straight forward system for use in evaluating the effectiveness of the novel technique used in the present study to induce sprouting.

Hypothesis

Both transplantation and sprouting paradigms are focusing on the elucidation of the stimuli necessary for hippocampal plastic responsiveness to injury (Dunnett et al., 1986 ; Gage et al., 1990). In the case of tissue transplantation, fiber outgrowth has an upper limit with respect to the extent of host reinnervation. This upper limit seems to be attained after approximately six months (Dunnett et al., 1986 ; Bjorklund et al., 1983). One focus of research in this instance should be on increasing the amount of host tissue reinnervation. The isolation of stimuli that are capable of expediting graft outgrowth, would be an asset to therapeutic advances in combating diseases of the cholinergic system.

This present proposal is an attempt to isolate one potential stimulus that could influence growth and repair in the CNS. This hypothesis is based on the concept of a post to presynaptic communication network that regulates axonal growth, as in the case of sprouting, and which can be directly manipulated by varying postsynaptic receptor activation. That is, it is hypothesised that postsynaptic receptor antagonism will result in the production of a growth signal that will induce postsynaptically directed growth of

presynaptic axons. In the context of lesion studies, it is hypothesised that postsynaptic receptor antagonism will mimic partial deafferentation and cause terminal sprouting of cholinergic fibers. This is conceivable given that the inhibition of normal transmitter-receptor interactions in situations of postsynaptic blockade are the same as those observed in states of partial denervation. In this way antagonism is a means of testing the hypothesis that receptor inactivity is a specific trigger of trophic agent release.

Support of hypothesis from PNS work

A similar approach to that taken in the present study has been employed in the PNS. Holland and Brown (1980) antagonized neuromuscular transmission in mice using repeated injections of the irreversible nicotinic blocker alpha-bungarotoxin. Repeated intramuscular injections were required with their "purified" sample of the irreversible drug because injections with a "pure" commercial sample resulted in receptor death (Holland et al., 1980). Doses consisted of a one microgram injection daily or two 0.5 microgram administrations daily. Four to eight days following initiation of injections Holland et al. (1980) assessed the level of sprouting that occurred. Motor nerve sprouting occurred within a few days of blockade such that presynaptic terminals demonstrated extensive sprouting at antagonized endplates. The conclusion reached suggested a role for inactive postsynaptic muscle fibers in producing terminal sprouting. This result was exciting because it was preceded by a couple of failed attempts. Pestronk and Drachman (1978) delivered alpha-bungarotoxin to a muscle paralysed by botulinum,

an antagonist of presynaptic transmission, and reported an inhibition of the previously studied botulinum induced sprouting (Duchen and Strich, 1968). In this case it was expected that sprouting would have occurred in the situation of combined botulinum and nicotinic receptor blockade given that each stimulus on its own is capable of inducing a sprouting response (Holland et al., 1980 ; Tonge, 1974 ; Duchen and Strich, 1968). Tonge (1978) investigated the effect of prolonged blockade of neuromuscular transmission on muscle endplates. Doses of the neurotoxin of Naja siamenis venom were delivered to soleus muscle of mice to produce a prolonged blockade of nicotinic Ach receptors. Nerve sprouting was not observed in this experiment. Neuromuscular transmission was only inhibited for two to three days. This limited duration of receptor blockade may have been the reason for the result obtained. If transmission could have been antagonized longer, sprouting may have been seen. Unfortunately multiple doses could not be given due to the fact that they induce excessive muscle damage (Tonge, 1978).

Holland et al., (1980) suggest that muscle inactivity, created by acetylcholine receptor blockade, is responsible for sprouting at the muscle endplate. However, these researchers further suggest that they cannot rule out a role for available acetylcholine (Ach) receptors. They pointed out that a single or twice daily dose of alpha-bungarotoxin is probably not sufficient to bind to all extrajunctional receptors. This suggests, in light of their clear sprouting result, that they presuppose that available Ach receptors could be of potential importance to trophic regulation, and the sprouting response seen in their study. This suggestion was first forwarded by Pestronk et al., (1978) who found that

nicotinic antagonism with alpha bungarotoxin inhibits the sprouting normally produced by botulinum toxin. Pestronk et al., (1978) argue for a role of availabe Ach receptors in sprouting regulation given that their combined botulinum/alpha bungarotoxin treatment failed to elicit sprouting as would have occurred in the botulinum condition alone; a condition in which Ach release is blocked but postsynaptic Ach receptors are not. The finding of Holland et al. (1980) clearly challenges this idea. In the context of the present proposal, perhaps the antagonism of Ach receptors and not the availability of these receptors to Ach activation is the key to the elicitation of sprouting. This approach toward sprouting is in line with that taken by Holland et al. (1980) in the PNS with the exception that muscarinic blockade in the hippocampus will not leave the postsynaptic cells inactive. During CNS muscarinic receptor antagonism, other neurotransmitter systems will remain intact. When nicotinic receptors are antagonized at the neuromuscular junction, as in the Holland et al. (1980) study, the post synaptic muscle is inactive.

It is predicted in the present study that, in the CNS, antagonized muscarinic receptor populations will induce a trophic signal to presynaptic axons initiating presynaptic growth to antagonized regions postsynaptically as was found in the PNS by Holland et al., (1980). The observation of sprouting in the undamaged hippocampus, as triggered in this way would be supportive of the present hypothesis that prolonged Ach receptor inactivity, through antagonism, is a factor that can initiate cholinergic fiber outgrowth. That is, muscarinic receptor antagonism alone will induce growth as opposed

to possibly enhancing growth following its induction by other means such as by signals consequent to embryonic septal cell implantation. Specifically, this study will investigate the existence of a potentially new stimulus of sprouting within the undamaged adult hippocampus. The present study will, in this way, contribute to knowledge concerning the origin of trophic signalling necessary to initiate sprouting in stable systems such as the hippocampus of adult rats.

Postsynaptic hippocampal Ach receptors

In the PNS, Holland et al. (1980) blocked postsynaptic nicotinic receptors using alpha-bungarotoxin and induced sprouting of axons. In the present study muscarinic receptors have been chosen as the target of antagonism. Of the two types of Ach receptors, muscarinic classes are in greater abundance in the hippocampus (Zilles, 1988). This characteristic makes them more easily accessible to pharmacological manipulations such that more muscarinic receptors could be targeted in a given area than possible with nicotinic receptors. Nicotinic receptors are believed to reside predominantly in presynaptic locations within the hippocampus and thus would not serve as an appropriate target for testing the present hypothesis that postsynaptic blockade can induce trophic signalling and subsequent presynaptic sprouting (Cheney, 1991).

Prolonged muscarinic receptor antagonism

The hypothesis regarding the induction of sprouting by muscarinic antagonism can

only be tested under a condition of prolonged blockade. If sprouting is to be induced by some trophic signal elicited through muscarinic blockade then the growth signal will have to be present long enough to elicit sprouting of presynaptic terminals. Trophic regulation by this means can only be maintained if the trigger, postsynaptic muscarinic receptor blockade, is persistent. The irreversible muscarinic receptor antagonist quinuclidinyl benzilate (QNB) was utilized to provide the necessary prolonged block of muscarinic receptors in this study.

A factor influencing the duration of muscarinic receptor blockade is the rate of muscarinic receptor turnover. A given receptor may only be antagonized for the length of time its in existence. Once the muscarinic antagonist is placed in the brain the limiting factor determining duration of action will be receptor turnover.

Sethy and Hyslop (1990) targeted muscarinic receptors using the irreversible muscarinic agonist BM-123. Oxotremorine, a muscarinic agonist, has been found to reduce the amount of Ach released at junctions in both central and peripheral nervous systems (Sethy and Hyslop, 1990). When BM-123 is administered, oxotremorine is not effective in reducing Ach release as determined by [3H] Ach release patterns. This is due to the unavailability of muscarinic receptors during the irreversible BM-123 block of existing receptor populations. The oxotremorine induced inhibition of Ach returns to normal by 144 hours after the initiation of the irreversible BM-123 blockade. The receptors that BM-123 bound to in the Sethy and Hyslop (1990) study, are the same ones that QNB bind. BM-123 blockade will also prevent QNB from acting at its receptor site

for approximately 144 hours (Russell, Smith, Booth, Jenden and Waite, 1986). This suggests that muscarinic receptors turnover in significant numbers in no more than six days. Given this rate of receptor turnover one can expect to maintain an effective muscarinic block using QNB for a maximum of six days, a sufficient time to induce sprouting in the CNS (Storm-Mathisen, 1974 ; Cotman et al., 1982 ; Mckeon et al., 1989 ; Gage et al., 1983 ; Benowitz et al., 1990).

Experiment 1

It is predicted in the following experiment that hippocampal injections of the irreversible muscarinic antagonist QNB will elicit sprouting, as indicated by an increase in AChE staining near the site of injection. Brain regions receiving a vehicle injection only should exhibit no sprouting of AChE positive fibers. It is further hypothesised that sprouting will occur given sufficient time. Animals will be assessed for AChE stain increases at four times: one, four, 14 and 21 days after QNB injection. It is expected, based on the rate of receptor turnover, that the antagonist should act at a receptor site for no more than six days (Sethy and Hyslop, 1990 ; Russell et al., 1986). Animals allowed to survive for a four day period should demonstrate sprouting while animals surviving for one day might not exhibit antagonist induced sprouting of AChE positive fibers unless the response is very rapid. Animals permitted to survive for 14 days may exhibit increased AChE staining but not to the degree of four day animals given the predicted termination of the sprouting signal after a maximum of six days. By 14 days it is expected that some

degree of retraction of sprouted fibers will occur given the absence of the muscarinic antagonism induced trophic signal following receptor turnover. By 21 days post injection, animals may not exhibit any AChE stain increases as a result of the treatment. Quinuclidinyl benzilate will not be bound to muscarinic receptors to maintain a sprouting stimulus by 21 days and sprouted fibers may retract or degenerate when the sprouting stimulus is terminated.

EXPERIMENT 1

METHOD

Subjects: The subjects used in the present study were 43 female Sprague Dawley rats weighing between 200 and 240 grams at experimental onset. All rats were obtained from Charles River Laboratories (Montreal, Quebec). They were housed individually in Plexiglas and wire cages with ad lib food and water administration. The housing area was maintained at a 12 hour on, 12 hour off light cycle.

Drug treatment: The muscarinic antagonist quinuclidinyl benzilate (QNB) was used to induce a prolonged blockade of muscarinic receptors in this study. This drug was purchased from Research Biochemicals Incorporated. Quantities of QNB were dissolved in a beta hydroxipropylcyclodextrin (36.6%) vehicle and stored at 4 degrees Celsius to maintain stability. Drug stability was estimated to be 48 hours based on the advice of the experts at Research Biochemicals incorporated (personal communication, 1990).

No in vivo studies utilizing QNB have been reported in the literature, so an effective dose could not be determined through this means. Ben-Barak and Dudai (1979) offer some indication of a possible effective QNB dose. They assessed the specific binding of [3H]QNB to homogenate of rat brain hippocampal formation. They found that saturation was reached at [3H]QNB concentrations of one to two nanomoles (nM) and that

similar amounts (1 nM) of atropine were required to displace the QNB. From this it seems that similar concentrations of atropine and QNB would be required to saturate the hippocampal region. The molecular weights of the two compounds are similar as well. Atropine has a molecular weight of 289.38 while the molecular weight of QNB is 337.38. Therefore, a study which utilizes intrahippocampal atropine administration would provide information as to an adequate QNB dose. Such a study was conducted by Leaton and Rech (1972) who found that locomotor activity of rats increased when given bilateral intrahippocampal atropine. The mode of atropine administration in this study consisted of tamping a cannula in the crystallized drug 100 times and then inserting this into an outer cannula. It was estimated, using a weight by difference technique, that doses ranged from 6 μg to 18 μg of the drug.

A dose of five μg of atropine administered bilaterally in the hippocampus in a study by Singh, Ott and Matthies (1974) was sufficient to impair acquisition of a 'Y' maze task. A drug effect was evident in their study and serves the purpose of offering a possible dose for use in the present study where an effective antagonizing dose is all that is required.

From the above mentioned studies an injection of between ten and 20 μg of QNB seemed a good starting point for the present study. To prepare a drug solution, QNB was mixed with the appropriate amount of vehicle and sonicated in a Bransonic 12 sonicator. This device used ultrasonic waves to break down the QNB solid into the finest particles possible. This produced a fine suspension.

The above calculated quantity of QNB (20 μg) was employed in a pilot study prior to this main study in two, one and 0.5 microliter (μl) volumes to determine the importance of volume for producing the hypothesised effect. The 20 μg/2 μl dose was most effective in a study in which AChE staining was measured 14 days following a QNB injection into one hemisphere and a beta hydroxipropylcyclodextrin (2 μl) vehicle into the other. Quinuclidinyl benzilate-injected hemispheres demonstrated increased AChE positive staining relative to their control, vehicle injected counterparts. These findings indicated that QNB (20 μg/2 μl) was sufficient to cause an increase in AChE positive staining at the region of injection in experimental hemispheres relative to controls. Based on this finding, it was decided to continue with this main study employing a 20 μg QNB dose in a 2 μl volume of vehicle. Equal volumes of vehicle were administered for control injections.

Procedure: All subjects received bilateral surgical injections into their hippocampi. Either the right or left hemisphere received QNB (20 μg/2 μl) while the other control hemisphere received an equal volume of 36.6% beta hydroxipropylcyclodextrin. Injections were administered during a stereotaxic surgical procedure during which animals were anaesthetized with Avertin (1 cc/kg). Aseptic techniques were followed throughout the surgeries. Prior to drawing any QNB from the drug-containing vial, the vial and contents were vigorously shaken manually to ensure that the drug was evenly suspended. Injections were delivered using a Hamilton microliter syringe mounted on the arm of a

stereotaxic apparatus. Surgical incisions were made into the skin above the skull of subjects using a scalpel blade, their skulls were drilled above the site of injection, the dura lining punctured using a fine needle and the syringe containing vehicle or QNB positioned. The syringe contents were delivered to a site -3.8 mm posterior, +/- 3.7 mm lateral and -3.7 mm ventral to bregma with the skull surface level. To prevent hippocampal tissue damage due to a high pressured injection of 2 µl volumes, injections lasted four minutes. The syringe plunger was lightly turned clockwise with a slight downward pressure for a duration of two minutes until all contents were in the hippocampus. Following this, the syringe was left in place for a further two minutes before it was removed from the brain slowly to prevent the injected substance from being extracted in a vacuum by a fast upward motion of the needle. Upon completion of injections to both sides of the brain, incisions were stapled closed with wound clips and a small amount of sulphur flowers antibiotic (powder) was applied to prevent infection Subjects were returned to their cages after surgery to recover.

During the post surgical period, subjects remained in their cages until they were sacrificed. The rats were sacrificed at either one, four, 14 or 21 days post surgery and the amount of sprouting assessed, using AChE staining as the dependent measure, between control and experimental hemispheres. At one of the four sacrifice times, subjects were treated with lethal intraperitoneal injections of sodium pentobarbital anaesthetic (1 cc at 65 mg per rat), then perfused through the left ventricle with 30 ml of 0.9% phosphate buffered saline. Tissue fixation was carried out using 300 ml of 4%

paraformaldehyde over approximately 15 minutes. Decapitation followed the chemical fixation procedure. The brain was subsequently removed from the skull and stored in 4% paraformaldehyde overnight at 4 degrees Celsius. The next day, brains were transferred to a cryoprotective solution of 25% sucrose and allowed to sink at a temperature of 4 degrees Celsius. After the brains sank they were frozen in a solution of 2-Methylbutane contained in a 500 ml beaker immersed in liquid nitrogen. Frozen brains were then sectioned using a cryostat microtome at -18 degrees Celsius. Coronal sections through the hippocampus were collected at a thickness of 40 μm and mounted on chrome alum coated slides for further processing.

Histochemistry: Once mounted on slides, the tissue sections were histochemically reacted using a stain for AChE. The tissue was incubated overnight in 200 ml of a stock solution of 50 mM sodium acetate, 4.0 mM copper sulphate crystals and 16 mM glycine set to a pH of 5.0. To this was added 0.006 g ethopropazine (inhibitor of nonspecific esterases) and 0.232 g acetylthiocholine iodide. The following day sections were rinsed six times in distilled water and developed for two minutes in a solution of 10% potassium ferricyanide, again rinsed three times in distilled water and reacted for one minute in a 1% silver nitrate solution before being rinsed six more times in distilled water. Sections were dehydrated in two baths of acetone for ten and 30 seconds respectively then placed in two fresh clearing baths of xylene for 60 seconds each to remove excess acetone from the tissue. Sections remained in the final xylene bath while being coverslipped using

Mikro kit transparent coverslipping medium.

Analysis: Comparisons of brains between experimental and control hemispheres were made regarding the level of AChE staining. Staining densities were assessed as one dependent measure in this study. These measurements were obtained using MCID, a computerized image analysis system (Imaging Research Inc., St. Catherine's, Ontario). Dimensional analysis using a Bausch and Lomb slide projector and a ruled scale made by Imaging Research Incorporated were also conducted for inclusion in the results. This involved obtaining a length measure (anterior to posterior plane) of stain increase throughout the hippocampus plus depth (dorsal to ventral plane) and width (medial to lateral plane) measures of staining at the darkest region of increase. The area demonstrating the greatest density of reaction was used because it best exemplified the reaction, in a given subject, to the treatment. The assessment of staining was carried out as follows:

1. Visual analysis: Sections from each animal were first inspected using the slide projector. The injection sites for each hemisphere were located and recorded in a paper trace of the tissue. If a stain difference between hemispheres existed the location of such was also recorded. If no clear injection sites or tracks were visible reaching the hippocampus the animal was categorized as questionable.

2. Density Analysis: If a stain difference existed between experimental and control hemispheres the density of the darkest region of difference was compared to equivalent regions in the opposite hemisphere. If sections were symmetrical on a given slide then this comparison was performed on the same tissue section. If however asymmetry of approximately greater than 0.5 mm existed within a tissue section on a given slide then symmetrical matches for sites being assessed were located from other sections and used for comparison. This procedure resulted in four relative optical density (ROD) values per subject as follows:

1. A QNB injection density value (density at a site of stain increase in the QNB injected hemisphere),

2. A symmetrically corresponding QNB control density value (density at a location in the vehicle hemisphere corresponding to the area measured in the QNB hemisphere),

3. A vehicle injection density value (density at the vehicle injection site), and

4. A symmetrically corresponding vehicle control density value (density at a location in the QNB treated hemisphere corresponding to the area measured in the vehicle injected hemisphere).

The comparisons were made using ROD as the dependent measure. Relative

optical density measurements were obtained by placing a computer generated cursor window over a site being assessed and allowing the computer to calculate a ROD reading. The same was then done for the symmetrically corresponding section of the opposite hemisphere using the same shaped window at the same location. The values obtained for each hemisphere were then normalized into a ratio format using the ROD of the corpus callosum (CC) as the denominator. This normalized value allowed a between subject comparison to be made by taking into account the baseline tissue density differences between subjects. The density at the CC varies slightly between animals reflecting baseline density differences. When this value is divided into hippocampal stain density values the result is a value reflective of AChE staining density with baseline intensity differences accounted for. Any baseline staining intensity differences (an unavoidable occurrence) between animals prevent raw ROD values from being comparable. Normalized values account for background tissue density and can therefore be compared between animals with differing levels of baseline densities.

Reporting density results:

DESCRIPTIVE STATISTICS: The normalized ROD scores of all animals of a given group were averaged and this mean score as well as a standard deviation and range are reported in the results. Graphical illustrations of group ROD means will include standard error bars.

ROD SAMPLES AS A PERCENT OF CONTROLS: Analysis of animals included calculating normalized ROD values for each subject's vehicle and drug injected hemispheres at the site of AChE increase if one existed. These values were compared with respective normalized ROD values of the symmetrical control sample contralateral to the injection. The mean normalized ROD scores of QNB and vehicle injection samples were reported as a percentage of the respective corresponding control ROD values.

In the case that no AChE stain differences between QNB and vehicle-injected hemispheres existed then ROD measures were taken at the injection sites. As per analysis of regions of staining increase, symmetrical sections were compared. If the injection site of a given hemisphere did not have a corresponding, symmetrical injection site in the opposite side on the same section then the corresponding symmetrical section was located and used for comparison. The same was then done for the vehicle injection site of the other hemisphere.

3. DIMENSIONAL ANALYSIS: The size of a stain increase was measured if one existed. That is, the extent of a the stain increase was measured both within and between sections.

A. length measure: The length (anterior to posterior plane) of stain increases in a hemisphere was measured and reported in millimeters (mm). This was done by counting the number of sections in which the stain difference persisted and multiplying by 40 μm,

the thickness of each section. This was the form of the between section measure. If no stain differences were observed, then of course subjects were not included on this measure.

B. Depth and Width Measure: The within section measure was calculated at the site judged to possess the greatest stain increase. The depth (dorsal to ventral plane) and width (medial to lateral plane) of the stained region was measured under a microscope slide projector using a linear scale. As with the length measure, if no stain differences existed between control and experimental hemispheres, then the subject in question was not included on this measure.

4. Injection/Stain Correlation: The location of any stain increase was correlated with the injection site of a given hemisphere. If the injection site was found on the same section as the greatest stain increase this was recorded. If the injection site was not located on the same section as the greatest stain increase then the distance between the injection site and stain increase was recorded.

5. STATISTICS: Statistical analysis was carried out using an SPSS-X statistical software package contained on the mainframe computer of Memorial University of Newfoundland. Student t-tests for dependent measures were performed on pooled control and vehicle injection sample ROD data to determine if differences existed between control

and vehicle injection measures. A between group analysis of ROD measures was conducted using the ANOVA and assessed QNB and vehicle injection sample scores by group (survival time) to determine if ROD measures of QNB and vehicle injection samples varied by group. This analysis was followed by post hoc comparisons of mean group ROD measures using the Tukey test. A MANOVA was used to calculate whether differences existed between the groups with respect to QNB and vehicle sample ROD scores, and hemisphere of treatment.

A within group analysis was computed in which each group was assessed individually using student t-tests that evaluated QNB injection versus vehicle injection sample ROD values. This set of tests was used to assess the extent of any difference existing within groups on experimental and control injection ROD measures. The results section of experiment one that immediately follows this methods section will deal with the results found in each individual group (four survival times) separately and will primarily focus on descriptive statistics as a tool to represent the findings. The results of the statistical analyses of experiment one will be found in the "summary results" section following the results of experiment two. This section will summarize the effects seen in both experiments one and two of the present study by presenting between and within group comparisons that characterize the phenomenon under study.

6. PHOTOMICROGRAPHS: A Wild Heerbrugg Photomicroscope M400 was employed to magnify and photograph sections. A Wild Leitz MPS46 Photoautomat was used to

regulate shutter speed of the 35mm camera accompanying the M400. Kodak T-Max 100 black and white film was used to capture section images.

INTRODUCTION TO RESULTS.

The results section of the present experiment set will describe observations made in the one, four, fourteen and twenty-one day survival groups in that order. Due to the fact that this study utilizes a novel approach to CNS sprouting, it was decided to format the results in such a way that they are descriptive in nature. This approach is considered appropriate when reporting findings gathered through a set of pilot experiments as is the case here. Result reporting for the respective groups will consist of a general statement about the results observed, a statement regarding injection accuracy, a list of areas receiving injections, an image analysis of injection sites or sites judged to bear increased staining as a result of the treatment and, a statement regarding hemispheric variation. Injection accuracy refers to the degree of certainty with which it can be declared that an injection entered the hippocampus. Injection tracks are used to determine if accuracy can be credited to a given injection. The localization of an injection track tended to get more difficult as the survival time increased. For this reason, results will be discussed with respect to injection accuracy such that all results will first be considered together and then again with questionable injection data removed. In the case of animals demonstrating a

difference in staining between control and experimental hemispheres, a dimensional analysis (length, width and depth measures) will be provided along with a statement assessing the location of injections relative to sites of stain increases.

ONE DAY SURVIVAL GROUP:

GENERAL RESULTS:

Of the nine subjects included in the one day survival group, none demonstrated any AChE staining increase in QNB injected hippocampi. Acetylcholinesterase staining patterns in controls did not differ from patterns observed in experimental hemispheres.

INJECTION ACCURACY:

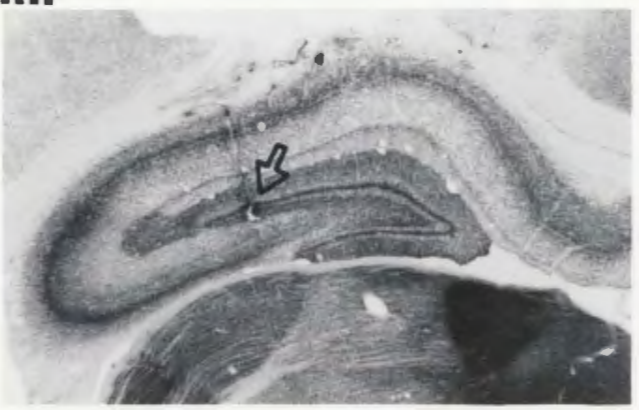
QNB INJECTIONS: Out of the nine subjects in total, eight were judged to have confirmed QNB injections into the hippocampus. The injection track of one subject was unable to be located due to tissue damage in the QNB injected hippocampus.

VEHICLE INJECTIONS: Out of the full complement of vehicle injections, seven were judged to have accurate placements such that tracks could be located entering the respective hippocampi. Two of the nine subjects were judged to have questionable vehicle injections. Figure 2 illustrates QNB and vehicle injection sites in a one day subject.

Figure 2. Photomicrograph of QNB treated (top) and corresponding vehicle treated (bottom) hippocampi from a one day survival subject.

Open arrows indicate injection site.
Abbreviations: RH = right hemisphere
LH = left hemisphere

RH



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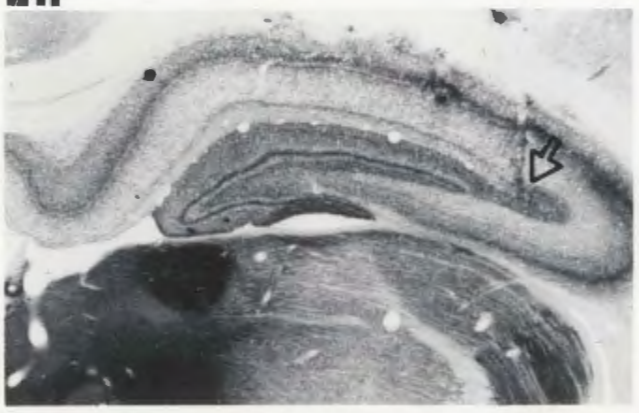


AREAS OF INJECTION:

QNB INJECTIONS: Of the eight QNB injections judged to have entered the hippocampus, five were found in the DG and one each in CA1, SLM and CA3.

VEHICLE INJECTIONS: Of the seven hippocampal vehicle injections identified, four were found in CA3 and one in each of CA1, DG and SLM.

DENSITY ANALYSIS:

QNB INJECTED REGIONS: A density analysis of QNB injected areas within the hippocampus was performed. Sampled QNB injected sites were found to have a mean normalized ROD value of 1.71 (SD = .30) with a range of 1.26 to 2.09. These calculations include image analysis measurements from all animals irrespective of QNB injection accuracy. The mean normalized ROD value of corresponding control sample areas was 1.74 (SD = .29) with a range of 1.27 to 2.08. Figure 3 illustrates the mean ROD scores of all one day survival group subjects irrespective of QNB injection accuracy. The mean QNB treated area ROD as a percent of the corresponding control ROD values was 98 percent.

With the scores of the subject demonstrating a questionable injection removed, the mean normalized ROD of the QNB injected targets was 1.77 (SD = .26) with a range of 1.37 to 2.08. The mean normalized ROD value of corresponding control samples was 1.79 (SD = .29) with a range of 1.26 to 2.08. Figure 4 illustrates the mean ROD scores of one day subjects with questionable QNB injections removed from the analysis. The

FIGURE 3. MEAN ROD SCORES OF 1 DAY SURVIVAL GROUP SUBJECTS IRRESPECTIVE OF INJECTION ACCURACY

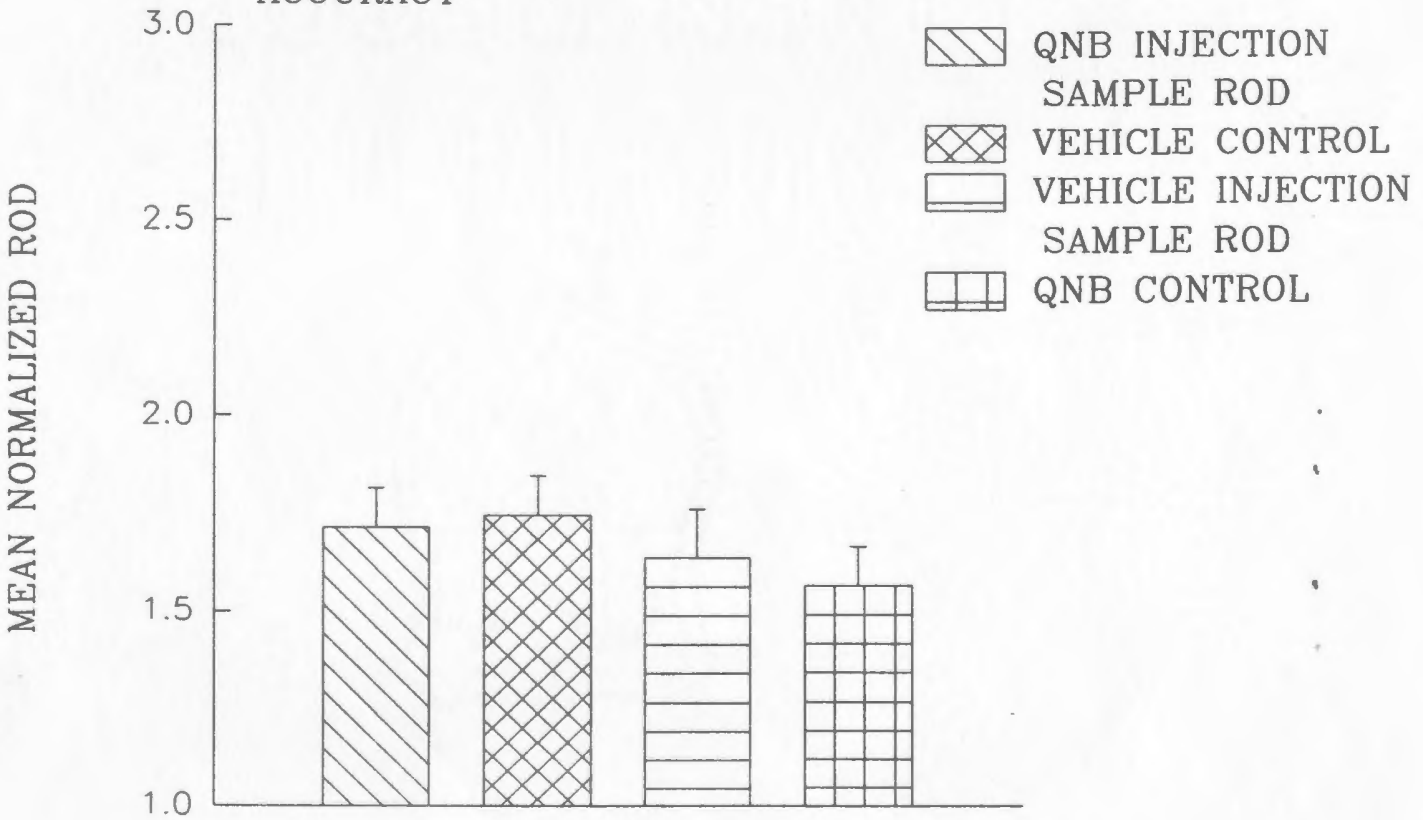
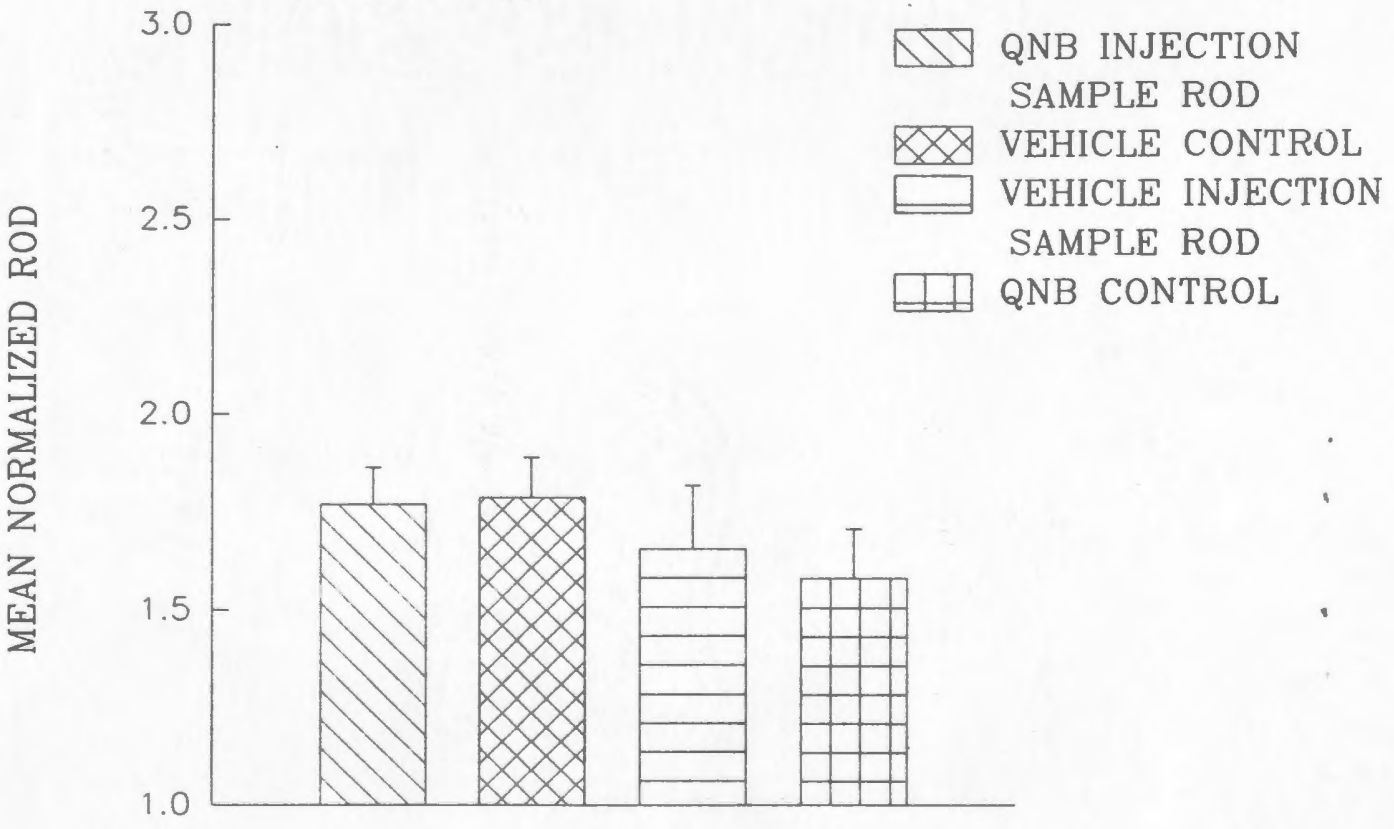


FIGURE 4. MEAN ROD SCORES OF 1 DAY SURVIVAL GROUP SUBJECTS WITH QUESTIONABLE INJECTION DATA REMOVED



mean QNB treated area ROD as a percent of the corresponding control ROD is 101 percent.

VEHICLE INJECTED AREAS: The mean normalized ROD value of vehicle injection sites was 1.63 (SD = .37) with a range ROD of 1.21 to 2.35. The mean normalized ROD value of corresponding control samples in the QNB hemisphere was 1.56 (SD = .29) with a range of 1.25 to 2.01. The mean normalized vehicle injection ROD was found to be 104 percent of the mean normalized ROD value of the corresponding control samples of the other hemisphere. These calculations include the ROD values of subjects with questionable vehicle injections. Figure 3 illustrates the mean ROD scores of subjects irrespective of vehicle injection accuracy. With the questionable scores removed from the analysis, the mean normalized ROD of vehicle injection sites was 1.66 (SD = .42) compared with 1.58 (SD = .33) for corresponding control hemisphere samples with a range ROD of 1.2 to 2.0. Figure 4 illustrates the mean ROD values of subjects in the one day survival group with questionable vehicle injections removed from the analysis. In this case, the mean normalized vehicle injection ROD was 105 percent of the normalized ROD of its corresponding QNB control samples.

HEMISPHERE VARIATION:

Of the nine subjects used in the one day survival group, four received left hemisphere QNB injections and five received right hemisphere QNB injections. Neither hemispherically differentiated group showed any enhancement of AChE staining at the

point of drug injection. No other noticeable difference between these subjects was observed.

CHARACTERISTIC INJECTION TRACK STAINING OF THIS GROUP:

Subjects of the one day survival group with clear injection tracks display increased staining within the track itself while not in surrounding tissue. See figure 2 for an illustration of this in left and right hippocampi of a subject.

FOUR DAY SURVIVAL GROUP:

GENERAL RESULTS:

Seventeen subjects formed the four day group in this experiment. Of the 17, 12 subjects (70.6 percent) demonstrated an AChE positive stain increase that correlated with the site of QNB injection in either the right or left hemispheres. Five animals did not demonstrate an AChE positive stain increase in either QNB or vehicle hemispheres.

INJECTION ACCURACY:

QNB INJECTIONS: Of the full complement of subjects, 15 had QNB injections judged to be in the hippocampus. Two subjects were judged to have had questionable QNB injections in that the injection tracks were not readily traceable or seen entering the hippocampus.

VEHICLE INJECTIONS: Eleven of the 17 subjects were judged to have obvious

and accurate hippocampal vehicle injections while six did not.

STAIN EFFECT AND ACCURACY OF INJECTION: Of the 15 subjects with confirmed QNB hippocampal injections, 11 were observed to demonstrate some degree of AChE positive stain increase at the site of injection. Four of fifteen did not. Of the two subjects with questionable QNB injections, one displayed an AChE positive stain increase while the other did not.

AREAS OF STAIN INCREASES:

Twelve subjects of the four day survival group were judged to possess an AChE positive stain increase in the QNB injected hemisphere relative to corresponding controls. From this number, 15 distinct areas of stain increase were observed such that each subject showed an increase in at least one region while three subjects had an increase in two areas.

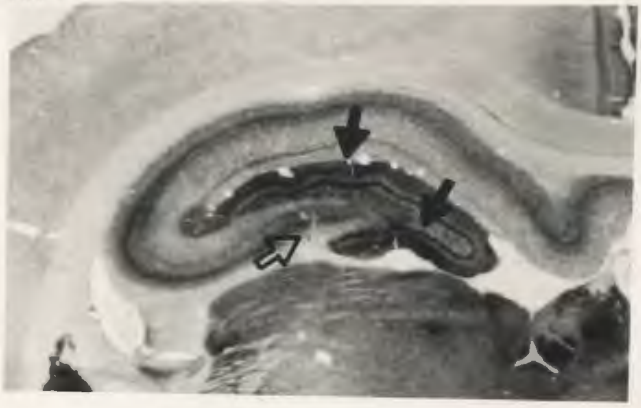
The largest number of AChE positive stain increases were seen in the DG. Eleven subjects demonstrated an increase in this region whether in the lateral or medial blade. In some cases AChE positive stain increases could be seen in the granule cell layer which is normally devoid of AChE staining. Four of the AChE stain increases were seen in CA1 of the hippocampus through strata oriens, pyramidale and superficial radiatum. See figure 5 for an illustration of QNB and vehicle injected hippocampal tissue of representative four day subjects.

Figure 5. Photomicrographs of four day survival subjects with AChE positive stain increases in the molecular layer of the DG (top left and lower left).
Adjacent pictures are corresponding vehicle treated hippocampi.
The middle photomicrographs are vehicle injected (right) and corresponding QNB treated (left) hippocampi of the subject displaying an AChE stain increase in the top left

photomicrograph. AChE staining in the vehicle injected hippocampus (middle right), its corresponding QNB side control (middle left) and the QNB injection control (top right) does not differ.

Abbreviations: RH = right hemisphere
LH = left hemisphere

RH



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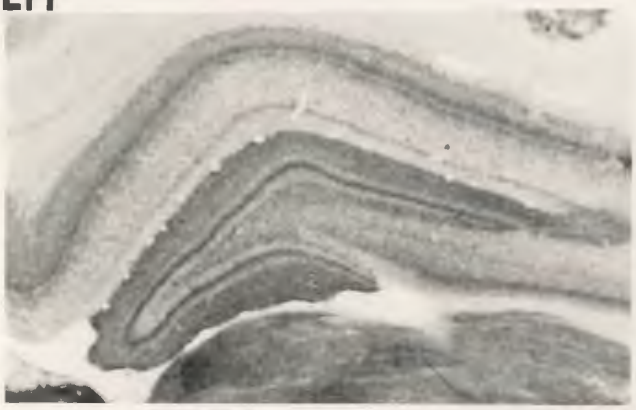
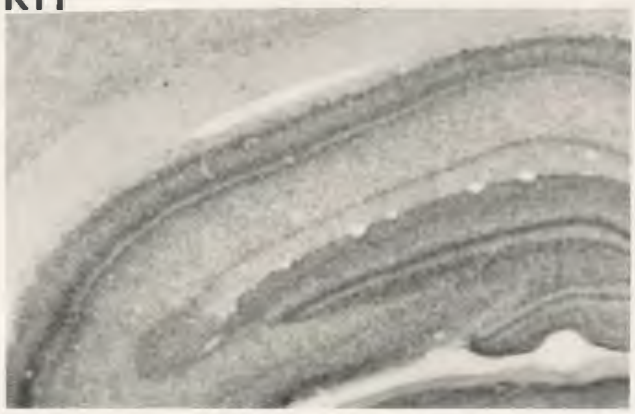


RH



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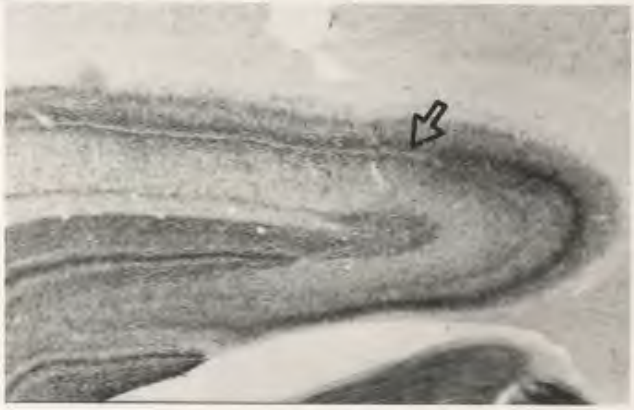

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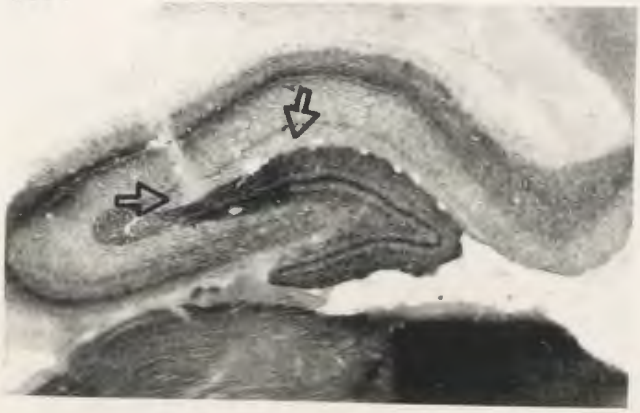


LH

AREAS OF INJECTION WITH NO OBSERVED STAIN INCREASE:

Five animals of the four day survival subjects did not exhibit an AChE positive stain increase. Of these, one had a QNB injection into region CA3. Another subject had an injection into the lateral blade DG while two others had injections yet no reaction in SLM.

DIMENSIONAL ANALYSIS:

WIDTH MEASURE: The width (medial to lateral plane) of observed AChE positive stain increases in QNB injected hemispheres was obtained for each subject demonstrating a stain difference at the point of greatest increase. Twelve subjects were found to exhibit an AChE positive stain increase in QNB injected hippocampi and 15 distinct sites of increase were observed in these 12. The mean width of the AChE positive stain increase at QNB injected sites was .79 mm (SD = .96) with a range of .11 mm to 4.0 mm. This mean was calculated using the results of a subject which demonstrated a diffuse increase in staining throughout the DG of 4.0 mm in width. With this extreme score removed from the analysis, the mean length is .56 mm (SD = .39) and the range is .11 mm to 1.39 mm.

DEPTH MEASURE: The mean depth (dorsal to ventral plane) of AChE positive stain increases was .223 mm (SD = .2397) including all animals. The range of depth measures was from .06 mm to .39 mm. Excluding the depth measure from the subject demonstrating a diffuse pattern of staining through the DG, the mean depth was .23 mm

(SD = .24) ranging from .06 mm to .39 mm.

DENSITY ANALYSIS:

QNB INJECTED STAIN INCREASES: The mean normalized ROD value for regions of observed AChE increase in QNB injected hemispheres was 2.36 (SD = .41) with a range of 1.88 to 3.06. The mean normalized ROD of the corresponding area in the other hemisphere was 1.87 (SD = .41) with a range of 1.24 to 2.50. Figure 6 illustrates the mean ROD scores of subjects illustrating an AChE stain increase at the QNB injection site. Quinuclidinyl benzilate injected samples demonstrating a stain increase had average ROD values 136 percent of those of corresponding controls areas.

Of the QNB injected subjects not displaying a stain increase, the mean normalized ROD value was 1.70 (SD = .42) with a range of 1.30 to 2.07. Subjects with stain increases have ROD values 149 percent of those noneffective QNB injected subjects. Those subjects receiving QNB injections yet not displaying a stain increase had normalized ROD values that were 98 percent of the corresponding control samples.

The above analysis of AChE stain increases includes the results of one subject that, although exhibiting an increase in staining, has a questionable QNB injection. With the questionable injection removed, the mean normalized ROD value for QNB samples became 2.55 (SD = .41) with a range of 1.88 to 3.06. The mean ROD value of corresponding control samples was 1.91 (SD = .39) with a range of 1.24 to 2.50. Figure 7 illustrates the mean ROD scores of four day survival group subjects with

FIGURE 6. MEAN ROD SCORES FOR ALL FOUR DAY SUBJECTS IRRESPECTIVE OF INJECTION ACCURACY

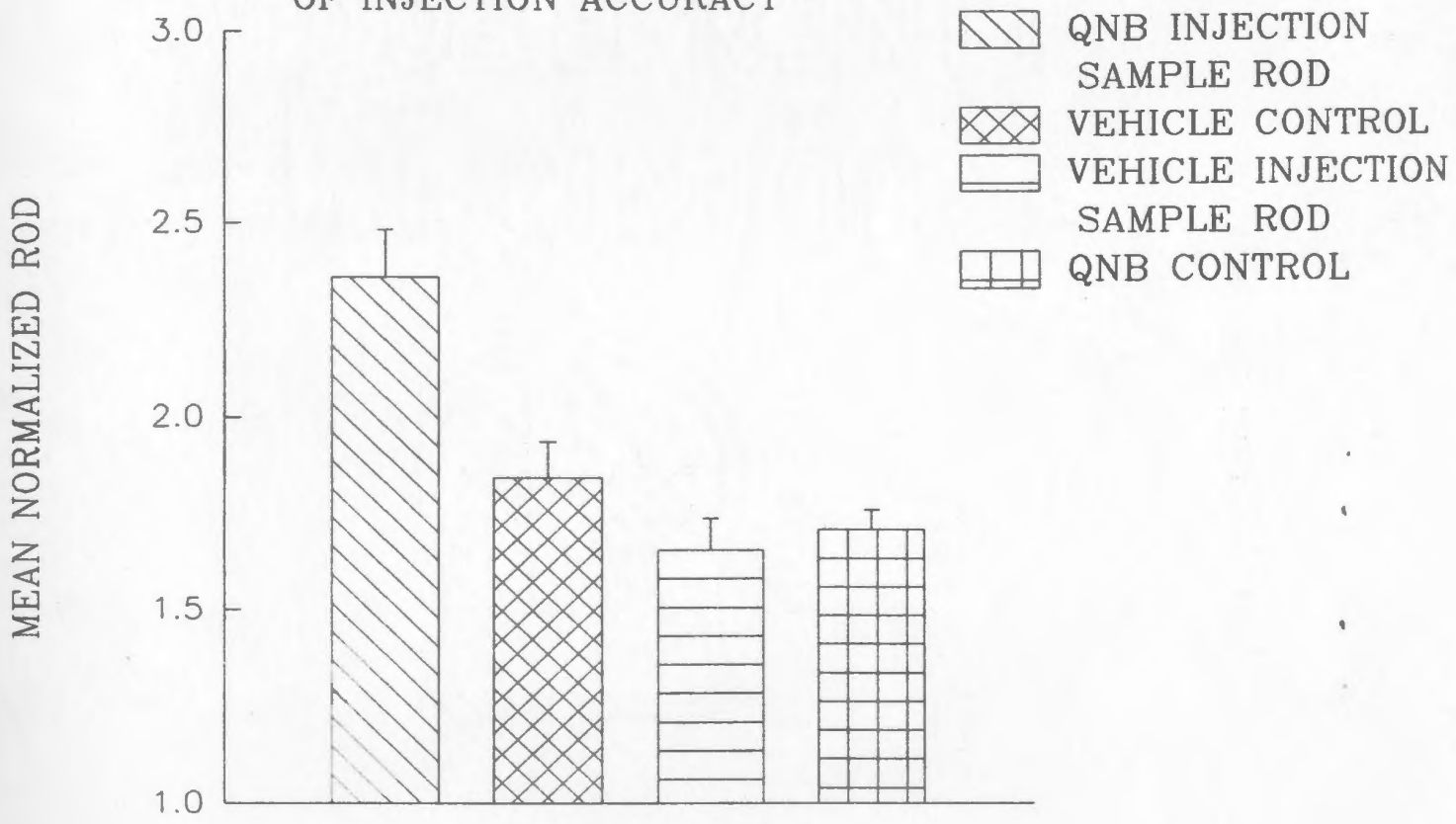
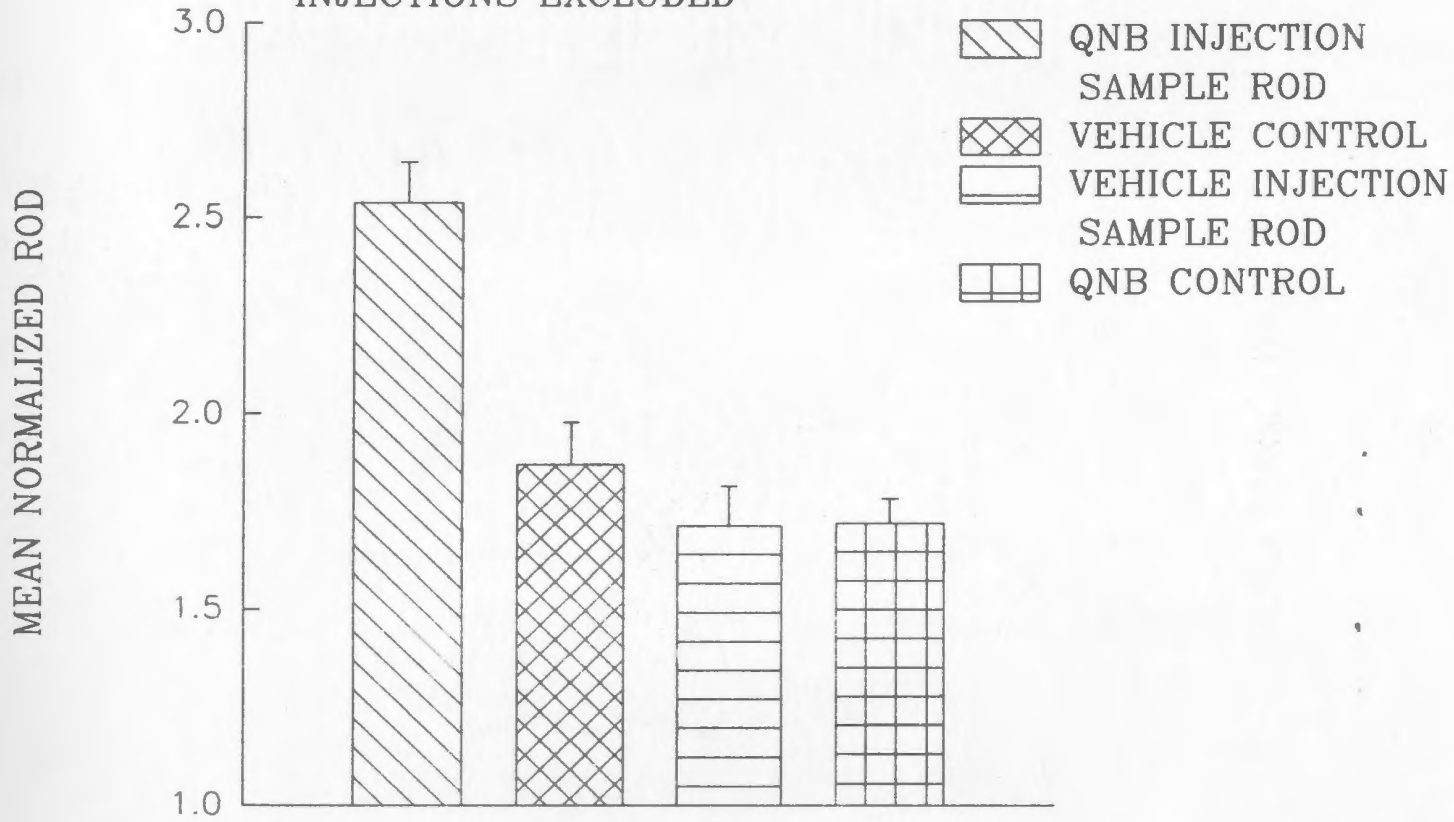


FIGURE 7. MEAN ROD MEASURES OF FOUR DAY SURVIVAL GROUP SUBJECTS WITH QUESTIONABLE INJECTIONS EXCLUDED



questionable injections removed. On average, QNB injected sample ROD values were 134 percent of corresponding vehicle control values.

VEHICLE INJECTED REGIONS: The mean normalized ROD value for sampled regions of vehicle injected hemispheres was 1.65 (SD = .32) with a range of 1.23 to 2.27. Corresponding control sites in the opposite hemisphere had a mean normalized ROD of 1.70 (SD = .20) with a range of 1.36 to 2.16. Figure 6 illustrates the mean vehicle injection and control ROD scores of four day subjects. The mean normalized ROD of vehicle injected samples was 97 percent of QNB injected control counterparts. These calculations were based on the entire pool of vehicle injected hemispheres irrespective of apparent vehicle injection accuracy. With those ROD values of questionable vehicle injections and their corresponding QNB controls removed, the mean normalized ROD of vehicle injected samples was 1.71 (SD = .34) while the same measure of corresponding QNB control samples was 1.72 (SD = .20). The range of ROD values for vehicle injected samples was 1.40 to 2.27 while the range for corresponding control ROD values was 1.52 to 2.16. Figure 7 illustrates the mean ROD values of four day subjects with questionable vehicle injection samples removed from the analysis. Vehicle injection sample ROD values were, on average, 97 percent of control QNB values.

LENGTH OF AChE INCREASES:

Of the 12 subjects demonstrating an increase in AChE positive staining, 15 areas

of increase within the hippocampus were noted. The mean length (anterior to posterior plane) of this increase through the hippocampus was found to be 658.7 μm (SD = 420.0 μm) with a range of 160 μm to 1680 μm.

LENGTH AND AREA OF INCREASE:

Of the 15 areas of AChE stain increase observed, 11 were found to be in the DG. The mean length of increase through this region was 687.3 μm (SD = 464.0 μm) with a range of 160 μm to 1680 μm. Four sites of stain increase were found in area CA1. The mean persistency of the increase was 580 μm (SD = 307.3 μm) with a range of 240 μm to 840 μm.

HEMISPHERE VARIATION:

Of the 12 subjects demonstrating an increase in AChE positive staining, nine received QNB injections in the right hippocampus while three received injections into the left hemisphere hippocampus. This included one right hemisphere injected subject with a questionable injection.

Three subjects receiving an injection of QNB into the left hemisphere yet not demonstrating an AChE stain increase did not have questionable QNB injections. Of two receiving right hemisphere QNB injections and not demonstrating an AChE positive stain increase, one had a questionable QNB injection.

FOURTEEN DAY SURVIVAL GROUP:

GENERAL RESULTS:

Nine animals were included in the 14 day survival group. Three (33.3 percent) demonstrated some degree of AChE positive stain increase in QNB injected hemispheres relative to corresponding vehicle controls. The regions of AChE stain increase correlated with the QNB injection sites. Six subjects did not demonstrate an AChE positive increase at the QNB injection site.

INJECTION ACCURACY:

QNB INJECTIONS: Of the three subjects demonstrating an increase in AChE positive staining, all were judged to possess accurate QNB injection sites.

Of the six subjects with no observed stain increase, two had questionable QNB injections, while four had QNB injections judged to be accurate.

VEHICLE INJECTIONS: Out of the three subjects exhibiting an increase in AChE positive staining two had vehicle injections that were judged to be questionable. Of the six subjects with no observed increase in AChE staining, two were judged to possess questionable vehicle control injections.

AREAS OF STAIN INCREASE:

A total of three subjects were observed to have increased AChE staining. Two

subjects demonstrated an increase in one area while another subject had an increase in two regions. Two areas of stain increase were located in the DG and one each in CA1 (through strata oriens, pyramidal and superficial radiatum) and the cortex respectively. Of the AChE positive stain increases in the DG, one was seen to penetrate the granule cell layer of the lateral blade, an area normally devoid of AChE staining. Figure 8 illustrates 14 day survival subjects demonstrating an AChE positive stain increase.

AREAS OF QNB INJECTION WITH NO OBSERVED INCREASE:

Of the subjects judged to possess accurate QNB injections yet no stain increase, five received an injection into CA1 while one received an injection in SLM lateral to DG.

DIMENSIONAL ANALYSIS:

WIDTH: The mean width (medial to lateral plane) of regions possessing an AChE positive stain increase was .63 mm (SD = .14) with a range of .50 mm to .78 mm.

DEPTH: The mean depth (dorsal to ventral plane) of increased AChE positive stained regions was .35 mm (SD = .31) with a range of .15 mm to .72 mm.

DENSITY ANALYSIS:

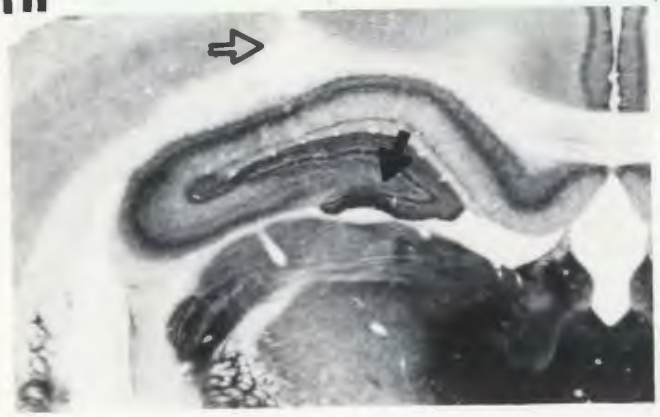
QNB INJECTED REGION SAMPLES: Normalized ROD values of QNB injected hemispheres were taken from regions of greatest stain increase and in the case of no stain increase, from injection sites. The mean normalized ROD value for all nine QNB

Figure 8. Photomicrographs of 14 day subjects with AChE positive stain increases in the medial and lateral blades of the DG (top and middle photomicrographs on the left respectively) and the cortex (bottom left). Adjacent photomicrographs on the right are corresponding vehicle injected hippocampi.

Black arrows indicate AChE positive stain increases and open arrows indicate injection sites or tracks.
The photomicrograph situated in the middle and left demonstrates a clear AChE positive stain increase in the molecular and granule cell layers. The latter is usually devoid of AChE positive fibers.

Abbreviations: RH = right hemisphere
LH = left hemisphere

RH



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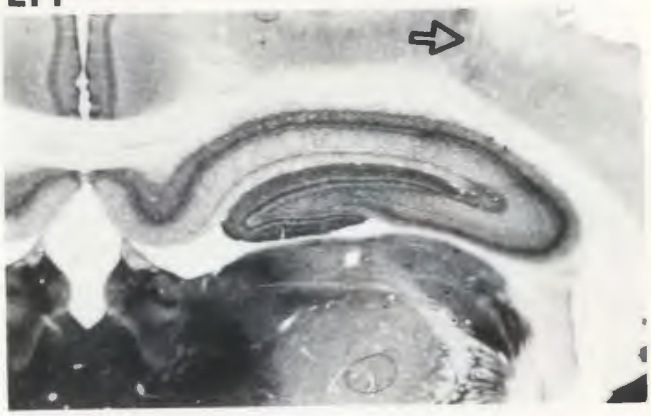

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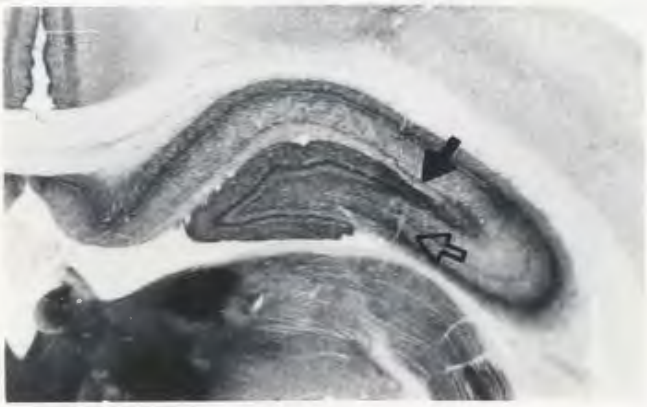
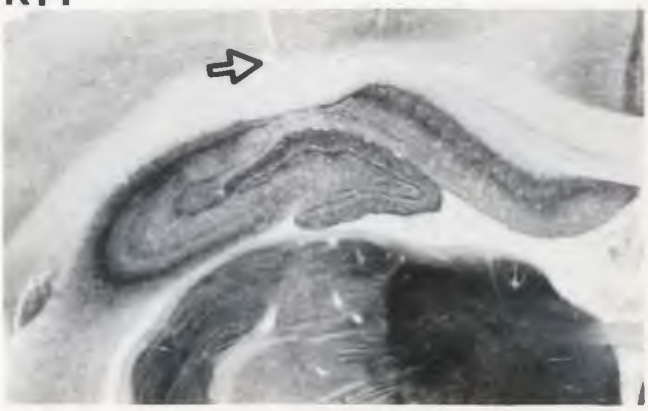


RH



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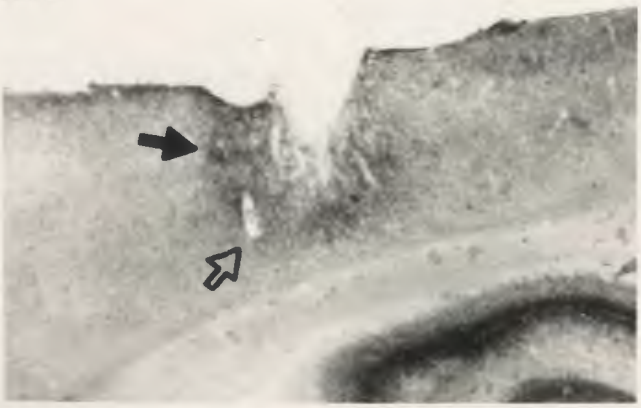
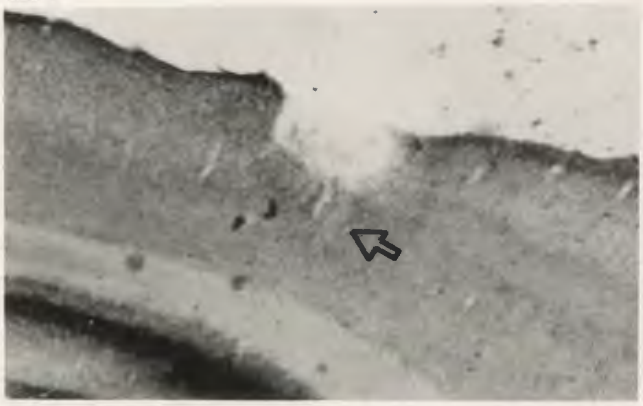


LH



injected hemisphere samples was 1.92 (SD = .37) with a range of 1.49 to 2.64. The same measure of corresponding vehicle control samples was 1.78 (SD = .54) with a range of 1.24 to 2.89. Figure 9 illustrates the mean ROD scores of all subjects irrespective of injection accuracy. The average normalized ROD of QNB injected regions sampled was 119 percent of the corresponding vehicle control samples.

Including only the three subjects demonstrating an AChE staining increase on QNB injected hippocampi, the mean normalized ROD value became, 2.13 (SD = .48) with a range of 1.68 to 2.64. The same measures of the corresponding vehicle control region samples produced a mean ROD of 1.38 (SD = .24) with a range of 1.24 to 1.66. The average ROD value of QNB injected samples observed to possess an increase was 154 percent of corresponding vehicle control samples.

Removing all samples taken from those subjects possessing questionable QNB injections the mean normalized ROD of QNB injected hippocampi was 1.97 (SD = .39) with a range of 1.49 to 2.64. The mean score of corresponding vehicle controls was 1.64 (SD = .39) with a range of 1.24 to 2.34. Figure 10 illustrates the mean ROD scores of 14 day survival subjects with questionable QNB injection results excluded. The average QNB ROD value became, in this case, 118 percent of control values.

VEHICLE INJECTED REGION SAMPLES: The mean normalized ROD value of vehicle injected hemisphere samples was 1.94 (SD = .74) with a range of 1.25 to 3.73. The same calculation for corresponding QNB control samples was 1.95 (SD = .51) with a range of 1.59 to 3.21. Figure 9 illustrates the mean ROD values of all 14

FIGURE 9. MEAN ROD SCORES OF ALL 14 DAY SURVIVAL GROUP SUBJECTS IRRESPECTIVE OF INJECTION ACCURACY

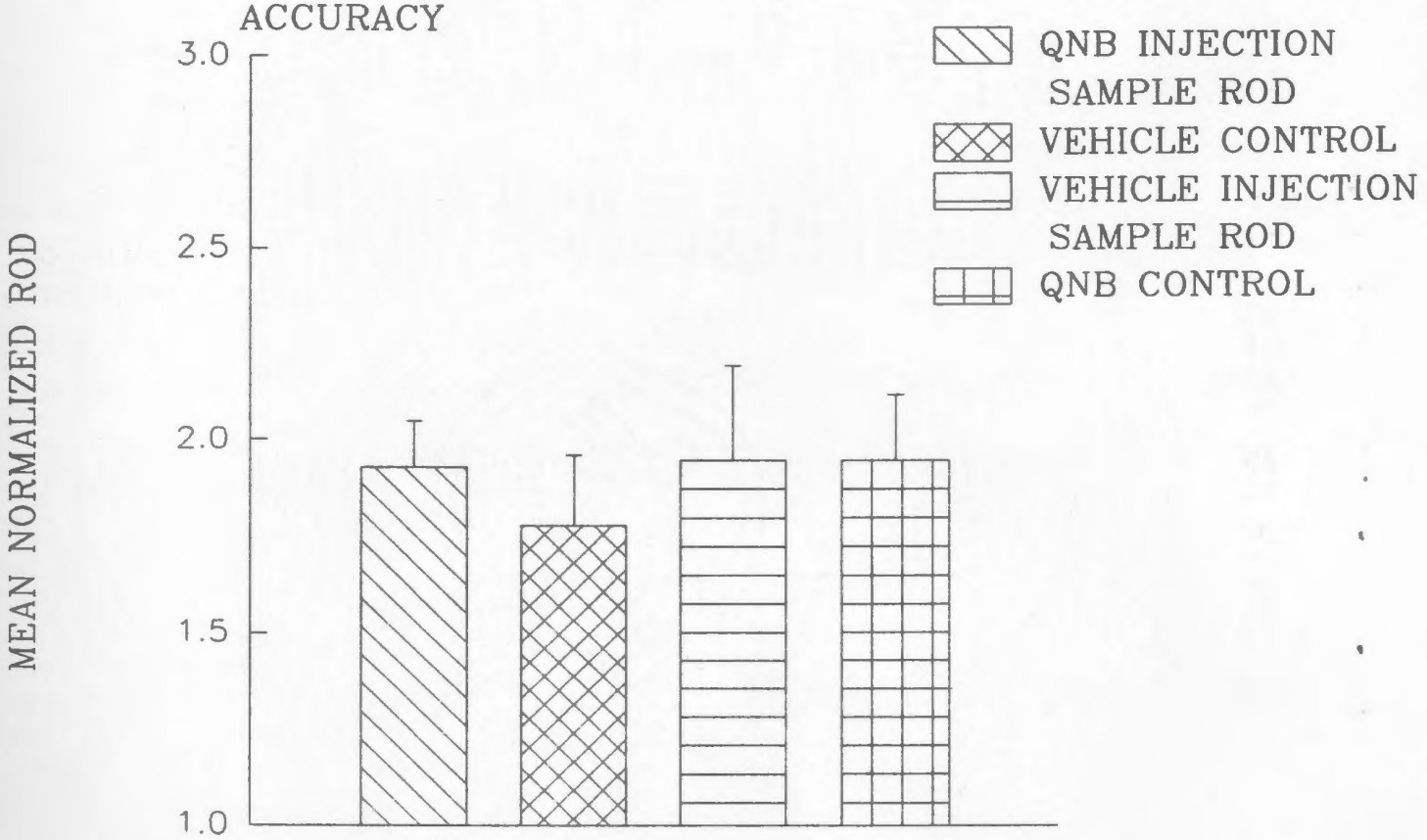
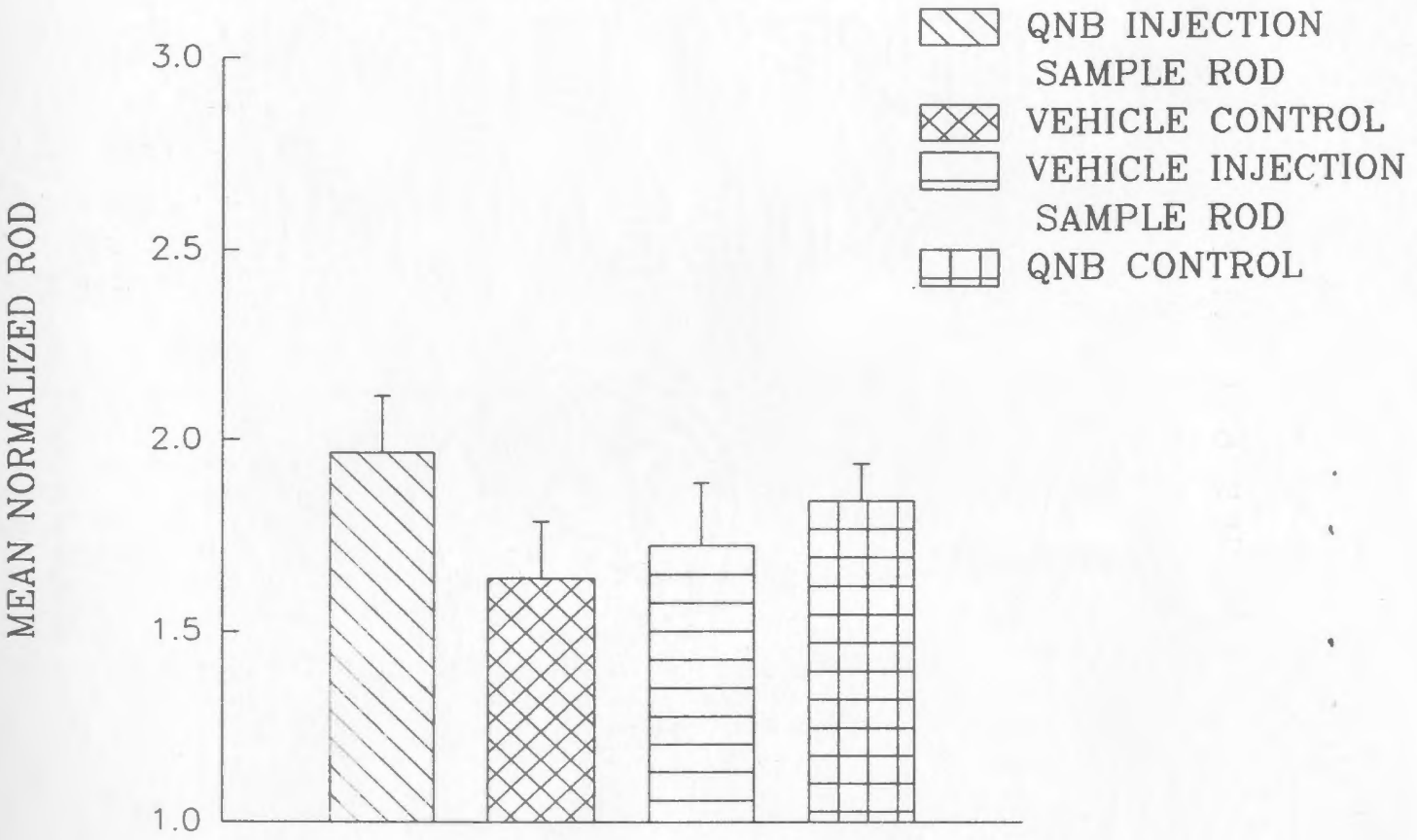


FIGURE 10. MEAN ROD SCORES OF 14 DAY SUBJECTS WITH ACCURATE INJECTIONS ONLY



day subjects. The average vehicle injection normalized ROD value was 99.4 percent of corresponding control values.

Removing the scores of all samples taken from subjects with questionable vehicle injections, the mean normalized ROD of vehicle injected hippocampi was 1.72 (SD = .40) with a range of 1.25 to 2.34. The same measure for corresponding QNB controls was 1.84 (SD = .21) with a range of 1.6 to 2.19. Figure 10 illustrates the mean ROD scores of subjects with questionable vehicle injection scores removed from the analysis. The normalized ROD values of vehicle injection sites sampled were on average, 93 percent of corresponding control values.

LENGTH OF STAIN INCREASE:

The mean length (anterior to posterior plane) of stain increases through the QNB injected hemisphere was 426.7 μm (SD = 220.30 μm) with a range of 280 μm to 680 μm.

HEMISPHERE VARIATION:

Of the three subjects demonstrating an increase in AChE positive staining, one received an injection of QNB into the left hemisphere. The other two received right hemisphere QNB injections.

TWENTY-ONE DAY SURVIVAL GROUP:

GENERAL RESULTS: Eight subjects were included in the 21 day survival group. Of these, none demonstrated an increase in AChE positive staining on QNB or vehicle injected hemispheres.

INJECTION ACCURACY:

QNB INJECTIONS: Six out of eight subjects had QNB injection tracks judged to be in the hippocampus. Two of eight QNB injections were judged to be questionable.

VEHICLE INJECTION: Of the eight subjects, three were judged as possessing questionable vehicle injections. The remaining five were judged to have accurate vehicle injections that entered the hippocampus.

AREAS OF INJECTION:

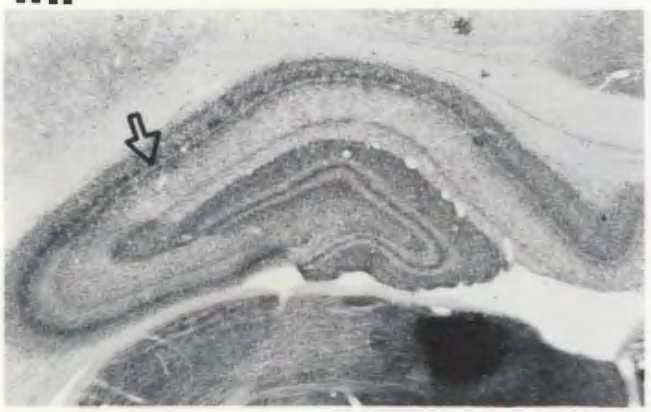
Of six subjects with confirmed QNB injection tracks, one was in SLM, two were in the DG and three were in area CA1.

Of the five accurate vehicle injections, one was found in CA3, two in CA1, one in CA4 and one in DG. Figure 11 illustrates QNB injection and vehicle injection sites of a 21 day survival subject.

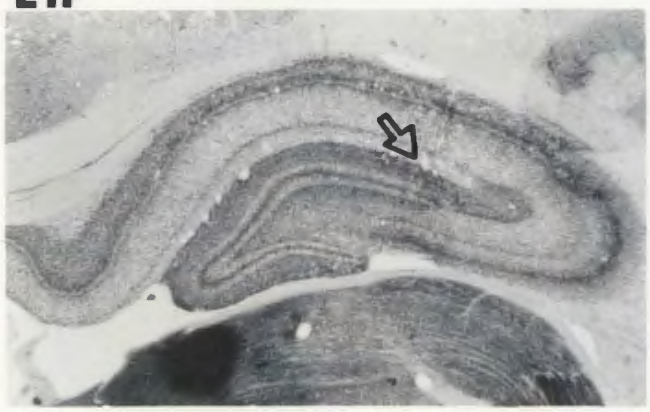
Figure 11. Photomicrographs of QNB injected (left) and corresponding vehicle injected (right) hippocampi of 21 day survival subjects.
Open arrows indicate injection sites where found.

Abbreviations: RH = right hemisphere
LH = left hemisphere

RH



LH



DENSITY ANALYSIS:

QNB INJECTION SAMPLES: The mean normalized ROD value of QNB injected regions of the 21 day survival group was 1.71 (SD = .25) with a range of 1.36 to 2.10. The mean normalized ROD value of corresponding vehicle control samples was 1.67 (SD = .32) with a range of 1.28 to 2.20. Figure 12 illustrates the mean ROD scores of all 21 day subjects. QNB injected regions sampled were on average, 102 percent of corresponding control normalized ROD values.

The above calculations were the result of pooling the image analysis data of all 21 day subjects regardless of QNB injection accuracy. With those samples derived from questionable injections removed from the analysis, the mean normalized ROD of QNB injected samples was 1.63 (SD = .21) with a range of 1.36 to 1.94. The corresponding vehicle control value on the same measure was 1.60 (SD = .29) with a range of 1.28 to 2.14. Figure 13 illustrates the mean ROD values of QNB injected samples with questionable injection scores removed. QNB injection ROD values were on average, 102 percent of corresponding control values.

VEHICLE INJECTION SAMPLES: The mean normalized ROD value of vehicle injected regions of the 21 day group was 1.60 (SD = .26) with a range of 1.34 to 2.20. The same calculation for corresponding QNB control samples was 1.63 (SD = .21) with a range of 1.37 to 2.10. Figure 12 illustrates the mean ROD scores of all 21 day survival subjects. On average, the mean ROD values of vehicle injection samples were 98 percent

FIGURE 12. MEAN ROD SCORES OF ALL 21 DAY SURVIVAL GROUP SUBJECTS IRRESPECTIVE OF INJECTION ACCURACY

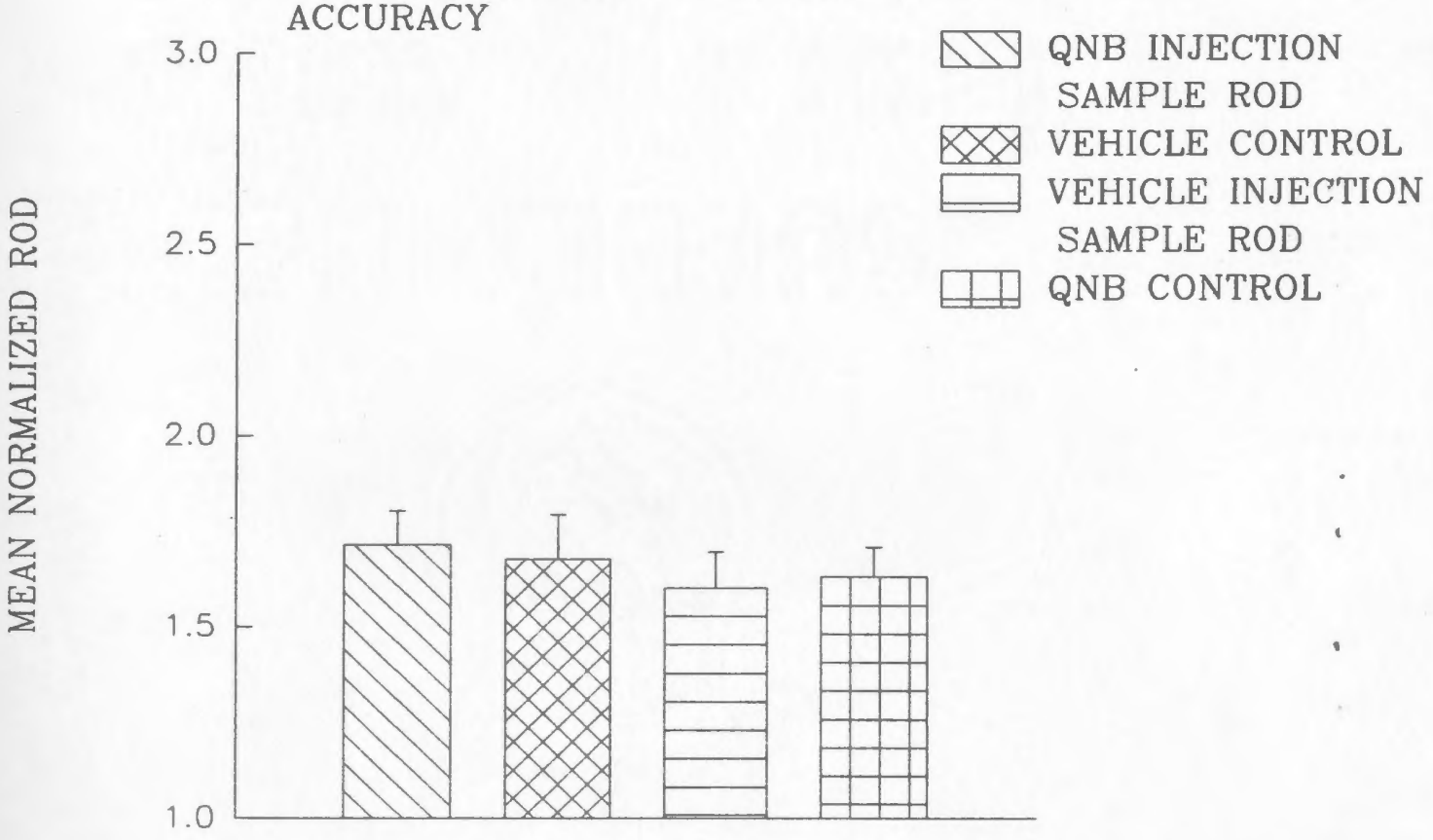
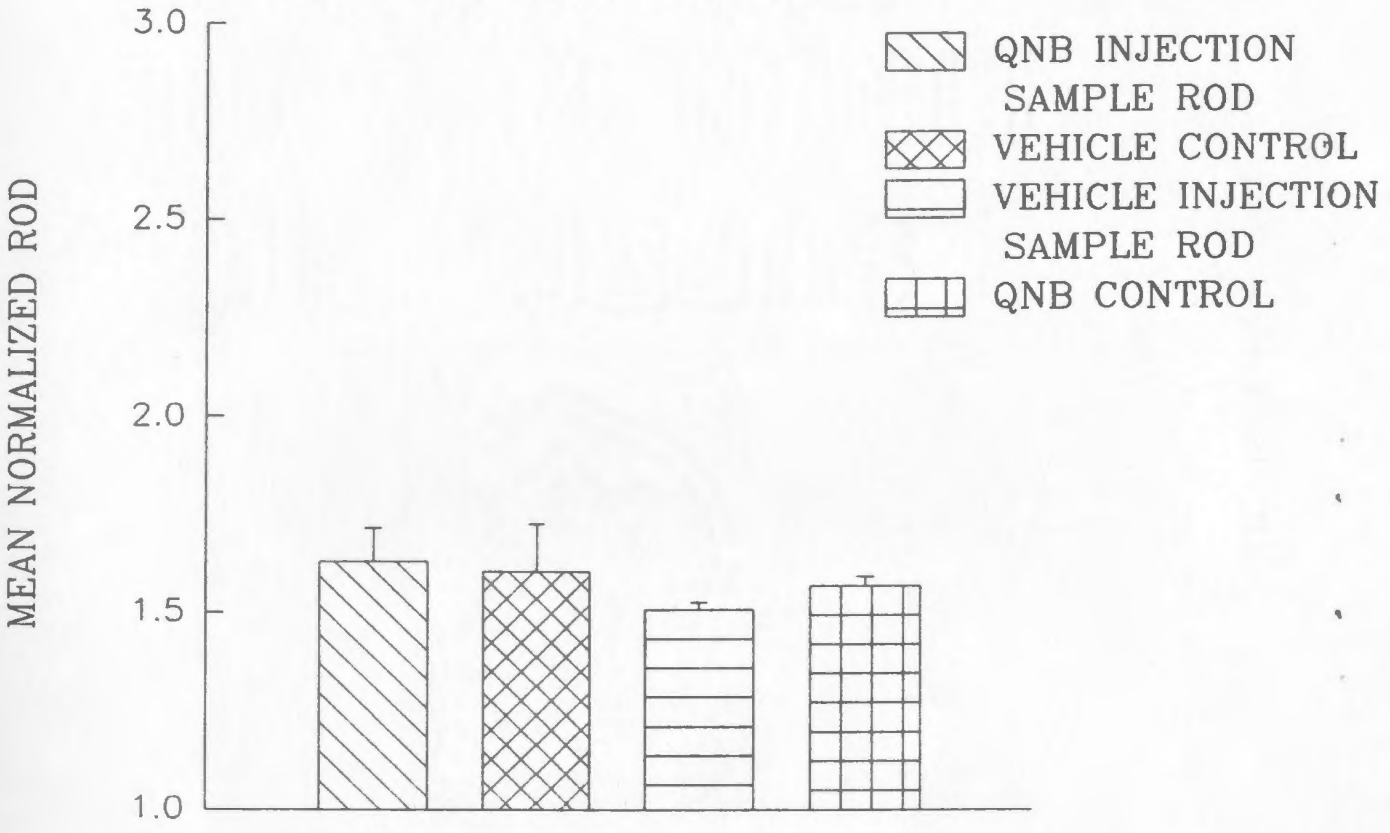


FIGURE 13. MEAN ROD SCORES OF 21 DAY SURVIVAL SUBJECTS WITH QUESTIONABLE INJECTION DATA EXCLUDED FROM ANALYSIS



of corresponding control values.

When the ROD values of subjects with questionable vehicle injections were removed from the analysis, the mean ROD value for vehicle samples was 1.51 (SD = .04) with a range of 1.46 to 1.57. Corresponding QNB control samples had a mean normalized ROD value of 1.56 (SD = .05) with a range of 1.52 to 1.65. Figure 13 illustrates the mean ROD scores of subjects with the results of questionable vehicle injections removed. Vehicle regions sampled have an average normalized ROD value that was 96 percent of corresponding QNB control ROD values.

HEMISPHERE VARIATION:

Two of eight subjects in the 21 day survival group received QNB injections into the left hippocampus while the other six received injections into the right hippocampus. Neither the left or right QNB injected hemispheres demonstrated an AChE positive stain increase.

EXPERIMENT TWO

INTRODUCTION

Thus far, the present study has demonstrated that prolonged muscarinic receptor blockade can induce AChE positive staining increases in the hippocampus of female adult rats of four and 14 day survival groups but not in one and 21 day survival groups. While this is true, the results suggest a degree of variability such that there were a number of subjects with clear QNB injections that did not demonstrate an AChE positive stain increase. There were also a large range of results on the dimensional analysis measures undertaken to define the extent of staining increases when they occurred. It was decided that this could possibly have been due to the QNB concentration used. Perhaps, the effect is dependent on enough muscarinic receptors being antagonized in a given area. While a 20 μg QNB concentration in 2 μl of vehicle was obviously adequate for the most part (70.5 percent of four day subjects in experiment one), it may be that coupled with other variables such as the location of targets, this QNB concentration was not as effective as a higher one may be. This possibility was investigated in experiment two.

The results of experiment one can only be generalized to a population of female rats of the Sprague Dawley strain. The possibility of sex related effects regarding responses to prolonged muscarinic blockade is not expected but at the same time, not immediately dismissable.

A concern when using female subjects is the occurrence of estrous effects on the

measure(s) under study. With the exception of the one day survival group, the manipulations of experiment one lasted at least a complete estrous cycle. Because of this it is likely that the estrous cycle was not a concern. The probability exists that periods of estrous were spread out randomly across each experiment such that the impact of its occurrence did not effect one group more than another. In experiment one, no effort was made to determine the stage of the estrous cycle rats under study were in. As well as assessing the effect of a higher concentration of QNB, experiment two attempted to address the concern of possible sex related differences regarding the increased AChE positive staining response to prolonged muscarinic antagonism. Further, the hemisphere of QNB treatment was also monitored as a possible source of staining differences in response to QNB injection.

It was predicted in experiment two that the 20 µg/2 µl dosage will induce AChE positive staining increases within treated hippocampi. It was also predicted that a higher dose (40 µg/2 µl) would, as well, induce an increase in staining and that this increase would be denser than increases produced by the lower dose. With respect to the sex of treated animals or the hemisphere of treatment, no differences were expected.

EXPERIMENT TWO

METHOD.

Subjects: The subjects used in experiment two were four male and four female Sprague Dawley rats. See experiment one methods section for details of weight, storage and maintenance.

Procedure: The subjects of this experiment were treated as per the procedure of experiment one with the following considerations. Half of each group of males and females received the usual 20 μg concentration of QNB in a 2 μl volume while the others received 40 μg of the drug in an equal volume. In half of the cases, QNB was injected into the right hemisphere while in the other half, QNB was injected into the left hemisphere. In all cases, the vehicle was injected into the hemisphere opposite to that in which QNB was injected.

STATISTICS: A statistical analysis was conducted on the data of experiment two. There were insufficient data points per treatment (sex, QNB concentration, hemisphere) to merit a comprehensive analysis of variables together; therefore, the variables were assessed individually. Student paired t-tests were computed for sex, QNB dose and hemisphere data with respect to density measure scores. Analysis of QNB injection ROD samples compared to vehicle injection ROD samples were reported in the "summary results" section along with other analyses that attempted to identify and characterize statistically the effect of the treatment both between and within the groups.

EXPERIMENT TWO RESULTS:

GENERAL RESULTS:

Eight animals sacrificed four days following QNB/vehicle treatment were used in this experiment. Of the eight, five (62.5 percent) demonstrated an AChE positive stain increase in QNB injected hippocampi relative to corresponding vehicle controls. Figure 14 illustrates representative AChE positive stain increases seen in experiment two.

INJECTION ACCURACY:

QNB INJECTIONS: Of the five subjects demonstrating an AChE positive stain increase, four were judged to have accurate QNB injections while one was not. Three subjects of the group were not found to demonstrate an AChE positive stain increase. One of these had a QNB injection judged to be reliable while the other two did not.

VEHICLE INJECTION. Of eight subjects included in the analysis, two were judged to have questionable vehicle injections. Of these, one was observed to demonstrate a stain increase in the QNB injected hippocampus.

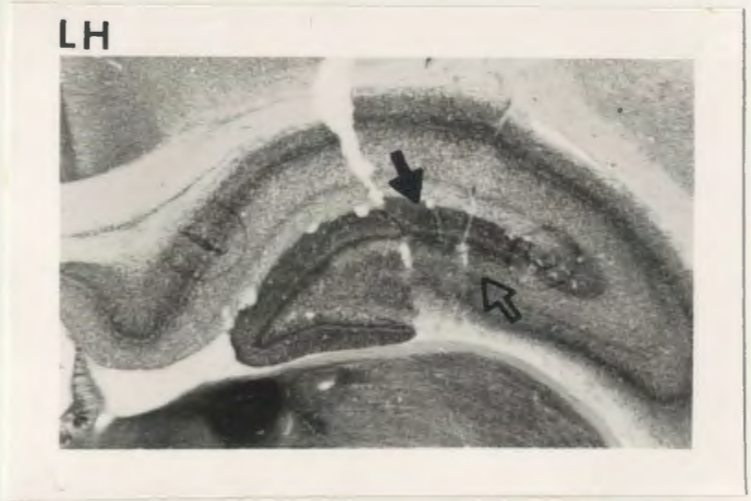
GENDER, DOSE, HEMISPHERE DIFFERENCES:

FEMALE/DOSE/HEMISPHERE: Of the four females used in this experiment, two demonstrated an increase in AChE positive staining. One of the two subjects exhibiting a positive stain increase received a 20 μg dose of QNB while the other

Figure 14. Photomicrographs of QNB (20 μg/2 μl) injected (top) and corresponding vehicle injected hippocampi of an experiment two 4 day male subject (Note: tear occurred during the mounting process).

Black arrow indicates AChE positive stain increases and the open arrows indicate injection sites or tracks. The DG in the QNB injected hippocampus (top) demonstrated darker AChE positive staining than the corresponding vehicle treated hippocampus. The white bands seen in the vehicle side molecular layer are absent in the treated hippocampus.

Abbreviations: RH = right hemisphere
LH = left hemisphere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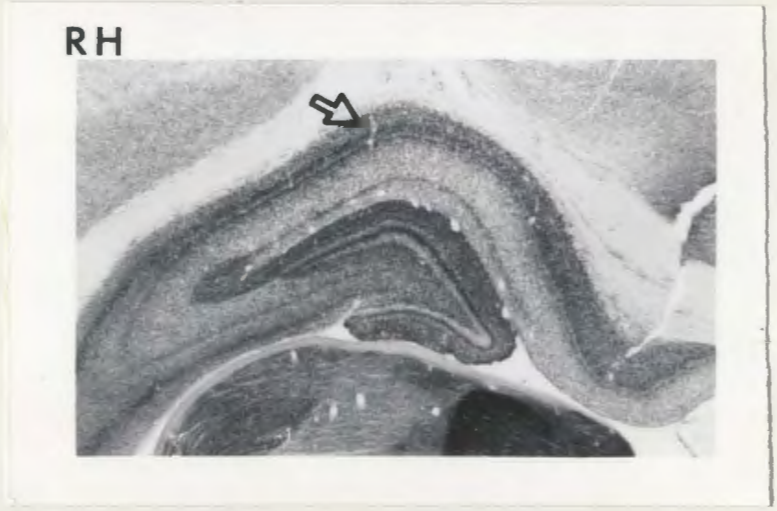

RH

received a 40 μg dose. Of the two instances of increase, one QNB injection was located in the right hippocampus while the other was in the left.

MALE/DOSE/HEMISPHERE: Of the four males used, three (75 percent) were found to demonstrate an AChE positive stain increase in the QNB injected hippocampus relative to corresponding controls. Of the three positive responses, two received 40 μg doses of QNB while the third received a 20 μg dose. Two of the increases were observed in the right hippocampus while one was observed in the left. Table 2 illustrates the results of experiment two by subject, sex, dose, hemisphere of injection, staining effect and injection accuracy.

AREAS OF STAINING INCREASE:

Of five subjects observed to demonstrate an increase in AChE positive staining in QNB injected hippocampi, six distinct areas demonstrating this increase were observed. Of these, two were in the DG (molecular layer and granule cell layer) and four were in area CA1 (through strata oriens, pyramidale and superficial radiatum).

Of the subjects not seen to exhibit an AChE stain increase, one had a definite QNB injection into the DG while two others had questionable QNB injections.

DIMENSIONAL ANALYSIS:

WIDTH: The mean width (medial to lateral plane) of AChE positive stain

Table 2. Experiment two results table.

SUBJECT	DOSE	SEX	QNB INJECTION	HEMISPHERE	AchE STAIN
1	40μg	F	IN	R	YES
2	40μg	F	IN	L	NO
3	40μg	M	IN	R	YES
4	40μg	M	?	L	YES
5	20μg	F	IN	L	YES
6	20μg	F	?	R	NO
7	20μg	M	IN	R	YES
8	20μg	M	?	L	NO

For each subject used in the experiment, the QNB dose (20μg/40μg), the subject's sex (M/F), the accuracy of QNB injection (IN/?), the hemisphere of injection (R/L) and the occurence of an AchE positive stain increase (YES/NO) are illustrated.

ABBREVIATIONS: F=female, M=male, IN= accurate QNB injection, ?=questionable QNB injection, R=right, L=left, YES=stain increase seen, NO=no stain increase seen.

increases in QNB injected hippocampi of experiment two was .54 mm (SD .46 mm) with a range of .14 mm to 1.44 mm.

DEPTH: The mean depth (dorsal to ventral plane) of AChE positive stain increases in QNB injected hippocampi was .14 mm (SD .0683) with a range of .06 mm to .22 mm.

IMAGE ANALYSIS:

QNB INJECTED HIPPOCAMPI SAMPLES: The mean normalized ROD value of QNB injected samples was 2.01 (SD = .44) with a range of 1.17 to 2.62. The same calculation for corresponding vehicle control samples yielded a mean of 1.54 (SD = .28) with a range of 1.26 to 2.02. Figure 15 illustrates the mean ROD scores of all experiment two subjects irrespective of QNB injection accuracy. The average QNB injected ROD value was 131 percent of vehicle control sample values.

The above analysis includes all QNB injected subject samples regardless of injection accuracy. With the scores of subjects possessing questionable QNB injections removed, the mean ROD value became 2.05 (SD = .24) with a range of 1.72 to 2.34. The mean of corresponding vehicle control samples became 1.50 (SD = .226) with a range of 1.27 to 1.84. Figure 16 illustrates the mean ROD scores of experiment two subjects with questionable QNB injections removed from the analysis. The average QNB injected ROD value is 136 percent of vehicle control sample ROD values.

If only subjects demonstrating An AChE positive stain increase were included in

FIGURE 15. MEAN ROD SCORES OF EXPERIMENT 2 SUBJECTS IRRESPECTIVE OF INJECTION ACCURACY

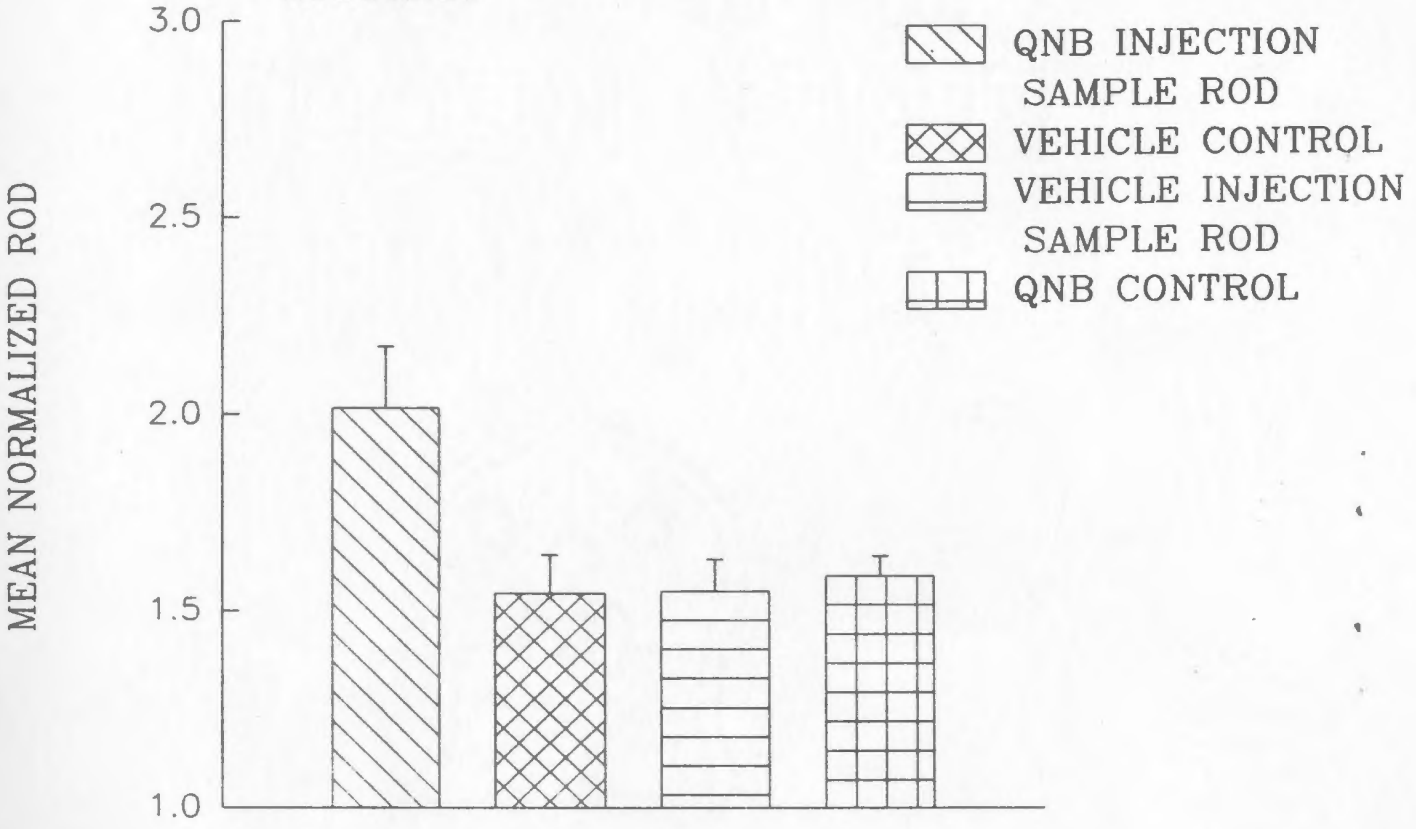
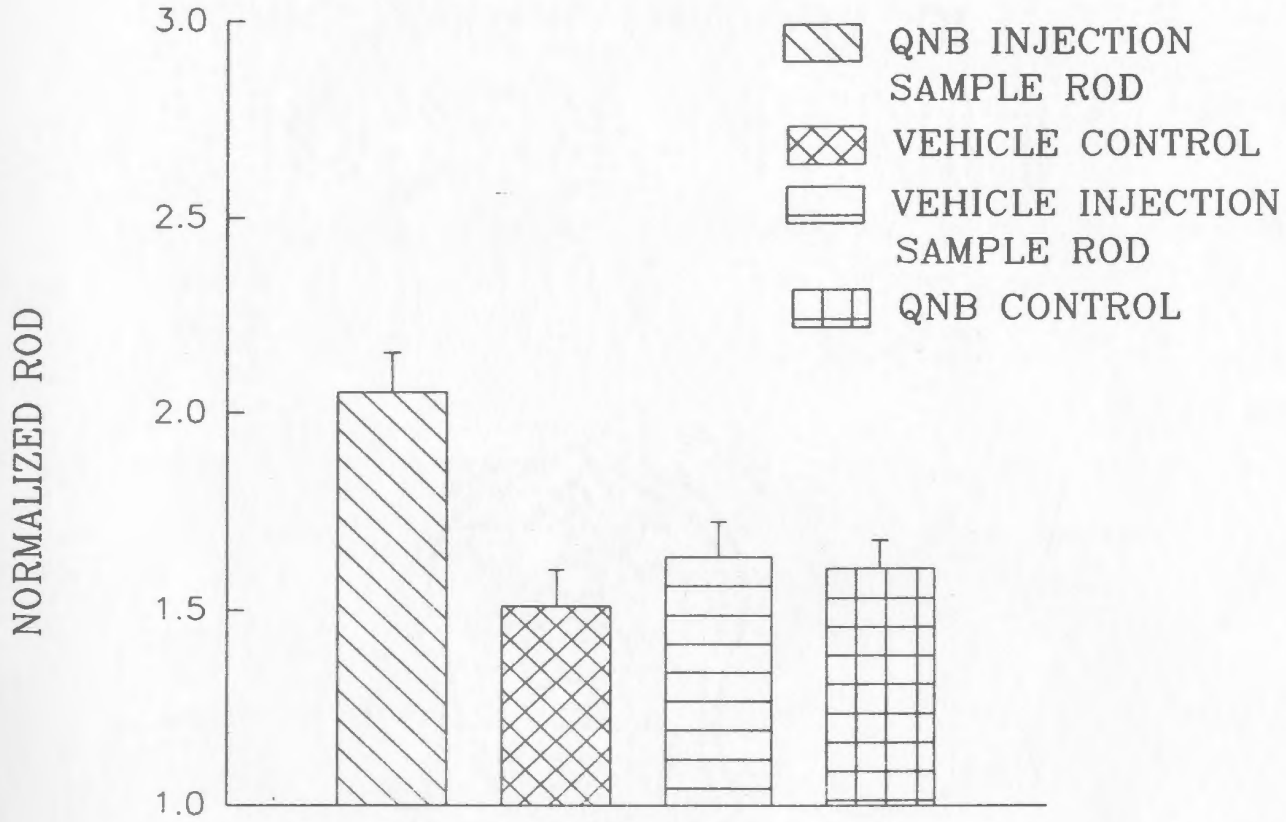


FIGURE 16.MEAN ROD SCORES OF EXPERIMENT 2 SUBJECTS WITH ACCURATE INJECTIONS



the analysis, the mean normalized ROD was 2.13 (SD = .34) with a range of 1.72 to 2.62. The same measure of corresponding vehicle controls was 1.58 (SD = .30) with a range of 1.27 to 1.84. The QNB injected samples are on average 135 percent of corresponding vehicle control values.

VEHICLE INJECTION SAMPLES: The mean normalized vehicle injection sample ROD was 1.54 (SD = .21) with a range of 1.31 to 1.96. The same calculations of the QNB control samples yielded a mean of 1.58 (SD = .13) with a range of 1.37 to 1.83. Figure 15 illustrates the mean ROD scores of experiment two subjects irrespective of vehicle injection reliability. On average, vehicle injection samples possessed a normalized ROD value which was 98 percent of corresponding controls.

The above calculations were based on the image analysis results of all animals pooled. If the questionable vehicle injection scores were removed from the analysis, the mean ROD of vehicle injection samples became 1.63 (SD = .19) with a range of 1.43 to 1.96. The mean normalized ROD value of corresponding QNB control samples became 1.60 (SD = .16) with a range of 1.37 to 1.83. Figure 16 illustrates the mean ROD scores of subjects with questionable vehicle injection sample scores removed. On average, vehicle injected samples had a normalized ROD value that was 102 percent of corresponding QNB control sample ROD values.

LENGTH OF OBSERVED AChE POSITIVE STAIN INCREASES:

Of the five animals demonstrating the AChE positive stain increase, the mean length (anterior to posterior plane) through the hippocampus was 526.7 μm (SD = 530.3 μm) with a range of 80 μm to 1520 μm.

AREA OF INCREASE AND LENGTH:

DG: Of the three sites of observed AChE increase in the DG, the mean length was 760 μm (SD = 723.3 μm) with a range of 80 μm to 1520 μm.

CA1: Three regions of stain increase observed in CA1 had a mean length of 293.3 μm (SD = 128.6) with a range of 200 μm to 440 μm.

STATISTICS:

Sex: A Student paired t-test was conducted to determine if a difference existed on the QNB injection ROD measure with respect to sex of the subjects. No differences were found between male and female subjects in experiment two with respect to QNB injection ROD measures (t = -1.66).

HEMISPHERE: A Student paired t-test was computed to determine if differences existed between hemispheres with respect to QNB injection sample ROD values. No significant differences were found on the QNB injection ROD measure between hemispheres of QNB treatment (t = -1.88).

SUMMARY ANALYSIS

AChE POSITIVE INCREASES: Figure 17 illustrates the percent of subjects in the respective groups that demonstrated an increase in AChE positive staining in the QNB injected hemisphere. Two survival time periods were found to show a stain increase while two did not. Both four (experiment one and two) and 14 day groups demonstrated a degree of staining increase in QNB injected hippocampi while 21 and one day groups did not. In the 14 day group, 33.3 percent of subjects displayed a stain difference favoring the QNB injected hippocampus. Of all subjects in the four day (experiment one) group, 70.6 percent of subjects demonstrated an AChE positive stain increase. A total of 62.5 percent of subjects demonstrated a stain increase in the four day group of experiment two. If the results of all subjects of the four day sacrifice condition are pooled from experiments one and two, then 68 percent of all four day subjects demonstrated some degree of AChE stain increase in QNB injected hippocampi.

INJECTIONS VERSUS CORRESPONDING CONTROLS:

QNB INJECTION: Figure 18 illustrates the QNB injection sample ROD values as a percent of corresponding vehicle control ROD values. In this analysis only subjects with accurate injection sites were included. One day QNB injection samples on average had ROD values 101 percent of control scores. Four day (experiment one) QNB injection

FIGURE 17.PERCENTAGE OF SUBJECTS FROM 4 AND 14 DAY SURVIVAL GROUPS DEMONSTRATING A STAIN INCREASE

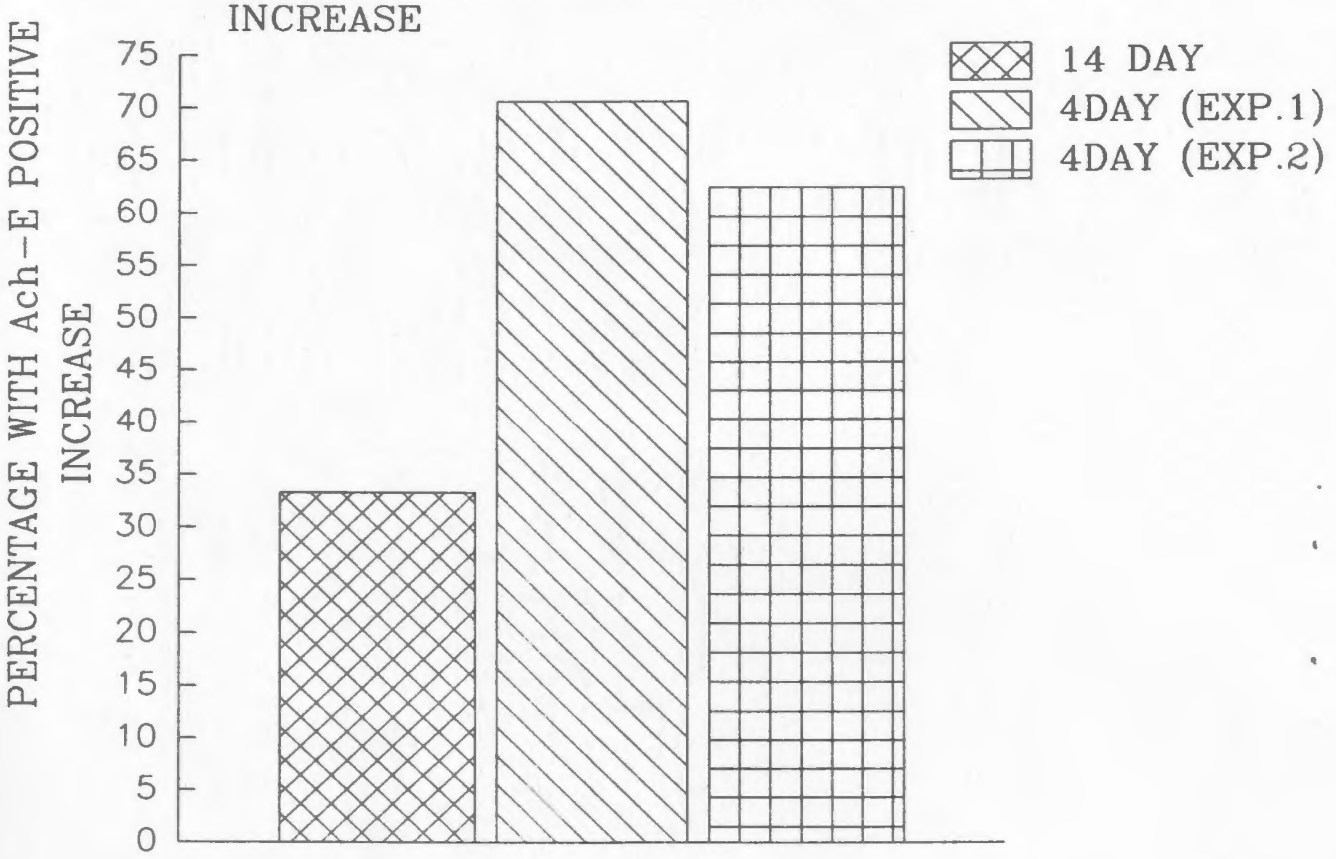
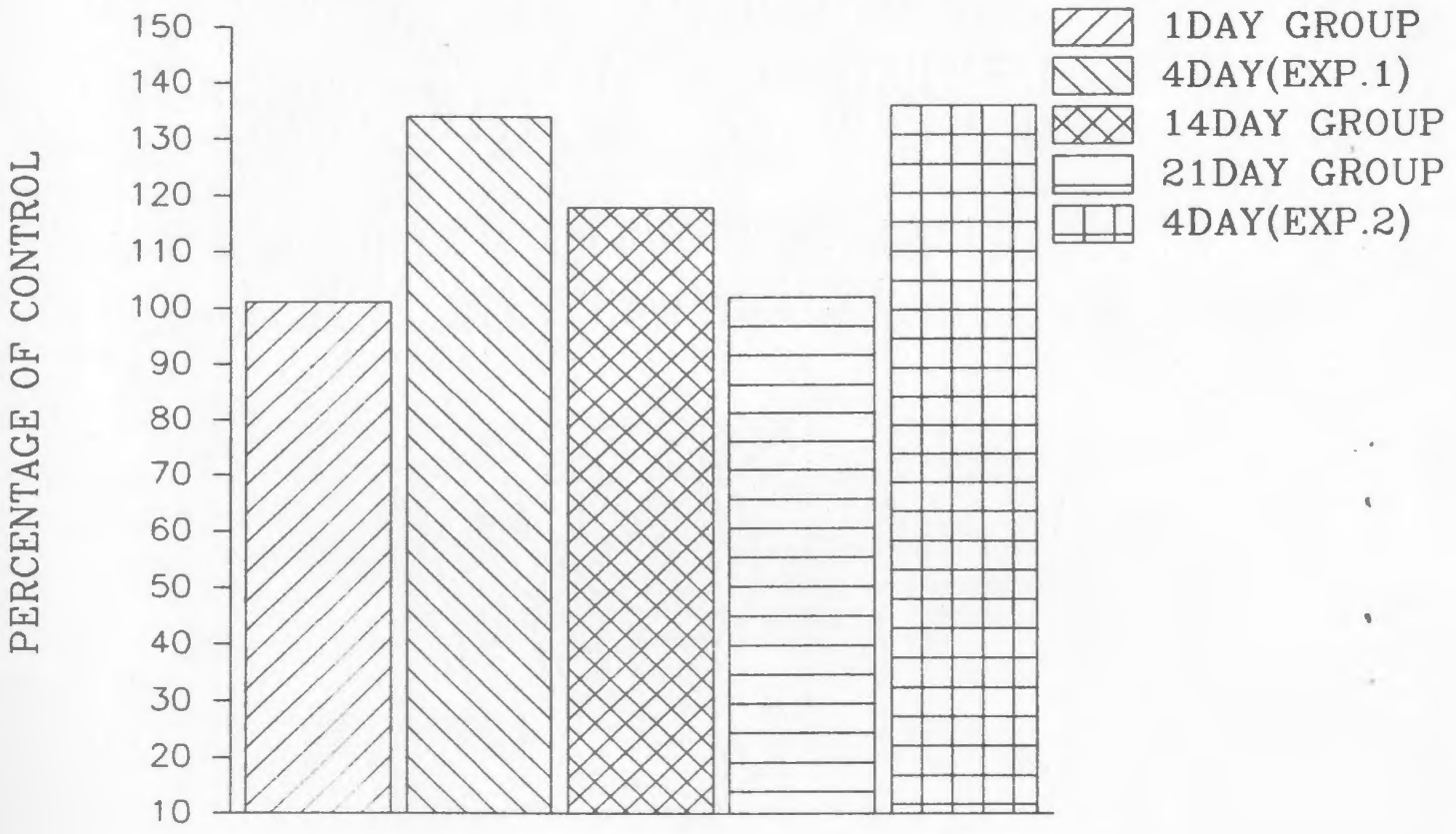


FIGURE 18. QNB INJECTION SAMPLE ROD VALUES AS A PERCENT OF CORRESPONDING VEHICLE CONTROL VALUES.



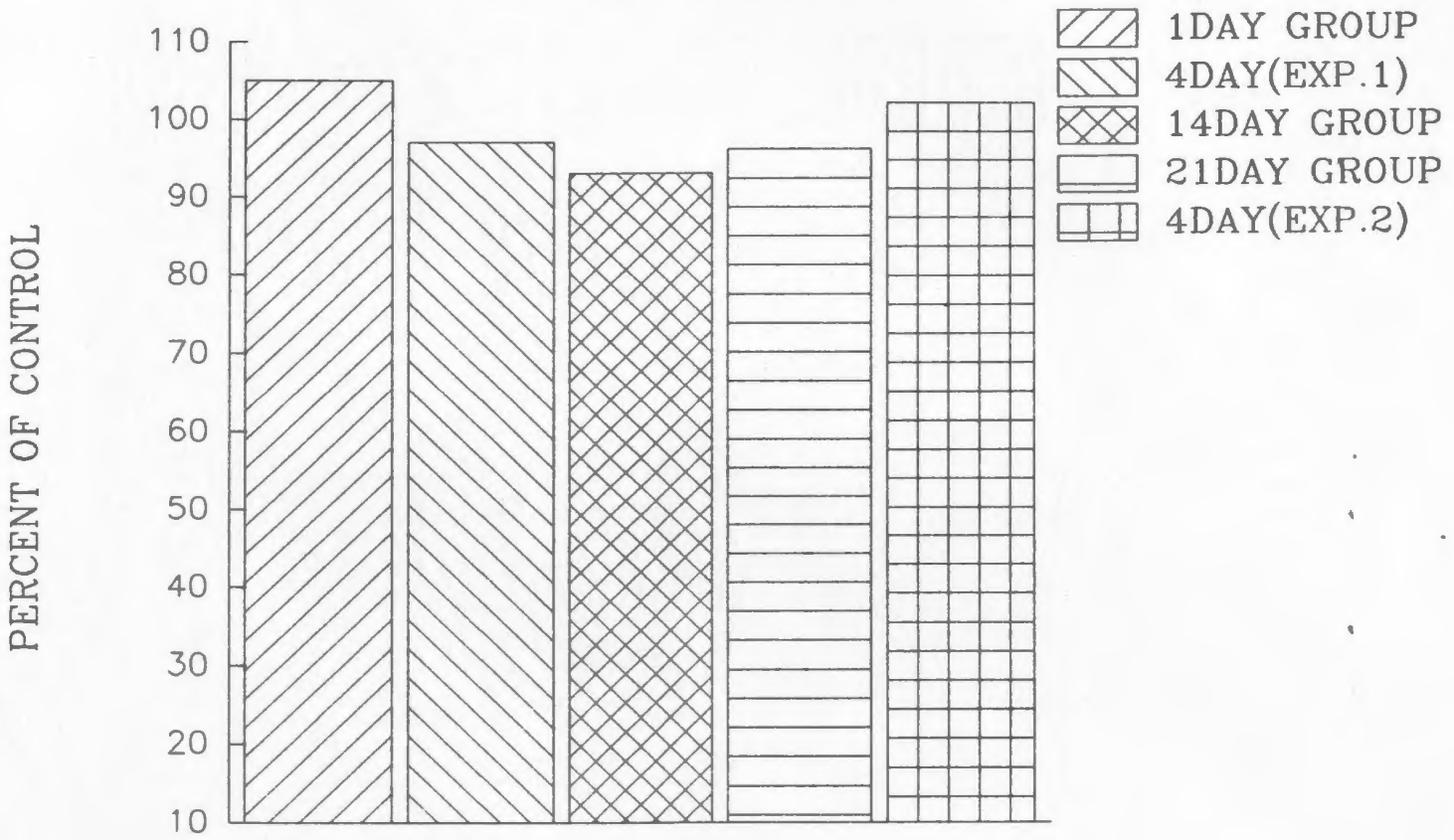
samples were found to be 134 percent of control values while four day (experiment two) QNB injection values were 136 percent of control values. Fourteen day QNB injection samples were found to be, on average, 118 percent of control sample ROD values while 21 day QNB injected samples were found to be 102 percent of control levels.

VEHICLE INJECTION: Figure 19 illustrates the vehicle injection ROD values as a percentage of their corresponding QNB control values. Again, only subjects with injections judged to be accurate were included. One day vehicle injection samples were found to be 105 percent of corresponding control values. Four day (experiment one) vehicle injection ROD samples were 97 percent of corresponding QNB control samples. Four day (experiment two) vehicle injected samples were found to be 102 percent of QNB control ROD scores. Fourteen and 21 day vehicle injection samples taken were determined to be 93 and 96 percent of QNB control values respectively.

STATISTICAL ANALYSES:

CONTROL FOR QNB INJECTION: For each subject in this study, the density analysis results consisted of four measures as outlined in experiment one methods section. This includes one QNB injection sample ROD, a vehicle injection sample ROD and two injection control sample ROD values (QNB control and vehicle control). The existence of this number of controls (3) makes data analysis cumbersome. It was decided therefore to compare QNB injection samples with vehicle injection samples only. To

FIGURE 19.VEHICLE INJECTION SAMPLE ROD VALUES AS A PERCENT OF CORRESPONDING QNB CONTROL SAMPLES



justify this, comparisons were calculated between a pooled (across all groups) vehicle injection sample mean and the pooled injection control sample mean using the Student t-test for dependent samples. The pooled vehicle injection ROD samples did not significantly differ from the means of the two injection control ROD values. The pooled vehicle injection ROD (mean = 1.67 , SD = .33) was not found to be significantly different from its control (mean = 1.76 , SD = .421)(t = -1.20). The pooled vehicle injection ROD (mean = 1.67 , SD = .333) was also not found to differ significantly from the QNB injection control ROD (mean = 1.67 , SD = .243 ; t = -.39). Because the vehicle injection samples did not differ significantly from the injection controls, all comparisons requiring a control value used the vehicle injection sample ROD.

BETWEEN GROUP ANALYSIS:

A statistical analysis of the density results of all subject scores from experimental and control conditions between groups (survival times) was conducted. This analysis included the normalized ROD data of all subjects of each group that demonstrated accurate QNB or vehicle injections. If the effects of QNB and the vehicle were to be evaluated in this study, then those subjects demonstrating that the injections of each were accurate best served the analysis. If those subjects with questionable injections were included, then a measure of the treatment effect would not truly have been assessed since these subjects may not have received the treatment.

QNB INJECTION ROD SAMPLES BY GROUP:

A one way ANOVA was conducted to analyze the QNB injection sample ROD results by group. This analysis produced significant results ($f(4,36) = 4.10$, $p = .007$) indicating that there was a significant difference between groups with respect to the QNB injection ROD measure. To identify the source of this difference, post hoc comparisons were conducted using the Tukey test. Significant differences ($p < .05$) were found, with respect to the QNB injection ROD scores, between the four day survival group of experiment one and the one and 21 day survival groups. The four day survival group of experiment one was not found to be significantly different from the four day group of experiment two or the 14 day group.

VEHICLE INJECTION ROD SAMPLES BY GROUP:

A one way ANOVA was conducted to analyze the vehicle injection sample ROD scores by group. As expected, the groups did not differ with respect to vehicle injection ROD scores ($f(4,27) = .397$).

HEMISPHERE DIFFERENCES:

QNB INJECTION ROD VALUES BY HEMISPHERE:

A MANOVA was conducted to analyze ROD measures of QNB injected samples with respect to the independent variables of treatment hemisphere and group. No statistical difference was found to exist between QNB injection ROD values obtained from

right and left hemispheres for each group. That is, there were no group differences on the QNB injection ROD measure with respect to hemisphere. ($F(3,32) = .79$)

VEHICLE INJECTION ROD VALUES BY HEMISPHERE:

A MANOVA was conducted to assess vehicle injection ROD values with respect to the independent variables of treatment hemisphere and group. No significant differences were found to exist between vehicle injection ROD samples collected from left or right hemispheres.($F(3,23) = 1.60$)

WITHIN GROUP ANALYSIS:

A student paired t-test was conducted on QNB injection ROD scores versus vehicle injection ROD scores of each group to determine if a difference existed between the two means. No significant difference was found between the QNB and vehicle injection samples of the one day group ($t = .89$). Within the four day survival group of experiment one, a significant difference was found to exist between QNB injection and vehicle injection ROD samples ($t = 2.90$, $p = .018$). Within the four day group of experiment two, the mean ROD values of the QNB and vehicle injection scores were found to be significantly different ($t = 9.98$, $p = .002$). The QNB and vehicle injection ROD mean values were not found to be significantly different within the 14 day survival group ($t = .89$). The 21 day survival group mean ROD values were not found to be significantly different between the QNB and vehicle injection samples ($t = 1.31$).

DISCUSSION.

The results of the present study support the hypothesis that prolonged muscarinic receptor antagonism by QNB is a sufficient stimulus for the elicitation of AChE positive fiber outgrowth near sites of injection in rat hippocampi.

Time course:

The time course for the occurrence of AChE positive staining increases in experiment one was determined by sacrificing animals at one, four, 14 or 21 days after treatment with QNB. Evidence of sprouting was observed at four and 14 days but not at one or 21 days following treatment. The observed AChE stain increases were demonstrated more often in the four day group (68 percent of subjects) while a less frequent AChE positive increase could be seen in 14 day animals (33.3 percent of subjects). The length (anterior to posterior plane) of the AChE positive stain increases through hippocampal tissue was greatest in the four day versus the 14 day subjects and the mean density of AChE stain increases were greater in four than 14 day subjects although this difference was non significant.

There is no literature addressing the time course of antagonist induced sprouting in the CNS. However, the optimal sprouting time of four days described in the present study is within the time frame of sprouting responses seen in other hippocampal sprouting

paradigms. It has been suggested that the initiation of sprouting within the CNS requires one to five days in the adult (Cotman et al., 1984 ; Gage et al., 1983). Two to four days following electrolytic lesion of the perforant path, Benowitz et al., (1990) observed an increase in growth associated phosphoprotein (GAP-43) levels within the molecular layer of the dentate gyrus. Growth associated phosphoprotein is a marker of regenerating axons and is used to label sprouting processes. One week following a selective lesion of the hippocampal granule cells, McKeon et al., (1989) observed a marked increase in AChE staining in the molecular layer adjacent to the lesioned granule cells. Storm-Mathisen (1974) measured AChE levels in the hippocampus following a unilateral lesion of the entorhinal cortex input into the dentate gyrus and reported that AChE positive stain intensification in the molecular layer was fully developed by eight days following transection of the perforant path in adult rats. Storm-Mathisen (1974) did not however, see stain increases at two or four days post lesion. These authors sampled animals at two, four and eight days after a perforant path lesion. This suggests that sprouting within the molecular layer of the DG occurs between four and eight days after a lesion of the perforant path. This time frame for sprouting initiation is in line with findings of the present study utilizing a muscarinic antagonist stimulus.

Cotman and Nieto-Sampedro (1984) suggest that unilateral entorhinal cortex lesions evoke synaptic growth over several weeks and its rate is contingent on the rate of clearance of degenerating synapses. One day after a unilateral lesion of the entorhinal cortex, 90 percent of the synapses in the outer molecular layer are lost. Three days

following the lesion, a massive regrowth of residual fiber systems in the dentate gyrus occurs (Cotman et al., 1984). This time frame for outgrowth initiation is consistent with that seen in the present study. It is unlikely that the sprouting observed in the present paradigm involved terminal degeneration prior to sprouting. The visualization of ultrastructural features of synapses in sprouting zones via electron microscopy would help address this question.

In the periphery, researchers have utilized a paradigm similar to that of the present study to elicit sprouting at the muscle endplate of the mouse. Holland et al. (1980) antagonized nicotinic receptors at the neuromuscular junction using the irreversible antagonist alpha-bungarotoxin. Sprouting of presynaptic terminals into the antagonized endplate was observed by four to eight days after injections with the antagonist. The time course of peripheral antagonist induced sprouting appears to parallel that of central antagonist induced sprouting displayed in the present study.

While AChE positive growth was observed at both four and 14 days in the present study, it was clearly absent at one and 21 days post treatment. The 1 day subjects demonstrated an increased darkening along the length of injection tracks. However, this is not attributed to a drug induced sprouting of AChE positive fibers but rather to blood accumulation in that region following surgery just 24 hours prior. This pattern of darkening is characteristic of one day animals and did not extend beyond the boundary of the injection track created by the injection cannula. This restricted darkening within the cavity formed by the cannula is support for the idea that one day darkening is not due

to sprouting. Stain increases seen within four day subjects regularly extended into tissue outside the injection track. If the increased darkening of one day subjects was due to sprouting it would be expected to have occurred with the same intensity beyond the cannula track into surrounding tissue as seen in four day subjects. Further, this increased darkening along the track was evident in both control and QNB injected hemispheres.

The absence of a sprouting response by 21 days post surgery is a consistent finding of the present study. This can most adequately be explained in context of the finite nature of the stimulus used. In the lesion induced sprouting paradigm, increased AChE can be demonstrated for months following the initiation of sprouting (Lynch et al., 1972 ; Storm-Mathisen, 1974 ; Woodruff and Baisden, 1989). In the case of unilateral entorhinal cortex lesions, the loss of entorhinal terminals in the molecular layer initiates the reinnervation of degenerated terminal spaces by adjacent septal terminal fields in the molecular layer (Cotman et al., 1984). These newly formed fields of innervation may persist due to persistence of the stimulus (loss of afferents from the entorhinal cortex or perforant path). In the present study however, the duration of QNB antagonism is finite given the ongoing process of receptor turnover. Once the life span of a receptor is surpassed it is replaced by another receptor of the same type. This new receptor will not be the source of an antagonist induced signal because QNB will not be present to bind to it. Quinuclidinyl benzilate binds to existing receptor populations upon its initial injection into the hippocampus and presumably any excess QNB will eventually be removed from the extracellular space and transported to peripheral organs in the circulatory system

where it is excreted from the body. Therefore, only receptors present at the time of injection will bind QNB and the length of the receptor/QNB association is dependent on receptor mortality. In the introduction, it was suggested that muscarinic receptors turnover every six days (Sethy et al., 1990). Given this, the antagonism of receptors by QNB could persist at most six days before new non-QNB bound receptors would develop and restore normal receptor functioning and effectively remove or "turn off" the proposed stimulus for growth. Acetylcholinesterase positive increases observed at 14 days following QNB treatment may in fact be due to residual terminal outgrowth formed between the period of one and six days during which time the sprouting stimulus was present. By 14 days, the percentage of subjects demonstrating an AChE positive increase has decreased considerably from the four day group percentage. It is possible that once the stimulus for sprouting is "turned off", retraction of sprouted fibers occurs. This seems likely given that this novel technique elicits sprouting in the undamaged hippocampal system of adults in which circuitry is presumably fully developed and functional. In this environment, sprouted fibers would have no utility in a stimulus "turn off" state given the existing functional connections, and would presumably retract.

Variability:

As discussed in the introduction, there are a number of studies which attempted to show that a blockade of postsynaptic nicotinic receptors will induce sprouting of presynaptic terminals in the PNS (Pestronk et al., 1978 ; Tonge, 1978). These

investigations did not support the idea that postsynaptic antagonism would induce terminal sprouting. The study of Holland et al. (1980) was the first to demonstrate that prolonged antagonism of postsynaptic nicotinic receptors induces sprouting at the muscle endplate in mice. The results of the present study are relatively robust while at the same time tending to be somewhat variable with respect to the magnitude of the AChE positive response observed between animals. Variability of the sprouting responses observed, while frustrating, is not terribly upsetting given the difficulty researchers had producing the effect in the PNS, an area traditionally attributed with greater plastic potential than the CNS (Oorschot and Jones, 1990 ; Gage et al., 1983).

There are a number of explanations for the variability seen. One source of variability could revolve around the drug preparation. When QNB was mixed with the vehicle beta hydroxipropylcyclodextrin, the former was not entirely dissolved in the latter. Sonication of the mixture led to as much of the drug going into solution as was possible. However, the drug/vehicle combination resembled a suspension which was somewhat viscous in texture. These characteristics may have led to varying doses for different injections given that it is difficult to control the exact dosage of QNB if it is both in suspension and solution in the same preparation. The viscous characteristic of the drug/vehicle combination may have also contributed to an injection spread in the hippocampus that was less than concentric or spherical. Consequently, the size of the area of drug action may have varied from animal to animal as was seen. To understand exactly how much the drug preparation affected drug spread within hippocampal tissue,

the physics of the drug spread would have to be directly evaluated. To investigate how the QNB/vehicle combination operated in hippocampal tissue a radiolabelled batch of QNB could be injected and the subsequent spread measured using autoradiographic imaging techniques and compared with the AChE reaction.

Area of injection versus receptor densities:

The area of the hippocampus into which the QNB was injected may well contribute to the presence or absence of an AChE positive response. Of the four day subjects in experiment one, 15 distinct areas of increase were observed. Eleven of these were found in the DG molecular, hilar or granule layers while four were observed in area CA1 (strata oriens, pyramidale and superficial radiatum only). All these areas are characteristically abundant in muscarinic receptors and AChE containing fibers. One exception which will be discussed later is the granule cell layer which exhibited AChE positive staining increases yet is low in muscarinic receptors and AChE levels (Swanson et al., 1987). The extension of AChE staining into only superficial CA1 stratum radiatum could be due to the low levels of AChE positive fibers in this region (Swanson et al., 1987). Also, below stratum radiatum there are low levels of muscarinic receptors that could bind QNB (Swanson et al., 1987). All other regions showing stain increases are rich in AChE positive fibers and muscarinic receptors.

Two injections in SLM and one each in CA3 and DG did not demonstrate AChE increases in experiment one. The one case of an AChE devoid DG injected region is not

readily explicable given the eleven incidences of increase in this region. The nonresponsive CA3 injection too is also surprising given the presence of muscarinic receptors and AChE in this region. Perhaps these findings are the result of variation in the injection routine in these cases. Alternatively, in the case of the CA3 injection of QNB, it is possible that normally high baseline AChE positive staining in this region masked the effect of injecting QNB into that area such that any "new" increases were undetectable against the background. This is supported by the fact that no CA3 stain increases were observed in any case.

Of the four day subjects in experiment two that demonstrated an AChE positive stain increase, six distinct areas of increase were recorded. Two were seen in DG molecular, hilar or granule layers while four increases were observed in CA1 strata oriens, pyramidale and superficial radiatum only. Again, these sites of stain increase fit with known muscarinic receptor and AChE distributions except in the case of an increase extending into the granule cell layer. The case of an AChE devoid injection into the DG of an experiment two subject cannot be explained given this regions ability to support antagonist induced increases in AChE staining. Injection variability in this case may be to blame.

Fourteen day survival subjects of experiment one demonstrated AChE positive increases in CA1, DG (molecular, hilar and granule layers) and, in one case, the cortex while revealing no response to injections into SLM and in five cases area CA1 (oriens and pyramidale). The AChE devoid CA1 injection areas in the 14 day subjects may

simply have resulted from the cessation of the QNB induced trophic signal by this time, as discussed above, or alternatively, injection variability.

Overall, injections into the molecular layer and hilus of DG in four and 14 day subjects appear to consistently demonstrate an AChE positive fiber increase. This is most probably due to the high density of muscarinic receptors in these regions for QNB to act upon (Swanson et al., 1987). Areas of CA1 exhibiting high to moderate muscarinic receptor densities are strata oriens, pyramidale and radiatum. These regions of CA1 all exhibited the ability to support AChE stain increases in response to QNB antagonism with the partial exception of stratum radiatum that exhibited increases in AChE superficially as though arising from sprouting AChE containing fibers in strata oriens or pyramidale. One area receiving a QNB injection that never exhibited an increase in staining was SLM. This finding fits well with what is known of muscarinic receptor densities within this area. Stratum moleculare or SLM contains no muscarinic receptors and few AChE positive fibers (Swanson et al., 1987).

Source of AChE positive fiber sprouting:

In the present study it is possible that the septal cholinergic projection to the hippocampus is the source of AChE positive fiber increases observed. To test this hypothesis the same paradigm should be repeated in conjunction with septal lesions. If the QNB induced sprouting is from septal fibers, then the QNB treatment should not produce an increase in AChE fiber outgrowth in a septal lesion condition.

The hippocampus has been shown to contain numerous AChE positive interneurons (Swanson et al., 1987). It may be that AChE positive interneurons in the hippocampus are the source of the AChE positive outgrowth seen (Swanson et al., 1987). These interneurons may be characterized by a transmitter other than Ach and yet be AChE positive. These interneurons are found throughout the hippocampus, however mainly in the hilus (McKeon et al., 1989). Given that they are predominantly found in the hilus, it is plausible that these interneurons are the source of AChE positive fiber outgrowth in that region reported in the present study. McKeon et al., (1989) lesioned granule cells of the DG and reported AChE positive but non-cholinergic sprouting into the molecular layer. Septal lesions in combination with granule cell lesions did not deter the occurrence of AChE positive sprouting (McKeon et al., 1989). McKeon et al., (1989) concluded that the source of AChE positive sprouting seen in their study is most probably AChE containing interneurons of the hippocampus.

Source of the trophic signal:

In the present study it is believed that the signal initiating AChE positive fiber outgrowth arose from the antagonized muscarinic receptor complex. The staining distributions tend to fit well with known localizations of muscarinic receptors in the hippocampus (Swanson et al., 1987). Given that the majority of muscarinic receptors in the hippocampus are postsynaptic (M1) in nature (Zilles, 1988), the trophic signal is believed to be in the post to presynaptic direction and the trigger of postsynaptically

directed growth of presynaptic terminals.

The presence of presynaptic receptors (thought to be M2 receptors) that would have been antagonized by QNB (as well as antagonizing the more abundant postsynaptic receptors) necessitates a discussion of presynaptic influences. Presynaptic muscarinic receptors are not thought to have played a role in the sprouting response seen in the present study as they are attributed with controlling Ach release from presynaptic terminals (Cooper, Bloom and Roth, 1986 ; Sokolovsky, 1984 ; North et al., 1985). Muscarinic antagonists have been found to increase Ach release by acting at presynaptic receptor sites while muscarinic agonists bound presynaptically have been shown to decrease Ach release in the hippocampus (Sethy et al., 1990 ; Cooper et al., 1986 ; Sokolovsky, 1984). Given that QNB antagonism increases Ach release when bound presynaptically (Sethy et al., 1990), the presynaptic muscarinic receptor can be implicated in facilitating sprouting only if Ach increases contribute to sprouting responses. Lipton, Frosch, Phillips, Tauck and Aizenman (1988) investigated the effects of Ach on neurite outgrowth in postnatal rat retinal ganglion cell cultures. These researchers reported that nicotinic antagonism of ganglion cells in culture initiates increases in process outgrowth within 24 hours of application. Normally, cells in retinal ganglion cell culture leak Ach (Lipton et al., 1988). Under such conditions neurite outgrowth is inhibited. Lipton et al. (1988) concluded that nicotinic antagonism elicits growth by removing the inhibitory effect of tonic levels of Ach in the culture. Lipton et al. (1988) suggest that in the retina, Ach inhibits growth processes. If the same is true for the hippocampus of adult rats then

antagonism of presynaptic receptors with QNB, which increases Ach release (Sethy et al., 1990 ; Sokolovsky, 1984 ; Cooper et al., 1986), may actually hinder growth. It is more plausible that postsynaptic signals subsequent to QNB antagonism of muscarinic receptors initiated AChE positive fiber outgrowth seen in the present study.

In the introduction, it was stated that M1 and M2 receptor populations occur with varying densities in different regions of the hippocampus (Zilles, 1988). The sites of consistent AChE positive fiber sprouting in the present study (molecular layer of the DG ; strata oriens of CA1) are more heavily populated with M1 receptors then with M2 receptors (Zilles, 1988). One exception is stratum pyramidale of area CA1. This area showed AChE positive stain increases and is abundant in M2 receptors (Zilles, 1988). The most robust AChE positive fiber sprouting of the present study was found in the molecular layer of the DG, an area with low densities of M2 receptors and high densities of M1 receptors (Zilles, 1988). From this it would appear that the most likely candidate for mediation of QNB induced trophic signals are M1 receptors.

In the PNS, blockade of nicotinic receptors was found to induce sprouting at the muscle endplate of mice (Holland et al., 1980). It may be, as presumed in the present study, that trophic signals originated from antagonized postsynaptic receptors. However, one alternative explanation is that sprouting was induced by muscle inactivity (Holland et al., 1980). In the state of prolonged nicotinic receptor blockade at the neuromuscular junction the postsynaptic muscle fibers are inactive due to the inability of Ach to bind to postsynaptic receptors. It may be that muscle inactivity led to the production of trophic

signals that induced sprouting at the endplate. In the present study this explanation is not possible given that there are numerous non-muscarinic receptors on postsynaptic surfaces which will continue to respond to neurotransmitter activation during muscarinic antagonism in the hippocampus. Trophic signal production in the present study must have arisen from a source other than postsynaptic inactivity.

Specificity of AChE positive fiber reinnervation:

The question arises as to the specificity of the AChE fiber growth with respect to sites of reinnervation. For the most part, areas exhibiting sprouting responses contain muscarinic receptors, the proposed source of trophic signals in the present study (Swanson et al., 1987). Therefore, it may be that the AChE positive fiber increases seen terminated at antagonized sites as guided by a signal consequent to muscarinic receptor antagonism. In this case, it is suggested that a trophic signal is generated by the QNB/muscarinic receptor interaction and that this interaction also acts to guide fiber outgrowth to sites of antagonized receptors as opposed to merely signalling a generalized growth of AChE positive fibers toward no specific target.

Alternatively, AChE containing fibers may have grown to non-antagonized sites also, presumably due to a generalized release of some trophic factor in the absence of specific guidance (such as from antagonized targets). In this case, the QNB antagonism of muscarinic receptors induces a trophic response but does not guide the sprouting fibers. Both possibilities may in fact be consequent to prolonged QNB antagonism of muscarinic

receptors in the hippocampus. One instance of support for the hypothesis that non-specific or non-guided sprouting of adjacent fibers is actually occurring may be the sprouting observed in the molecular layer of the DG. In some cases, AChE fiber increases could be seen in an area normally devoid of AChE and containing few muscarinic receptors: the granule cell layer. In control AChE stained preparations, the granule cell layer appears as a white band between the moleculare and hilar regions. In some QNB treated cases, clear increases in staining were seen in this region presumably arising from adjacent areas rich in endogenous AChE containing fibers (see figures 5, 8 and 14). Because the granule layer contains few muscarinic receptors for QNB to have bound to, it is not likely that signals conducive to sprouting into this region arose from the granule layer itself. It is possible that the sprouting response to a trophic signal consequent to QNB binding to muscarinic receptors is general in nature and not specifically directed towards sites of QNB antagonism. This could conceivably be accomplished through a general or diffuse release of some trophic factor during QNB antagonism of muscarinic receptors. Alternatively, the AChE fiber increases evident in the granule layer of some subjects may well terminate at antagonized sites. To establish whether QNB antagonism guides sprouting as well as initiating it, techniques that allow the dual visualization of QNB binding sites and sprouted terminals would be required. In this way, it could be determined if there is a strong correlation between QNB binding sites and AChE positive sprouted terminals.

Alternative explanations:

The hypothesis behind the present study was that QNB antagonism of hippocampal muscarinic receptors induces AChE positive fiber increases as a result of sprouting cholinergic fibers. A systematic review of the possible alternative explanations would help clarify and strengthen the results that tend to support this hypothesis.

One obvious alternative explanation for the AChE positive fiber increases would be that mechanical damage from the injection of QNB into the hippocampus caused the sprouting observed. Perhaps the force of the injections were such that cell damage in the hippocampus resulted in the sprouting response observed. The injection then would effectively act as a lesion. This possibility was controlled for however by 1.) a careful injection routine and 2.) the use of within animal controls. The QNB and vehicle were injected slowly over a four minute period. If the injection procedure caused damage induced sprouting in experimental hemispheres, then the same should have been seen in control hemispheres - it was not.

The increased AChE staining seen at four and 14 days after QNB treatment are hypothesized to be the result of sprouting cholinergic fibers. One alternative explanation is that the AChE positive fiber increases were due to an increase in glial and macrophage responsiveness in the region of the injection. This is unlikely given that the present study employed an inhibitor of non-specific esterases (ethopropazine) in the AChE stain procedure to rule out the occurrence of esterases associated with glial responses (McKeon et al., 1989). Further, if esterase-producing glial responses occurred in the QNB injected

hemispheres they would have been expected to occur with similar frequency in control hemispheres - they did not.

Hemisphere and sex differences:

Experiment two investigated the variables of sex and hemisphere in the QNB induced sprouting response. The results suggest that the response to QNB blockade is not contingent on the hemisphere of treatment. Quinuclidinyl benzilate induced AChE positive stain increases were observed in both left and right hemispheres.

The occurrence of AChE positive fiber sprouting also did not appear to be contingent on sex. The results of experiment one showed that female Sprague Dawley rats could exhibit an increase in AChE staining in response to QNB antagonism. Experiment two demonstrated that males too could support AChE fiber sprouting in response to QNB. Within the scope of this study, sex differences do not seem to exist with respect to the ability of animals to demonstrate sprouting responses to QNB antagonism. Future research utilizing the paradigm of the present study could investigate whether other strains of rats and even other species of animals are equally capable of exhibiting sprouting under similar treatment conditions.

Effect of QNB concentration:

Experiment two also looked at varying levels of QNB concentration with respect to AChE positive fiber outgrowth. Varying the QNB dose between 20 μg or 40 μg was

not a determining factor for the occurrence of AChE positive fiber outgrowth in the present study. Sprouting was observed equally under the two drug conditions in both male and female rats. It may be that AChE positive sprouting will occur when an unknown proportion of receptors in a given area are antagonized and that both drug doses satisfy this demand. The proportion of receptor antagonism then would presumably be directly related to trophic factor activation. To assess whether sprouting is contingent on levels of receptor binding, tissue culture techniques would be beneficial. A coculture of muscarinic-rich hippocampal cells and septal cholinergic cells could be treated with radiolabelled QNB and subsequent muscarinic binding/cholinergic sprouting correlated.

Mediation of muscarinic antagonist induced sprouting:

It has been shown that NGF exists in highest CNS concentration in the hippocampus (Collins and Crutcher, 1985). Entorhinal cortex destruction has been shown to result in reactive synaptogenesis characterized by sprouting of cholinergic fibers from the inner one third of the molecular layer filling the deafferented outer molecular layer (Lynch et al., 1972 ; Stanfield and Cowan, 1982 ; Woodruff et al., 1990 ; McKeon et al., 1989). Crutcher (1987) has demonstrated that NGF-like activity is increased in the fascia dentata of the rat following perforant path (PP) lesions. It is likely that the sprouting of cholinergic fibers observed following entorhinal cortex lesions is dependent on NGF (Gage, Buzsaki and Armstrong, 1990). The present study demonstrated an AChE positive stain increase that indicated the occurrence of cholinergic fiber sprouting

in tissues receiving injections of QNB. The trophic factor(s) mediating this response can not be determined based on the present study. However, given the implication of NGF in other forms of hippocampal sprouting it is plausible that this trophic agent is mediating sprouting in the present paradigm as well. Crutcher et al., (1982) have shown that NGF concentrations in the hippocampus are elevated following fimbria-fornix lesions. Given that prolonged QNB exposure to hippocampal tissue may mimic this denervated state it is possible that QNB antagonism too could increase NGF concentrations.

Gage et al. (1988) have found that glial cells may mediate NGF activity in the hippocampus. Fimbria-fornix and PP lesions separately induce increases in glial fibrillary acidic protein immunoreactivity (GFAP-IR). It has been suggested that this increase in GFAP-IR is reflective of astrocytic cell activation and is a necessary requirement for the occurrence of sprouting (Gage et al., 1988). Given the implication of glial cell activity in sprouting responses, it is possible that increases in astrocyte activation would accompany the sprouting response observed in the present study (Gage et al., 1988). Future research into this question with respect to the present paradigm should involve immunoreacting antagonist treated tissue for both NGF and GFAP to determine if NGF and astrocyte activation are increased during QNB induced sprouting.

Markers of sprouting hippocampal cholinergic fibers:

Acetylcholinesterase has been used as an indicator of growth of cholinergic fibers in numerous studies including the present experiments (Lynch et al., 1972 ; Storm-

Mathisen, 1974 ; Dunnett et al., 1982 ; Dunnett et al., 1986 ; Stanfield et al., 1982 ; Bjorklund et al., 1983 ; McKeon et al., 1989). While AChE is not a direct indicator of cholinergic neurons, it correlates well with ChAT activity, a direct marker of Ach synthesizing neurons (Swanson et al., 1987). In future, investigations of muscarinic antagonist induced cholinergic sprouting could involve reacting tissue for both AChE and ChAT and in this way compare the usefulness of both markers in assessing cholinergic fiber outgrowth within the framework of the present paradigm.

The above mentioned means of evaluating sprouting cholinergic fibers via biochemical markers are invaluable techniques for assessing changes in the cholinergic fiber system. Another approach to evaluating the proliferation of fiber systems involves reacting treated tissue for markers of axonal outgrowth and regeneration. Growth associated phosphoprotein (GAP-43), also known as B-50, is expressed by neurons during states of axonal outgrowth (Benowitz et al., 1990). Growth associated phosphoprotein is transported to distal segments of developing axons and can be labelled via immunocytochemical techniques (Goslin, Schreyer, Skene and Banker, 1988). Growth associated phosphoprotein would be a useful marker in future studies evaluating synaptic changes subsequent to prolonged muscarinic antagonism. Used in association with biochemical markers of cholinergic activity, GAP-43 could add useful insight into the time course and magnitude of structural changes in neurons during growth conditions and could determine if more than cholinergic fibers are sprouting in response to that stimulus.

Practical application of antagonist induced sprouting:

Prolonged muscarinic antagonist induced sprouting in the CNS as illustrated in the present experiments is a novel approach to understanding CNS plastic changes. Once the mechanisms underlying the sprouting responses observed are elucidated, this technique could conceivably be employed towards some therapeutic ends. Cholinergic transplantation paradigms in which embryonic septal tissue is implanted into the deafferented hippocampus of rats could conceivably benefit from antagonist induced sprouting. One of the negative aspects of tissue grafts with respect to therapeutic application is that they exhibit an upper limit with respect to the amount of reinnervation attained. Cholinergic cell transplants into the damaged hippocampus attain maximal reinnervation of the target by approximately six months (Bjorklund et al., 1983 ; Dunnett et al., 1986). When used in combination with muscarinic antagonism, cholinergic grafting techniques may display more enduring growth past the usual upper limit Presumably, once transplanted cells extend their axons into the denervated tissue, any trophic signal generated by the loss of normal innervation ceases. Muscarinic antagonism in the hippocampus could maintain a growth inducing signal that could allow transplanted fibers to extend further into that structure. This could be achieved by infusing a muscarinic antagonist into the hippocampus following graft implantation and assessing the extent of outgrowth relative to controls infused with vehicle only.

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