**Neural and Behavioral Effects of Intracranial 192 IgG-Saporin in Neonatal Rats:
Sexually Dimorphic Effects?**

by

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ABSTRACT

The consequences of neonatal cholinergic lesions were examined in male and female rats. Rats were injected intracranially with 600 ng of 192 IgG-saporin at 7 days of age and examined behaviorally and histologically at 21, 45 and 90 days of age. 192 IgG-saporin profoundly reduced p75-, and to a lesser extent, choline acetyltransferase-immunoreactive cells in the basal forebrain. Presumptive sympathetic ingrowths into the hippocampus were not apparent until 45 days of age, and they were not significantly longer at 90 days. 192 IgG-saporin increased the time females spent on the open arms of the elevated plus maze but had no effect on males. Lesioned rats also showed a mild impairment in the acquisition of the Morris water maze, but did not differ from controls on the probe trial. Generally, the effects of neonatal cholinergic lesions were not sex dependent and are unlikely to model Rett syndrome.

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INTRODUCTION

There is a large body of literature implicating the cholinergic basal forebrain in learning and memory functions. Specifically, rodent memory processes can be facilitated or impeded by cholinergic agonists or antagonists respectively (Murray & Fibiger, 1985; Whishaw, O'Connor & Dunnett, 1985; Murray & Fibiger, 1986). In addition, age-related deficits in rodent mnemonic abilities have been associated with concomitant reductions in cortical and hippocampal choline acetyltransferase (ChAT) activity, acetylcholinesterase (AChE) fiber density and hippocampal muscarinic receptor numbers (Lippa, Pelham, Beer, Critchett, Dean & Bartus, 1980; Strong, Hicks, Hsu, Bartus & Enna, 1980). Evidence obtained from postmortem studies of patients with Alzheimer's disease, a neurodegenerative disorder associated with impaired cognitive processes, demonstrated reduced ChAT activity and AChE fiber density in cortex and hippocampus as well as reduced numbers of cholinergic cells in the basal forebrain when compared to age-matched controls (Coyle, Price & DeLong, 1983; Beach, Honer & Hughes, 1997). Taken together, these data formed the cholinergic deficit hypothesis of Alzheimer's disease, and researchers began trying to reproduce cholinergic deficits experimentally to further investigate the disease. Unfortunately this model proved problematic in that the lesion techniques available were rudimentary and relatively nonspecific for cholinergic neurons. However in 1991 Wiley, Oeltmann and Lappi published a seminal paper on the selective destruction of central cholinergic neurons via a novel immunotoxin called 192 IgG-saporin. The development of this immunotoxin has allowed

researchers to specifically target central cholinergic systems in the basal forebrain and further characterize their roles not only in learning and memory, but also in attention, arousal and the neurobiology of aging. In contrast, very few studies have evaluated the functional and biological consequences of cholinergic lesions on brain development. This is surprising considering that at least one neurodevelopmental disorder, the putatively X-chromosome linked Rett syndrome, is associated with forebrain cholinergic dysregulation. The proposed study will evaluate the sexually dimorphic effects of basal forebrain cholinergic lesions in neonatal rats. Special attention will be given to the histological assessment of lesion effects and post-lesion recovery of cholinergic markers over three developmentally relevant time points. The temporal kinetics of sympathetic hippocampal ingrowth development, one of the biological sequelae of cholinergic septal lesions, will also be examined.

Experimentally Induced Cholinergic Deficits: Excitotoxins and Transections

The rodent cholinergic basal forebrain consists of the medial septum, the ventral and horizontal limbs of the diagonal band of Broca and the nucleus basalis complex (comprising the ventral pallidum, globus pallidus and nucleus basalis magnocellularis), the latter being analogous to the human nucleus basalis of Meynert. The basal forebrain nuclei account for the vast majority of cholinergic innervation to the hippocampus and cortex, with the medial septum and the vertical limb of the diagonal band sending projections mainly to the hippocampus, the nucleus basalis area projecting mainly to the cortex and the horizontal limb of the diagonal band sending a small projection to the olfactory bulbs (Mesulam, Mufson, Wainer & Levey, 1983; Amaral & Witter, 1995). Since the loss of cholinergic projections to

the hippocampus and cortex and the loss of cholinergic cell bodies in the nucleus basalis of Meynert are thought to underlie some of the major cognitive deficits in Alzheimer's disease, animal models were designed to disrupt the functional integrity of basal forebrain connections in adulthood, either pharmacologically (excitotoxins) or mechanically (electrolytic or transection lesions). Indeed, it was subsequently demonstrated that basal forebrain cholinergic lesions did produce marked behavioral impairments on tasks involving learning and memory components, such as the Morris water maze and the radial arm maze (Hepler, Wenk, Cribbs, Olton & Coyle, 1985; Murray & Fibiger, 1985; Dokla & Thal, 1988; Riekkinen, Sirvio & Riekkinen, 1990). Nevertheless, the lesion studies outlined below contain method-dependent inconsistencies which have cast doubt on the involvement of the cholinergic basal forebrain in learning and memory *per se*.

ChAT is the enzyme responsible for the biosynthesis of acetylcholine (ACh) and is used as an index of cholinergic activity. Administration of the amino acid excitotoxin ibotenic acid to the rat nucleus basalis area causes reductions in cortical ChAT activity of as much as 60% as compared to control values (Wenk & Olton, 1984; Wenk, Markowska & Olton, 1989). Behaviorally, ibotenic acid-induced nucleus basalis lesioned rats have shown impaired choice accuracy in the radial arm maze and a positively reinforced T-maze task, as well as deficits in the acquisition of a passive avoidance task and the Morris water maze (Salamone, Beart, Alpert & Iverson, 1984; Hepler et al., 1985; Mayo, Kharouby, LeMoal & Simon, 1988; Riekkinen et al., 1990). These deficits are similar to those seen after administration of the central muscarinic receptor antagonist atropine sulfate (Whishaw et al., 1985), and can be reversed with the muscarinic receptor agonist pilocarpine or the AChE inhibitor

physostigmine (Murray & Fibiger, 1985; Murray & Fibiger, 1986; Dokla & Thal, 1988). Since hippocampal ChAT activity was unaffected in all of these studies, the cortical cholinergic projection from the nucleus basalis area was initially thought to be a requisite component of intact spatial and nonspatial memory systems.

Although the behavioral impairments produced by ibotenic acid have been attributed to the loss of cholinergic cell bodies in the nucleus basalis area as determined by reductions in post-lesion cortical ChAT levels, a comparison of ibotenic acid and another amino acid excitotoxin, quisqualic acid, has revealed that this interpretation is inconsistent with the data. Several studies have shown that lesions of the nucleus basalis area produced by quisqualic acid are far more selective for cholinergic neurons and result in greater reductions of cortical ChAT activity as compared to ibotenic acid lesions. Surprisingly though, quisqualic acid treated rats show fewer and less severe behavioral deficits (Robbins, Everitt, Marston, Wilkinson, Jones & Page, 1989; Wenk et al., 1989; Markowska, Wenk & Olton, 1990; Connor, Langlais & Thal, 1991; Rugg, Dunbar, Latimer & Winn, 1992; for review, see Dunnett, Everitt & Robbins, 1991). Thus the cognitive deficits seen as a result of excitotoxic lesions of the nucleus basalis are not correlated with cholinergic cell loss *per se*, but can be correlated with the extent of nonspecific (i.e. non-cholinergic) subcortical damage. For example, Wenk et al. (1989) found that while both ibotenic and quisqualic acid treated rats were impaired for the first two days of spatial alternation testing, on subsequent testing days the impairment persisted for ibotenic acid treated rats while the performance of quisqualic acid treated rats had improved to control levels. This occurred despite the fact that quisqualic acid produced a greater reduction in cortical ChAT activity as compared to ibotenic acid

(73% vs. 54% reduction). Riekkinen, Riekkinen and Riekkinen (1991) found similar results: ibotenic, but not quisqualic, acid lesions impaired acquisition of the Morris water maze in rats, even though quisqualic acid produced a significantly greater cholinergic lesion as compared to ibotenic acid. Finally, a subsequent study found that rats with deafferentation of the frontal cortex made significantly more working memory errors in a fully baited radial arm maze than either ibotenic or quisqualic acid lesioned rats, although a performance deficit did emerge in the ibotenic acid lesioned group in the half-baited version of the radial arm maze, a task requiring more complex discriminative abilities (Ammassari-Teule, Amoroso, Forloni, Rossi-Arnaud & Consolo, 1993). The quisqualic acid lesioned rats were behaviorally indistinguishable from controls despite reductions similar to the deafferented rats in *in vivo* fronto-cortical ACh release and ChAT activity, which were significantly greater than the reductions exhibited by both ibotenic acid and control rats. The behavioral similarities between deafferented rats and rats with fimbria/fornix transections suggested that the septohippocampal cholinergic system may be more involved in mnemonic functions than ascending cortical cholinergic projections.

The majority of medial septal neurons projecting to the hippocampus do so via the fimbria/fornix (Mesulam et al., 1983; Amaral & Witter, 1995). Transection of the fimbria/fornix axotomizes these cells, resulting in a loss of cholinergic cell markers such as ChAT and AChE staining in the hippocampus and medial septum (Gage, Bjorklund & Stenevi, 1983; O'Brien, Svendsen, Isacson & Sofroniew, 1990; Tuszyński, Armstrong & Gage, 1990). However this technique does not reliably produce a persistent lesion effect. For example, one study found that although ChAT levels in the medial septum decreased to 82%

of control levels the day following fimbria/fornix transection, by 6 months post-lesion ChAT activity had risen to 146% of control levels, suggesting that intraseptal sprouting had occurred (Hage, Frotscher & Naumann, 1996). At survival times of 6 months or more, some degree of recovery is also seen in hippocampal ChAT levels (Gage et al., 1983; Hage et al., 1996). Despite the memory impairments reported in rats after fimbria/fornix transection, this lesion technique is nonspecific for cholinergic fibers: transection effectively axotomizes all fibers passing through the fimbria/fornix, raising the question once again of whether or not the behavioral deficits detected can be attributed exclusively to cholinergic hypofunction in the hippocampus. In order to spare fibers of passage, intraseptal injections of ibotenic and quisqualic acid were used to further evaluate the functional significance of the cholinergic septohippocampal projection.

Injections of ibotenic acid into the medial septum/diagonal band have been shown to reduce hippocampal ChAT levels to approximately 40% of control levels, with concomitant impairments of spatial memory as assessed by the Morris water maze (Hagan, Salamone, Simpson, Iverson & Morris, 1988; Riekkinen et al., 1990). Mean escape latencies were longer and less time was spent in the correct quadrant during probe trials of rats receiving medial septal lesions. In a detailed study, Nilsson and Gage (1993) compared the performance of aged rats with the performance of medial septal, nucleus basalis, and combined medial septal/nucleus basalis lesioned young rats on the Morris water maze both prior to and after atropine sulfate challenge. In their first experiment, naive aged rats were characterized as either impaired or unimpaired when compared to naive young rats in the Morris water maze. The rats were then given an atropine sulfate challenge which moderately impaired the

performance of the young rats, but severely impaired the performance of both the impaired and unimpaired aged rats. The second experiment involved testing young rats with lesions of either the medial septum, the nucleus basalis or the combined medial septal/nucleus basalis (dual lesion group) in the Morris water maze before and after atropine sulfate challenge. Initially the dual lesioned rats took more time to acquire the task. However all rats (except one in the dual lesioned group) were performing at comparable levels just prior to atropine sulfate administration. After drug challenge, the septal lesioned rats (i.e. both medial septal and dual lesion groups) showed greatly increased escape latencies and reduced numbers of annulus crossings as compared to the non-septal lesioned group (i.e. nucleus basalis lesion and control). Thus both naive aged rats and septal lesioned young rats showed similar sensitivity to blockade of central cholinergic neurotransmission as demonstrated by impaired performance on the Morris water maze. Collectively these data suggested that impaired spatial memory could indeed result from cholinergic hypofunction in the septohippocampal system.

Although lesions of the basal forebrain produce behavioral deficits on spatial and reference memory tasks, lesion techniques such as fimbria/fornix transections and excitotoxins are generally nonselective for cholinergic neurons. The ethylcholine mustard aziridinium ion AF64A has been shown to produce comparatively large cholinergic depletions via internalization at the high affinity choline uptake site (Walsh, Chrobak, Tilson, Dehaven & Hanin, 1985; Mouton, Meyer, Dunn, Millard & Arendash, 1988; Speiser, Reicher, Gitter & Cohen, 1989; Brake & Pappas, 1994; for review see Fisher & Hanin, 1986; Hanin, 1990), yet administration of this toxin also results in extensive nonspecific tissue damage, confounding

interpretation of post-lesion behavioral impairments (McGurk, Hartgraves, Kelly, Gordon & Butcher, 1987; Armstrong & Pappas, 1991). Indeed, especially after the demonstration that quisqualic acid was more effective than ibotenic acid at reducing cortical ChAT activity while producing fewer behavioral side effects in a number of mnemonic tasks, a selective cholinergic lesion method was needed before any definitive conclusions about the function of the cholinergic basal forebrain could be drawn.

Experimentally Induced Cholinergic Deficits: 192 IgG-Saporin Lesions

In 1991, Wiley et al. published a seminal paper regarding a highly selective cholinergic immunotoxin called 192 IgG-saporin. 192 IgG is a monoclonal antibody to p75, the low-affinity nerve growth factor (NGF) receptor expressed exclusively on cholinergic neurons in the basal forebrain. The antibody is coupled to the toxin saporin, derived from the seeds of the plant *Saponaria officinalis*. Once granted access to the cell via internalization of 192 IgG at the p75 receptor site, saporin enzymatically disables ribosomes and arrests protein synthesis, resulting in cell death. 192 IgG-saporin has proven highly effective as a cholinotoxin, producing near total destruction of p75-positive cholinergic cells in the adult rat basal forebrain when injected intracerebroventricularly (i.c.v.), while sparing the cells of other transmitter systems (Wiley et al., 1991; Heckers, Ohtake, Wiley, Lappi, Geula & Mesulam, 1994; Waite, Wardlow, Chen, Lappi, Wiley & Thal, 1994; Leanza, Nilsson, Wiley & Bjorklund, 1995; Robner, Schliebs, Hartig & Bigl, 1995; Walsh, Kelly, Dougherty, Stackman, Wiley & Kutscher, 1995). For example, Leanza et al. (1995) found that i.c.v. injection of 2.5 µg of 192 IgG-saporin decreased the number of p75-positive cells in the

medial septum/vertical limb of the diagonal band and the nucleus basalis magnocellularis by 81% and 80% respectively, and a 5 μ g dose decreased the number of cells by 96% and 90%. ChAT activity in parietal cortex and hippocampus was reduced by as much as 80% and 90% of control values respectively (5 μ g group), while ChAT levels in other areas such as brain stem and cerebellum remained unaffected. Similarly, Walsh et al. (1995) found that i.c.v. injection of either a 2 μ g or a 4 μ g dose of 192 IgG-saporin produced a 60% or an 80% reduction in high-affinity choline transport in the hippocampus, and a 65% reduction in high-affinity choline transport for both doses in frontal cortex. High-affinity choline transport in the striatum was unaffected. In a separate study, AChE fiber density has been reduced to less than 1% of control values in the dentate gyrus and neocortex following i.c.v. injection of 2.7 μ g of the toxin (Vnek, Kromer, Wiley & Rothblat, 1996). Indeed, previously published results from this laboratory have shown that neonatal administration of as little as 400 ng of 192 IgG-saporin produces persistent reductions of cortical and hippocampal ChAT levels of 52% and 84% respectively (Pappas, Davidson, Fortin, Nallathamby, Park, Mohr & Wiley, 1996).

When compared with other conventional lesion techniques, 192 IgG-saporin has proven to be at least as effective at creating cholinergic lesions in the basal forebrain as fimbria/fornix transection or excitotoxins. The efficacy of both 192 IgG-saporin and the excitotoxin ibotenic acid, injected directly into the medial septum and the nucleus basalis magnocellularis, was evaluated using a delayed non-matching to position task (Robinson, Wenk, Wiley, Lappi & Crawley, 1996). Although a significantly greater lesion in the hippocampus and cortex was obtained with 192 IgG-saporin as assessed by ChAT activity, behaviorally the ibotenic acid and immunotoxin lesioned rats were comparably impaired on

the task. Hage et al. (1996) have shown that 192 IgG-saporin lesions not only produce larger cholinergic lesions when compared to fimbria/fornix transections, but more persistent ones as well. Following fimbria/fornix transection, ChAT levels in the medial septum decreased by 18% on the first day, but then increased by 23% on the second day, and by 3 weeks post-lesion had returned to normal. This group also reported an initial dramatic decrease in hippocampal ChAT levels (approximately 80%), however by 6 months post-lesion ChAT levels in the hippocampus had increased to about half of control values, indicating that some degree of recovery had occurred. The rise in ChAT levels could be explained by intraseptal sprouting of surviving neurons in the absence of their proper target. In contrast, intrahippocampal injections of 192 IgG-saporin caused a 53% reduction of septal and an 84% reduction of hippocampal ChAT levels. No recovery was seen for at least 3 weeks following 192 IgG-saporin, suggesting that administration of this toxin had effectively resulted in cell death.

While there is no doubt that i.c.v. administration of 192 IgG-saporin produces large, persistent, highly selective cholinergic lesions, conflicting data has been reported on its behavioral effects. Some laboratories have found that 192 IgG-saporin treated rats show performance deficits in the Morris water maze, passive avoidance retention and delayed non-matching to position tasks (Berger-Sweeney, Heckers, Mesulam, Wiley, Lappi & Sharma, 1994; Leanza et al., 1995; Steckler, Keith, Wiley & Sahgal, 1995; Walsh et al., 1995). Other laboratories have reported no significant effects of the toxin on learning and memory, even though comparably sized lesions have been produced (Pappas et al., 1996; Vnek et al., 1996).

Site-specific lesions of the basal forebrain may afford a clearer interpretation of these behavioral data.

192 IgG-saporin lesions of the nucleus basalis magnocellularis have been shown not to disrupt performance on delayed spatial alternation or passive avoidance despite producing significant cortical ChAT depletions (Wenk, Stoehr, Quintana, Mobley & Wiley, 1994). High-affinity choline uptake can be reduced in a dose-dependent manner in the terminal fields of the medial septum (i.e. the hippocampus, the cingulate and the entorhinal cortex) following intraseptal injections of 192 IgG-saporin (Walsh, Herzog, Gandhi, Stackman & Wiley, 1996). Behaviorally, the septal lesioned rats showed dose-dependent deficits in a delayed non-matching to position task. These rats were maximally affected by the 1 hour delay, but at the 8 hour time point their performance was not distinguishable from controls. In contrast, a comparison of i.c.v. and intraseptal injections of 192 IgG-saporin using the delayed non-matching to position task demonstrated that i.c.v. administration of 192 IgG-saporin disrupted choice accuracy whereas intraseptal administration did not (Steckler et al., 1995). The behavioral data correlated with reductions in hippocampal ChAT activity in both groups: 57% reduction for intraseptal vs. 75% reduction for i.c.v. administration. The behavioral side effects produced by i.c.v. 192 IgG-saporin were indistinguishable from those produced by acute i.c.v. administration of the choline uptake inhibitor hemicholinium-3, suggesting that the impairment following i.c.v. 192 IgG-saporin was in fact due to cholinergic hypofunction. In a separate study, injections of 800 ng of 192 IgG-saporin into the hippocampi of both mature and aged rats produced similar reductions of hippocampal ChAT activity in both age groups (Bannon, Curzon, Gunther & Decker, 1996). However, only aged rats showed

subsequent performance deficits in the Morris water maze, presumably due to a pre-existing hypofunction in the aged septohippocampal system.

Other studies have compared the behavioral effects between lesion sites in distinct nuclei of the basal forebrain. For example, lesions of the medial septum were shown to impair the acquisition, but not the asymptotic phase, of the uncued Morris water maze, whereas nucleus basalis magnocellularis lesions impaired both the acquisition and asymptotic phases; combined medial septal/nucleus basalis lesions impaired both cued and uncued navigation (Berger-Sweeney et al., 1994). In contrast, another study showed that spatial learning in rats with 192 IgG-saporin lesions of either the medial septum, the nucleus basalis, or the combined medial septum/nucleus basalis, was unaffected by the immunotoxin in the Morris water maze. However mild impairments in all three lesion groups were apparent in the radial arm maze (Dornan, McCampbell, Tinkler, Hickman, Bannon, Decker & Gunther, 1996). Thus, despite the availability of a selective cholinotoxin, the lack of conformity in behavioral data suggests that cholinergic neurotransmission in the basal forebrain is not solely responsible for mediating spatial learning and memory in the rat.

Hippocampal Sympathetic Ingrowths

Hippocampal reorganization in the form of sympathetic sprouting has been reported following fimbria/fornix transection (for review, see Crutcher, 1987). These noradrenergic fibers, often associated with blood vessels and putatively originating from the superior cervical ganglion, grow into NGF-rich subnuclei of the hippocampus (i.e. the dentate gyrus and stratum lucidum) in a pattern reminiscent of the topographical organization of the

septohippocampal projection (Gage et al., 1983). Although the underlying mechanisms of this reorganization are unknown, it has been postulated that passive NGF accumulation in the hippocampus as a result of cholinergic denervation could induce sprouting of sympathetic axons (Yu, Wiley & Perez-Polo, 1996).

The sympathetic sprouting response can be elicited by several types of lesions, including electrolytic, aspiration and transection lesions (Loy, Milner & Moore, 1980), as well as 192 IgG-saporin lesions (Pappas et al., 1996). However regardless of the type of lesion, disruption of hippocampal afferents arising in or passing through the medial septal area is a necessary condition for the manifestation of this phenomenon (Loy et al., 1980). In addition, the sympathetic sprouting response is sexually dimorphic and age dependent. Females exhibit a more robust response than males if the fimbria/fornix is transected at 2 weeks of age or later (Milner & Loy, 1980). However when the fimbria/fornix is transected in infancy (i.e. during the first postnatal week), the sprouting response is similar in both sexes (Milner & Loy, 1980).

Recently, the effect of ingrowths on cholinergic receptors has been studied by comparing septal lesioned rats (i.e. hippocampal cholinergic denervation with sympathetic ingrowth) with superior cervical ganglionectomized rats and dual lesioned rats (i.e. hippocampal cholinergic denervation without sympathetic ingrowth) (Roberson, Kolasa, Parsons & Harrell, 1997). Although cholinergic denervation (septal and dual lesioned groups) increased the number of muscarinic ACh receptors in the dorsal hippocampus as compared to control and ganglionectomized rats, the presence of hippocampal ingrowths (septal lesions) induced an elevation in muscarinic receptor number above that observed for cholinergic

denervation alone (dual lesions, no ingrowths). Interestingly, muscarinic receptor affinity was reduced in rats with ingrowths as compared to dual lesioned rats.

The functional significance of hippocampal sympathetic ingrowths is unknown, however this issue has been incorporated into an ongoing study in this laboratory. Neonatal rats were given either i.c.v. injections of 192 IgG-saporin (i.e. hippocampal cholinergic denervation with sympathetic ingrowths), subcutaneous injections of 6-hydroxydopamine (6-OHDA) (i.e. forebrain and sympathetic noradrenergic lesion) or both (i.e. hippocampal cholinergic denervation without sympathetic ingrowths). If hippocampal ingrowths were behaviorally compensatory, it could be expected that dual lesioned rats would exhibit behavioral impairments in the Morris water maze, delayed spatial alternation or the radial arm maze when compared to rats with 192 IgG-saporin lesions alone. In fact, no differences in platform latency between any groups were found in the Morris water maze, and the only difference between groups seen in the delayed spatial alternation task was the tendency of cholinergically lesioned rats (i.e. both 192 IgG-saporin and dual lesions) to perseverate to one side of the maze. Although the cholinergically lesioned rats made more working memory errors in the radial arm maze, all groups made comparable reference memory errors. It was concluded that neonatal cholinergic lesions impair working memory and that sympathetic ingrowths do not functionally compensate for this impairment.

Neonatal Cholinergic Lesions

The consequences of cholinergic lesions on the developing brain have received minimal attention. Rodent cholinergic basal forebrain afferents reach their cortical and

hippocampal targets at roughly the time of birth, but do not attain a mature pattern of connectivity until approximately 4 weeks post-partum (Coyle & Yamamura, 1976; Koh & Loy, 1989). A battery of behavioral tasks have been used to evaluate, in adulthood, rodents receiving cholinergic basal forebrain lesions in infancy. For example, electrolytic lesion of the nucleus basalis magnocellularis in the one day old mouse pup, while producing only transient cortical cholinergic denervation, produces persistent abnormalities of cortical morphology and cytoarchitecture (Bachman, Berger-Sweeney, Coyle & Hohmann, 1994). These abnormalities were correlated with behavioral deficits in adulthood, namely impairments in the Morris water maze and in retention of a passive avoidance task. Neonatal ibotenic acid administration to two day old rat pups produced a persistent, although modest, reduction in cortical ChAT activity (i.e. 18% less than control value at 8 months and 25% less than control value at 12 months post-lesion) (Sengstock, Johnson, Jantzen, Meyer, Dunn & Arendash, 1992). Behaviorally, adult rats showed performance deficits in the retention aspect of a passive avoidance task and the acquisition aspect of an active avoidance task. These rats were also impaired in a spatial alternation task and made more reference and working memory errors in the radial arm maze. Pappas et al. (1996) examined the effects in adulthood of i.c.v. 192 IgG-saporin administration to 7 day old rat pups. The immunotoxin produced a persistent loss of 52% of cortical and 84% of hippocampal ChAT activity, yet these rats were unimpaired in the Morris water maze or in a delayed spatial alternation task. However lesioned rats were less active and spent less time on the open arms of an elevated plus maze, suggesting increased timidity. A separate study confirmed that administration of 192 IgG-saporin produced reductions in ChAT levels which persisted for at least 8 months post-lesion

(Leanza, Nilsson, Nikkah, Wiley & Bjorklund, 1996b). Similar to the Pappas study, these rats showed no impairment in the Morris water maze. However subsequent administration of atropine sulfate or a re-lesion of the basal forebrain with 192 IgG-saporin disrupted effective search behavior in the Morris water maze, indicating that a total or near-total destruction of cholinergic afferents may be necessary before severe behavioral effects emerge.

Despite the lack of behavioral effects produced by neonatal 192 IgG-saporin lesions in adulthood, there is a possibility that neonatal cholinergic lesions give rise to developmental changes that disappear with maturation. Hohmann, Brooks and Coyle (1988) studied the histological effects on mouse pups of electrolytic lesions of the nucleus basalis magnocellularis performed within one day of birth. While ChAT levels in cortex were reduced by nearly 90% as compared to controls at postnatal day 5, the lesion was transient: ChAT levels began to rise by at latest postnatal day 7 and had returned to normal levels by postnatal day 30. Morphological changes, coinciding spatially with the loss of AChE fiber staining, were detected in the cortex of lesioned mice on postnatal day 8. These included a delay in emergence of cortical layer IV and of pyramidal cells in layer V, as well as a blurring of the laminar boundaries of the cortex, especially between layers III and IV and layers IV and V. The normalization of cortical cholinergic markers attenuated the severity of these morphological changes. A subsequent study compared the histological and behavioral effects of 192 IgG-saporin administration at both postnatal day 1 and postnatal day 7 on juvenile rats (Ricceri, Calamandrei & Berger-Sweeney, 1997). When assessed at 20 days of age, lesions at postnatal day 1 produced only a slight ChAT reduction in cortex, while postnatal day 7 lesions produced large ChAT depletions in cortex, hippocampus and septum. Behaviorally

postnatal day 1 lesions had no obvious effect, while postnatal day 7 lesioned rats were impaired in the acquisition of a passive avoidance task (assessed at postnatal day 15 and 16) and were less apt to explore than control rats in a hole board test (assessed at postnatal day 19). Similar to the Sengstock (1992), Pappas (1996) and Leanza (1996b) studies, these investigators found no evidence of abnormal cortical morphology.

Interestingly, none of the above studies have examined sex-dependent effects of neonatal cholinergic lesions despite sex-dependent differences in the development and activity of the cholinergic system. For example, during development the cholinergic septohippocampal pathway reaches functional maturity faster in females than in males, and female levels of p75 mRNA are consistently higher than in males during the first two weeks of life (Loy & Sheldon, 1987; Kornack, Lu & Black, 1991). Leanza et al. (1996b) did assess female rats in their study, however they pooled both males and females on all behavioral and biological assessments. Pappas et al. (1996) also assessed both male and female rats neurochemically, but did not find any sex-dependent differences. However these rats were only examined in adulthood. Consequently, despite the apparent lack of sex-dependent effects, further studies are needed to evaluate differences in sex-dependent response to neonatal cholinergic lesions during maturation.

Rett Syndrome

Understanding the developmental role of the cholinergic system in both males and females is especially important considering that forebrain cholinergic dysregulation is a major biological correlate of Rett syndrome, a putatively X-chromosome linked disorder of young

girls. The etiology of Rett syndrome is unknown and there is currently no known genetic, biochemical or pathological marker for this disease, thus diagnostic criteria are solely clinical, based on the disease profile and exclusion of other possible disorders. Rett syndrome is characterized clinically by a normal pre- and perinatal period of development, followed by a rapid decline in psychomotor skills anywhere between five months and four years of age (Hagberg, 1995). One of the first apparent symptoms of Rett syndrome is acquired microcephaly, after which girls quickly lose purposeful hand and language skills, develop psychomotor retardation, stereotypic hand movements, as well as gait and truncal apraxia. Secondary symptoms include apnea and/or hyperventilation, seizures, growth retardation (possibly a secondary symptom of malnutrition due to oral motor dysfunction), scoliosis and muscle spasticity (Hagberg, 1995). The extent to which Rett syndrome is an inherited disorder is still unclear. Although Rett syndrome does occur only in females and shows concordance in monozygotic twins, most cases of the disorder appear to be sporadic. Indeed, the risk of giving birth to a second girl with Rett syndrome is less than 0.4% (Dr. D. Glaze, personal communication, Ontario Rett Syndrome Assn. conference, November 1997).

The past decade has been extremely productive in terms of research into the biological substrates of this disease. The Rett brain is characterized by a significantly reduced cerebral volume, especially in the caudate nucleus and midbrain, with a preferential decrease in grey matter (Reiss, Faruque, Naidu, Abrams, Beaty, Bryan & Moser, 1993). Decreases in cell size and increases in cell packing density are apparent throughout the brain, with no evidence of gliosis or active degeneration (Bauman, Kemper & Arin, 1995). However reductions in the number of cholinergic cells in the basal forebrain as well as melanin-containing cells in the

substantia nigra have been documented (Kitt & Wilcox, 1995). As a result of the decline in cholinergic cell numbers, ChAT activity is reduced in the cortex, hippocampus, thalamus and basal ganglia (Wenk, Naidu, Casanova, Kitt & Moser, 1991). This disruption of cholinergic neurotransmission may underlie some of the cognitive impairments of Rett syndrome patients. Inconsistent and variable changes have also been reported in markers for dopamine, norepinephrine and serotonin (for review see Wenk, 1997). Recently, morphological abnormalities in cortical dendrites of Rett syndrome girls have been identified. These abnormalities include shortened apical and basal cortical pyramidal dendrites, as well as reduced numbers of dendrites and dendritic spines (Belichenko, Oldfors, Hagberg & Dahlstrom, 1994; Armstrong, Dunn, Antalffy & Trivedi, 1995). This is consistent with a report of reduced microtubule-associated protein-2 staining in the neocortex of Rett brain (Kaufmann, Naidu & Budden, 1995). Interestingly, a lack of cortical projections from transmitter systems such as dopamine and acetylcholine during ontogeny may serve to explain abnormalities in dendritic morphology. Should these projections not reach the cortex, cortical pyramidal cells may not receive the necessary stimulation to form appropriate dendritic architecture. These data suggest that Rett syndrome is a neurodevelopmental as opposed to a neurodegenerative disorder involving the dysregulation of a number of neural systems including, most notably at present, the forebrain cholinergic system.

While post mortem studies of Rett tissue are helpful in elucidating the impact of the disorder on the human brain, animal models offer the advantage of experimental manipulation of disease parameters in a controlled environment. Unfortunately, due to difficulty in the past of obtaining specific lesions of neurotransmitter systems such as ACh, animal lesion models

were not always representative of the disease states they tried to mimic. Even with the advent of a highly specific lesion method, the neonatal 192 IgG-saporin lesion model does not mimic all of the severe behavioral and pathological correlates of Rett syndrome. However it does replicate some of the major biological changes and thus deserves to be evaluated as a possible animal model for Rett syndrome.

Research Proposal

The proposed study assessed the impact of neonatal 192 IgG-saporin administration during development. Rats were lesioned at postnatal day 7 and examined both behaviorally and histologically at three developmentally relevant time points corresponding to late infancy (postnatal day 21), adolescence (postnatal day 45) and early adulthood (postnatal day 90). Behavioral analysis determined the extent of performance impairments and whether or not they ameliorated with maturation. Histological analysis determined the size and specificity of the lesion throughout the developmental period. Length of hippocampal sympathetic ingrowths were monitored at each time point. Additionally, a systematic comparison of both males and females on all parameters measured in this study provided a unique opportunity to determine whether the effects of neonatal 192 IgG-saporin administration are sexually dimorphic.

METHODS

Subjects and Design

Pregnant dams were obtained from Charles River and kept on a 12:12 hour reversed light/dark schedule in a humidity controlled room with food and water provided *ad libitum*. The pups were culled on postnatal day 1, with even numbers of each sex kept in each litter whenever possible to ensure an equal distribution of sexes and lesion conditions across litters. After weaning, all rats were group housed, except for the youngest rats who were not weaned before sacrifice.

Time point 1 (postnatal day 21):

28 male and 28 female rats were injected i.c.v. with either 192 IgG-saporin or vehicle at postnatal day 7. Rats were tested in the elevated plus maze at postnatal day 19. After one day of rest, half of the rats (7 male and 7 female controls, 7 male and 7 female lesioned) were sacrificed by decapitation and their brains were removed for future study. The remaining animals (7 male and 7 female controls, 7 male and 7 female lesioned) were perfused, with the right cerebral hemisphere saved for cryostat sectioning and the left cerebral hemisphere embedded in paraffin for future study.

Time point 2 (postnatal day 45):

28 male and 28 female rats were injected i.c.v. with either 192 IgG-saporin or vehicle at postnatal day 7. Rats were tested in the elevated plus maze at postnatal day 39. Starting

at postnatal day 40, rats were tested in the Morris water maze over 3 consecutive days. After one day of rest, half of the rats (7 male and 7 female controls, 7 male and 7 female lesioned) were sacrificed by decapitation and their brains were removed for future study. The remaining animals (7 male and 7 female controls, 7 male and 7 female lesioned) were perfused, with the right cerebral hemisphere saved for cryostat sectioning and the left cerebral hemisphere embedded in paraffin for future study.

Time point 3 (postnatal day 90):

28 male and 28 female rats were injected i.c.v. with either 192 IgG-saporin or vehicle at postnatal day 7. Rats were tested in the elevated plus maze at postnatal day 84. Starting at postnatal day 85, the rats were tested in the Morris water maze over 3 consecutive days. After one day of rest, half of the rats (7 male and 7 female controls, 7 male and 7 female lesioned) were sacrificed by decapitation and their brains were removed for future study. The remaining animals (7 male and 7 female controls, 7 male and 7 female lesioned) were perfused, with the right cerebral hemisphere saved for cryostat sectioning and the left cerebral hemisphere embedded in paraffin for future study.

Surgery

In order to prevent maternal cannibalism the dam was removed from the home cage prior to surgery and was not returned until all pups had fully regained consciousness. On postnatal day 7 pups were anesthetized with a 5% halothane/oxygen mixture, and then maintained on a 2% halothane/oxygen mixture throughout the surgical procedure. The pup was placed in a plaster body mould attached to a Kopf stereotaxic apparatus. The skull was

exposed and two holes were drilled bilaterally 1.8 mm from Bregma. A 10 μ l Hamilton syringe with a 30 gauge needle tip, attached to the stereotaxic apparatus via a Chaney adapter, was lowered 3.5 mm from the dura. Pups received a bilateral 1.5 μ l injection into the lateral ventricle of either a 200ng/ μ l solution of 192 IgG-saporin (300 ng per side, for a total of 600 ng), or vehicle (sterile phosphate buffered saline). The needle was lowered and raised over an interval of 2 minutes in order to limit intracerebral tissue tearing. Injections were infused over an interval of 2 minutes and the solution was given an additional 2 minutes to diffuse away from the injection site before retraction of the needle. The incision was closed with a small amount of Vetbond tissue glue (3M) and the pup was kept warm until it regained consciousness, after which it was returned to the home cage.

Elevated Plus Maze

At 19 days, 39 days and 84 days of age, rats were tested in the elevated plus maze. 19 day old rats were tested on a conventional sized mouse plus maze made of grey Plexiglas. The entire maze measured 55 cm across and was elevated 50 cm from the floor. The arms were 5 cm wide by 25 cm long and the sides walling the closed arms were 20 cm tall. 45 and 90 day old rats were tested on a conventional rat elevated plus maze made of white Plexiglas. The entire maze measured 104 cm across and was elevated 50 cm from the floor. The arms were 14 cm wide by 45 cm long and the sides walling the closed arms were 40 cm tall. Briefly, rats were placed at the center of the maze and given 10 minutes to explore. The experimenter recorded number of open and closed arm entries as well as time spent in open arms, closed arms and in the center area of the maze. An entry was recorded only if all four

of the rat's paws crossed the threshold of an arm. All testing was performed during the rats' dark cycle (between 0900 and 1700 hours) under reduced lighting. The experimenter was blind to the rats' identities during the testing period.

Morris Water Maze

Only 45 day and 90 day old rats were tested in the Morris water maze, as 21 day old rats were deemed too young to successfully navigate the maze. The maze consisted of a large white polypropylene pool 1.6 m in diameter filled to approximately 15 cm from the top with tepid water (20 ± 1 C). Large black visual cues were placed on the walls to facilitate spatial mapping of the room. On the day following elevated plus maze testing, rats began a series of 5 training sessions in the Morris water maze. Rats were given two sessions per day (a.m. and p.m.) for a total of five sessions over a three day period. Briefly, the pool was separated into four equal quadrants by four equidistant starting points designated north, south, east and west. A clear Plexiglas platform was placed approximately 2 cm below the water line in the southeast quadrant of the pool and was maintained in that position for all sessions for each rat. A training session consisted of a forced swim from each of the starting points, giving a total of 4 swims per session. Starting point order was varied quasi-randomly between each session. If the rat failed to locate the platform within 90 seconds, the rat was placed on the platform by the experimenter and allowed to rest for approximately 15 seconds, giving rats the opportunity to look around the room. Latency to find the hidden platform was recorded for each swim and averaged for each training session.

After the fifth training session, rats were given a no-platform probe trial. The hidden platform was removed and rats were placed in the pool at the north starting point. Rats were allowed to swim freely for 60 seconds. Probe trials were recorded using a Sony VHS video camera. Swim paths for each rat were traced onto paper from the video recording. Path length was determined by tracing swim paths with a cartographer's wheel. Swim speed was calculated by dividing total path length by the duration of the trial (60 seconds). The experimenter was blind to the rats' identities during the testing period.

Tissue Preparation

Rats were deeply anesthetized with sodium pentobarbitol and perfused as follows: 21 day old pups were perfused with 30 ml of heparinized saline followed by 30 ml of 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4; 45 day old rats were perfused with 45 ml of heparinized saline followed by 60 ml of 4% paraformaldehyde; 90 day old rats were perfused with 100 ml heparinized saline followed by 250 ml 4% paraformaldehyde. Brains were removed and post-fixed for 24 hours in 4% paraformaldehyde, after which they were separated into right and left cerebral hemispheres by a midline scalpel cut. The left hemisphere, including all of the cerebellum, was paraffin embedded and set aside for future study, while the right half was washed first in 0.1M PB, then in 10% sucrose in 0.1M PB, and finally in 20% sucrose in 0.1M PB in preparation for sectioning in a Reichert-Jung cryostat. Both 16 μ m and 40 μ m coronal sections were taken from areas corresponding to plates 13 to 22 of Paxinos and Watson (1986). 16 μ m sections were mounted onto gelatin coated microscope slides and stored at -20 C until needed. 40 μ m sections were floated in a

cryoprotectant solution (37.5% sucrose, 37.5% glycerol in 0.1M PB) in polystyrene well plates and stored at -20 C until needed.

Before staining, 16 μ m sections were warmed, dried, and a ring of rubber cement was placed around the tissue on the slide to create a well in which to put staining solutions. 40 μ m sections were brought up to room temperature and washed in 20% sucrose, 10% sucrose and twice in 0.1M PB.

AChE Histochemistry

Histochemistry for the visualization of AChE-containing fibers was performed only on 16 μ m sections. Briefly, slides were collected in 0.1M PB and given 3 x 5 minute washes in 0.1M sodium acetate. Sections were incubated overnight in the dark in a solution containing 0.68% sodium acetate, 0.1% cupric sulfate, 0.12% glycine and 0.001% acetylthiocholine iodide, pH 5.0. The next morning sections were rinsed 2 x 5 minutes in 0.1M sodium acetate and then reacted in a 1% sodium sulfide solution, pH 7.5. Sections were allowed to develop for 10 minutes, then were given 2 x 5 minute washes in 0.1M sodium acetate. Sections were then rinsed with water, dehydrated in a series of ascending alcohols, cleared with clearene and coverslipped.

Immunohistochemistry

16 μ m sections were stained with monoclonal antibodies raised in mouse against p75 (1:75 dilution; Boehringer Mannheim), dopamine β -hydroxylase (DBH; 1:2000; Chemicon) and tyrosine hydroxylase (TH; 1:15 000; Sigma). 40 μ m sections were stained with the same

p75 (1:125) and DBH (1:4000) antibodies as well as two other monoclonal antibodies raised in mouse against ChAT protein (1:5000; Chemicon) and parvalbumin (1:25 000; Sigma). Secondary antibody was a biotinylated anti-mouse Ig raised in sheep (Amersham); tertiary antibody was a streptavidin biotinylated horseradish peroxidase complex (Amersham). Both secondary and tertiary antibodies were used at a dilution of 1:100 for all immunohistochemical procedures.

Briefly, sections were rinsed in 10mM phosphate buffered saline (PBS), pH 7.2. Sections were incubated overnight at room temperature in primary antibodies diluted to working concentrations with 0.3% lambda carrageenan, 0.3% bovine serum albumin and 0.3% triton X-100 in PBS. Lambda carrageenan and bovine serum albumin are used to block nonspecific antibody binding; triton X-100 is a surfactant used to recover membrane components under non-denaturing conditions. The next morning sections were rinsed 2 times in PBS and incubated in secondary antibody for 2 hours at room temperature, then rinsed twice again and incubated in tertiary antibody for 2 hours. After 2 rinses in PBS, sections were reacted for 10 minutes in 0.02% 3,3'-diaminobenzidine, 0.6% ammonium nickel sulfate, 0.01% hydrogen peroxide in 50mM Tris buffer, pH 7.4. 16 μ m sections were rinsed in PBS and allowed to dry before dehydration, clearing and coverslipping; 40 μ m sections were mounted onto microscope slides, allowed to dry, and then were dehydrated, cleared and coverslipped.

Histological Measurements

Sections were visualized with an Olympus BH-2 microscope coupled to a Sony CCD video camera, and digitized with a commercial imaging software package (MCID Imaging Research). The digitized image from the microscope was displayed on a monitor, and cells were counted by positioning the cursor over the cell and clicking the mouse button. Cell counts from each nucleus were averaged over several sections as follows: medial septum, vertical limb of the diagonal band and horizontal limb of the diagonal band, 2 sections each; ventral pallidum, 3 sections; globus pallidus, 5 sections; nucleus basalis magnocellularis, 4 sections. The computer software was equipped for length measurements by calibrating the number of pixels in an image to a given length from a microscope calibration bar. Hippocampal ingrowths were measured by positioning the cursor over the beginning of the ingrowth, holding down the mouse button, and dragging the cursor along the length of the ingrowth. Ingrowth length in the lower blade of stratum moleculare was averaged over 4 sections; ingrowth length in all other subfields of the hippocampus was averaged over 3 sections. The experimenter was blind to the rats' identities during all measurement procedures.

RESULTS

Post-Surgical Survival

Of the 103 rat pups injected with 192 IgG-saporin, 3 male pups died during the surgical procedure. Of the remaining 100 lesioned pups, 14 did not survive to the behavioral testing phase of the experiment. Three males died between 7 and 9 days after surgery and 5 were euthanized one to two weeks after surgery due to a general failure to thrive. Four female pups died between one and two weeks after surgery, and one was euthanized one week after surgery due to a general failure to thrive. One lesioned pup of unknown sex was cannibalized by its mother 3 days after surgery. Of the 91 vehicle injected pups, none died during the surgical procedure and only one female pup died after surgery. Cause of death was unknown, although the pup appeared to recover from surgery normally until developing an apparent circulatory problem in the hind legs roughly one week after the surgical procedure.

Weight

Analysis of variance using age (21, 45 or 90 days), gender (male or female) and surgery condition (vehicle control or 192 IgG-saporin) as factors revealed a significant main effect of surgery condition ($F(1,153)=13.78$, $p<0.001$). 192 IgG-saporin treated rats weighed 25% less than controls at 21 days of age and 7% less than controls at 45 and 90 days of age. The age by surgery condition interaction was not significant. However, a significant age by gender interaction ($F(2,153)=146.45$, $p<0.001$) was found. Males and females had similar

Figure 1. Group means for body weight. 192 IgG-saporin treated rats had significantly lower body weights than controls ($F(1,153)=13.78$, $p<0.001$). Males were significantly heavier than females at 45 and 90 days of age, but not at 21 days of age ($F(2,153)=146.45$, $p<0.001$; Tukey's HSD, $p<0.05$).

Body Weight

average weights at 21 days of age, but by 45 days of age males weighed almost 50% more than females, and by 90 days of age were nearly twice as heavy as females (Tukey's Honestly Significant Difference (HSD), $p < 0.05$). Graphical representation of group means is shown in Figure 1.

Elevated Plus Maze

Analysis of variance using age, gender and surgery condition as factors revealed a significant main effect of age on total number of arm entries ($F(2, 157) = 68.43$, $p < 0.001$). The 21 day old rats made 83% fewer total entries than either 45 day old rats or 90 day old rats (Tukey's HSD, $p < 0.05$). No significant interactions or main effects of either surgery condition or gender were found.

Number of entries into both closed and open arms were expressed as percentages of total arm entries for statistical analysis. Neither 192 IgG-saporin nor gender had an effect on entries into closed arms. However post hoc analysis of the significant main effect of age ($F(2, 156) = 14.44$, $p < 0.001$) indicated that at 21 days, rats made approximately 24% more of their total entries into closed arms than at 45 or 90 days (Tukey's HSD, $p < 0.05$). Group means are shown in Figure 2. Similar to closed arm entries, neither 192 IgG-saporin treatment nor gender had an effect on open arm entries. However at 21 days, rats made approximately 54% fewer of their total entries into open arms than at 45 or 90 days ($F(2, 156) = 14.07$, $p < 0.001$; Tukey's HSD, $p < 0.05$). Group means are shown in Figure 3. No interactions for either closed or open arm entries were found.

Time spent in each of the three portions of the maze (i.e. closed arms, open arms,

Figure 2. Group means for closed arm entries and closed arm time in the elevated plus maze. Top panel: 21 day old rats made significantly more of their entries into closed arms than 45 or 90 day old rats ($F(2,156)=14.44$, $p<0.001$; Tukey's HSD, $p<0.05$). Bottom panel: 192 IgG-saporin significantly reduced time spent in closed arms ($F(1,156)=5.36$, $p<0.03$). 21 day old rats spent significantly more time in the closed arms than 45 or 90 day old rats ($F(2,156)=18.36$, $p<0.001$; Tukey's HSD, $p<0.05$).

Closed Arm Entries

Control 192 IgG-saporin

Closed Arm Time

Figure 3. Group means for open arm entries and open arm time in the elevated plus maze. Top panel: 21 day old rats made significantly fewer open arm entries than 45 or 90 day old rats ($F(2,156)=14.07$, $p<0.001$; Tukey's HSD, $p<0.05$). Bottom panel: 192 IgG-saporin significantly increased time spent by females, but not males, on the open arms ($F(1,156)=5.71$, $p<0.02$; Tukey's HSD, $p<0.05$). 21 day old rats spent significantly less time on the open arms than 45 or 90 day old rats ($F(2,156)=7.15$, $p<0.005$; Tukey's HSD, $p<0.05$).

Open Arm Entries

Control 192 IgG-saporin

Open Arm Time

center area) was expressed as a percentage of total time for statistical analysis. 192 IgG-saporin significantly reduced time spent in closed arms by 8% as compared to control rats ($F(1,156)=5.36$, $p<0.03$). Gender had no effect on time spent in closed arms, but a significant main effect of age was found ($F(2,156)=18.36$, $p<0.001$). At 21 days, rats spent 19% more time in the closed arms than at 45 days, and 28% more time in the closed arms than at 90 days (Tukey's HSD, $p<0.05$). There were no significant interactions for the closed arm time variable. Group means are shown in Figure 2.

A significant gender by surgery condition interaction was found on time spent on the open arms of the maze ($F(1,156)=5.71$, $p<0.02$). 192 IgG-saporin treatment increased the amount of time spent by females on the open arms by approximately 127%, while having no effect on time spent by males on the open arms (Tukey's HSD, $p<0.05$). 192 IgG-saporin increased time spent on the open arms by 62% over all groups ($F(1,156)=5.03$, $p<0.03$), and females spent 75% more time on the open arms than did males ($F(1,156)=6.66$, $p<0.02$). In addition, a significant main effect of age indicated that 21 day old rats spent approximately 66% less time on the open arms than either 45 or 90 day old rats ($F(2,156)=7.15$, $p<0.005$; Tukey's HSD, $p<0.05$). Group means are shown in Figure 3.

Immunotoxin treatment had no effect on time spent in the center area of the maze. While females spent almost 25% less time in the center area than males did, this effect did not achieve statistical significance ($F(1,156)=3.65$, $p=0.058$). A significant main effect of age indicated that at 21 days rats spent 60% and 73% less time in the center than at 45 or 90 days respectively, and at 45 days spent 35% less time in the center than at 90 days ($F(2,156)=21.32$, $p<0.001$; Tukey's HSD, $p<0.05$). Group means are shown in Figure 4.

Figure 4. Group means for center area time in the elevated plus maze. 21 day old rats spent less time in the center than 45 or 90 day old rats, and 45 day old rats spent less time in the center than 90 day old rats ($F(2,156)=21.32$, $p<0.001$; Tukey's HSD, $p<0.05$).

Center Time

Morris Water Maze

Platform location latency was analyzed using a repeated measures analysis of variance with age (45 or 90 days), gender (male or female) and surgery condition (vehicle control or 192 IgG-saporin) as the between subjects variables and session (1 to 5) as the within subjects variable. A significant main effect of session was found ($F(4,416)=193.54$, $p<0.001$), indicating that latencies decreased over the course of the testing period (i.e. the data demonstrated a learning curve). No significant interactions involving session and the between subjects variables, or involving the between subjects variables alone, were found (see Figures 5 and 6 for graphical representation of group means). Significant main effects of 192 IgG-saporin treatment ($F(1,104)=4.11$, $p<0.05$), gender ($F(1,104)=7.50$, $p<0.01$) and age ($F(1,104)=10.64$, $p<0.005$) were found. Across all 5 sessions, 192 IgG-saporin increased platform location latency by 10%, females had 14% longer latencies than males and 45 day old rats had 17% longer latencies than 90 day old rats.

Inspection of the maze acquisition data indicated that it would be clarified by a breakdown in terms of session despite the fact that there were no significant interactions involving the within subjects variable “session”. The data would be more interpretable in terms of, for example, performance variables. Thus post hoc tests for differences in means of surgery condition, gender and age were performed at each of the 5 sessions. Tukey’s HSD indicated that 192 IgG-saporin treatment significantly increased platform location latency only at session 1 (by 15%; $p<0.05$); all other differences were not significant (session 2, increase 0.1%; session 3, increase 10%; session 4, increase 17%; session 5, increase 4%) (Figure 7). Females had longer latencies than males at session 1 (11% increase), session 2 (23% increase)

Figure 5. Group means of 45 day old rats for platform location latency in the Morris water maze.

MWM 45 Day: Males **Females**

Figure 6. Group means of 90 day old rats for platform location latency in the Morris water maze.

MWM 90 Day: Males **Females**

Figure 7. Graph of control vs. 192 IgG-saporin platform location latency in the Morris water maze. 192 IgG-saporin increased platform latency only at session 1 ($F(1,104)=4.11$, $p<0.01$; Tukey's HSD, $p<0.05$).

Morris Water Maze: Control vs 192 IgG Saporin

Figure 8. Graph of male vs. female platform location latency in the Morris water maze. Females had significantly longer platform latencies at sessions 1, 2 and 5 ($F(1,104)=7.50$, $p<0.01$; Tukey's HSD, $p<0.05$).

Morris Water Maze: Male vs Female

Figure 9. Graph of 45 vs. 90 day platform location latency in the Morris water maze. 45 day old rats had significantly longer platform latencies at sessions 2, 3, 4 and 5 ($F(1,104)=10.64$, $p<0.005$; Tukey's HSD, $p<0.05$).

Morris Water Maze: 45 vs 90 Days

and session 5 (27% increase) ($p < 0.05$), but differences at session 3 (4% increase) and session 4 (7% increase) were not significant (Figure 8). 45 day old rats had significantly longer platform latencies than 90 day old rats at all sessions except session 1 (Tukey's HSD, $p < 0.05$). At sessions 2 and 3, 45 day old rats showed an increase in platform latency of 16% and 18% respectively over 90 day old rats; by session 4 the increase had risen to 31%, and by session 5 45 day old rats had 39% longer platform location latencies than 90 day old rats (Figure 9).

Probe trial data was analyzed using a repeated measures analysis of variance with age (45 or 90 days), gender (male or female) and surgery condition (vehicle control or 192 IgG-saporin) as between subjects factors and quadrant (northeast, northwest, southeast, southwest) as the within subjects variable. No main effects of the between subjects variables were found. There was a significant main effect of quadrant ($F(3,318)=255.20$, $p < 0.001$), with post hoc tests showing that overall, the rats preferred the target quadrant (southeast) above all others (Tukey's HSD, $p < 0.05$) (Figures 10 and 11).

Swim speed was calculated from probe trial data. 192 IgG-saporin treated rats swam on average roughly 6% faster than control rats ($F(1,106)=4.31$, $p < 0.05$). In addition, 90 day old rats swam approximately 14% faster than their 45 day old counterparts ($F(1,106)=20.69$, $p < 0.001$). No main effect of gender nor any interactions were found (Figure 12).

Cell Counts

Analysis of variance for all cell counts was performed using age (21, 45 or 90 days), gender (male or female) and surgery condition (vehicle control or 192 IgG-saporin) as

Figure 10. Graph of probe trial data from 45 day old rats in the Morris water maze. All rats showed a preference for the target quadrant ($F(3,318)=255.20$, $p<0.001$; Tukey's HSD, $p<0.05$).

Morris Water Maze Probe Trial: 45 Days

Figure 11. Graph of probe trial data from 90 day old rats in the Morris water maze. All rats showed a preference for the target quadrant ($F(3,318)=255.20$, $p<0.001$; Tukey's HSD, $p<0.05$).

Morris Water Maze Probe Trial: 90 Days

□ NE Quadrant ■ SE (Platform) Quadrant
▨ NW Quadrant ▤ SW Quadrant

Figure 12. Graph of swim speeds in the Morris water maze. 192 IgG-saporin significantly increased swim speed ($F(1,106)=4.31$, $p<0.05$). 90 day old rats swam significantly faster than 45 day old rats ($F(1,106)=20.69$, $p<0.001$).

Morris Water Maze Swim Speed

factors. Graphical representation of group means for p75- and ChAT-positive counts in basal forebrain nuclei can be found in Figures 13 and 14 respectively.

Medial Septum

Significant main effects of both immunotoxin treatment and gender were found on p75-positive cell numbers in the medial septum. Treatment with 192 IgG-saporin reduced p75-positive cell number in the medial septum by approximately 90% ($F(1,70)=136.61$, $p<0.001$). This effect was consistent across age groups and genders. Male rats had roughly 40% more p75 positive cells than females ($F(1,70)=5.88$, $p<0.02$). This effect was consistent across age groups. No significant interactions were found.

Similar to the results obtained with p75-positive cells, 192 IgG-saporin treatment reduced ChAT-positive cell numbers by approximately 80% ($F(1,70)=94.04$, $p<0.001$) (see Figure 15 for photographic illustration). This effect was consistent across age groups and genders. Male and female rats did not differ in number of ChAT-positive cells; no other group differences or interactions were found.

192 IgG-saporin reduced parvalbumin-positive cell counts in the medial septum by 30% overall ($F(1,64)=4.73$, $p<0.04$). Post-hoc analysis of the main effect of age ($F(2,64)=21.57$, $p<0.001$) with Tukey's HSD indicated that 21 day old rats had fewer cells than 45 and 90 day old rats (53% and 75% fewer respectively, $p<0.05$), and 45 day old rats had fewer cells than 90 day old rats (45% fewer, $p<0.05$). No main effect of gender nor any interactions were found (Figure 16).

Figure 13. Group means for p75-positive cell counts in the basal forebrain. 192 IgG-saporin severely reduced cell numbers in all nuclei. Males had significantly more cells in the septum ($F(1,70)=5.88$, $p<0.02$) and VLDB ($F(1,70)=5.04$, $p<0.03$) than females. 21 day old control rats had significantly more cells in the N basalis than 45 or 90 day old control rats ($F(2,72)=10.03$, $p<0.001$; Tukey's HSD, $p<0.05$). 90 day old rats had significantly more cells than 45 or 21 day old rats in the VLDB ($F(2,70)=4.78$, $p<0.02$; Tukey's HSD, $p<0.05$). Abbreviations: HLDB, horizontal limb of the diagonal band; N basalis, nucleus basalis magnocellularis; VLDB, vertical limb of the diagonal band; V pallidum, ventral pallidum. Legend: crosshatch indicates control; black indicates 192 IgG-saporin.

P75^{NTR}-IR Cells

Figure 14. Group means for ChAT-positive cell counts in the basal forebrain. 192 IgG-saporin severely reduced cell numbers in all nuclei. Males had significantly more cells in the N basalis ($F(1,72)=4.53$, $p<0.04$) than females. 90 day old rats had significantly more cells in the HLDB than 21 day old rats ($F(2,71)=5.15$, $p<0.01$; Tukey's HSD, $p<0.05$). 90 day old rats had significantly more cells in the V pallidum than 45 day old rats ($F(2,71)=4.01$, $p<0.03$). 21 day old rats had significantly more cells in the globus pallidus ($F(2,72)=13.23$, $p<0.001$) and N basalis ($F(2,72)=4.55$, $p<0.02$) than 45 or 90 day old rats (Tukey's HSD, $p<0.05$). Abbreviations: HLDB, horizontal limb of the diagonal band; N basalis, nucleus basalis magnocellularis; VLDB, vertical limb of the diagonal band; V pallidum, ventral pallidum. Legend: crosshatch indicates control; black indicates 192 IgG-saporin.

ChAT-IR Cells

Figure 15. Photomicrographs of ChAT- and p75-positive cells in the medial septum and nucleus basalis magnocellularis. Top four photographs (A-D): ChAT-positive cells in the medial septum/vertical limb of the diagonal band of female rats. A: 21 day old control. B: 21 day old lesioned. C: 90 day old control. D: 90 day old lesioned. Bottom four photographs (E-H): p75-positive cells in the nucleus basalis magnocellularis of male rats. E: 21 day old control. F: 21 day old lesioned. G: 90 day old control. H: 90 day old lesioned.

Figure 16. Group means for parvalbumin-positive cells in the medial septum. 192 IgG-saporin significantly reduced parvalbumin-positive cells ($F(1,64)=21.57$, $p<0.04$). 90 day old rats had more cells than 45 day old rats, and 45 day old rats had more cells than 21 day old rats ($F(2,64)=21.57$, $p<0.001$; Tukey's HSD, $p<0.05$).

Parvalbumin-IR Cells: Medial Septum

Diagonal Band of Broca

A significant main effect of surgery condition was found on p75-positive cell numbers in the vertical limb of the diagonal band of Broca ($F(1,70)=285.75$, $p<0.001$). 192 IgG-saporin treatment reduced p75-positive cell numbers in the vertical limb by more than 90%; this effect was consistent across gender and age groups. There was a main effect of gender ($F(1,70)=5.04$, $p<0.03$). Males had approximately 25% more p75-positive cells than females at 21 and 45 days, but only 11% more cells at 90 days; the age by gender interaction was not significant. Post-hoc analysis of the main effect of age ($F(2,70)=4.78$, $p<0.02$) indicated that 90 day old rats had significantly more (35%) p75-positive cells than 45 day old rats or 21 day old rats in the vertical limb (Tukey's HSD, $p<0.05$). 192 IgG-saporin treatment also reduced ChAT-positive cell numbers in the vertical limb by more than 85% ($F(1,69)=128.78$, $p<0.001$), an effect which was consistent across gender and age groups. No other group differences or interactions in ChAT-positive cell numbers were found.

Immunotoxin treatment reduced p75-positive cell numbers in the horizontal limb of the diagonal band of Broca by 86% across groups ($F(1,71)=374.76$, $p<0.001$). While the reductions were generally greater at 21 days than at 45 (90%) or 90 (83%) days, and females had greater reductions (90%) than males (83%), no interactions were significant. ChAT-positive cell numbers were reduced by 78% ($F(1,71)=204.61$, $p<0.001$) in the horizontal limb. Similar to p75-positive cell reductions, 21 day old rats generally had more cell loss (i.e. greater than 85%) than older rats (i.e. less than 80%), but the age by surgery condition interaction was not significant. No gender effects were found, but there was a significant main effect of age on ChAT-positive cell number in the horizontal limb ($F(2,71)=5.15$, $p<0.01$).

Tukey's HSD indicated that 90 day old rats had 43% more ChAT-positive cells than 21 day old rats ($p < 0.05$). No other significant effects were found.

Area of the Nucleus Basalis

The area of the nucleus basalis was subdivided into three separate nuclei for counting: the ventral pallidum, the globus pallidus and the nucleus basalis magnocellularis. 192 IgG-saporin significantly reduced p75-positive cell numbers across groups in the ventral pallidum, globus pallidus and nucleus basalis magnocellularis by 85% ($F(1,72)=206.86$, $p < 0.001$), 82% ($F(1,72)=274.53$, $p < 0.001$) and 90% ($F(1,72)=693.36$, $p < 0.001$) respectively (see Figure 15 for photographic illustration). A significant age by surgery condition interaction for p75 cells in the nucleus basalis magnocellularis ($F(2,72)=10.03$, $p < 0.001$) indicated that while cell number declined with age in control rats (i.e. 21 day old control rats had 21% more p75-positive cells in the nucleus basalis magnocellularis than either 45 or 90 day old rats; Tukey's HSD, $p < 0.05$), cell number did not differ significantly with age in 192 IgG-saporin treated rats (Tukey's HSD, $p < 0.05$). No main effect of gender was found in any of the three nuclei.

The immunotoxin had a similar main effect on ChAT-positive cell counts in the three nuclei. Cell numbers were reduced across groups in 192 IgG-saporin treated rats by 29% in the ventral pallidum ($F(1,71)=41.09$, $p < 0.001$), 57% in the globus pallidus ($F(1,72)=116.47$, $p < 0.001$) and 73% in the nucleus basalis magnocellularis ($F(1,72)=203.32$, $p < 0.001$). Post hoc tests of the significant age by surgery condition in the globus pallidus indicated that while cell number declined with age in control rats, cell number did not significantly differ with age in 192 IgG-saporin treated rats (Tukey's HSD, $p < 0.05$). A main effect of gender was seen

only in the nucleus basalis magnocellularis, with males having a significantly greater number (19% more) of ChAT-positive cells than females ($F(1,72)=4.53$, $p<0.04$). This trend was apparent in ChAT-positive cells in the ventral pallidum as well, but did not achieve statistical significance ($F(1,71)=3.77$, $p=0.056$). No other interactions were found, but there was a main effect of age in all three nuclei. Post-hoc tests indicated that at 90 days rats had significantly more ChAT-positive cells in the ventral pallidum than at 45 days, but not 21 days (Tukey's HSD, $p<0.05$); rats at 45 days had significantly fewer ChAT-positive cells in the globus pallidus than at 21 days but not 90 days (Tukey's HSD, $p<0.05$); rats at 45 days had significantly fewer ChAT-positive cells in the nucleus basalis magnocellularis than at 21 days but not 90 days (Tukey's HSD, $p<0.05$).

Caudate

The immunotoxin reduced numbers of ChAT-positive cells in the caudate by 22% across groups ($F(1,72)=19.99$, $p<0.001$) (see Figure 17 for graphical representation of group means; see Figure 18 for photographic illustration). There was no main effect of gender, but a significant main effect of age was found ($F(2,72)=20.87$, $p<0.001$), with Tukey's HSD indicating that 21 day old rat caudate contained over 40% more ChAT-positive cells than either 45 or 90 day old rat caudate ($p<0.05$).

AChE Fiber Density

Visual inspection of the tissue sections indicated that AChE fiber density in hippocampus and cortex was almost completely abolished in the 192 IgG-saporin treated rats

Figure 17. Group means for ChAT-positive cells in the caudate. 192 IgG-saporin significantly reduced ChAT-positive cells relative to controls ($F(1,72)=19.99$, $p<0.001$). 21 day old rats had significantly more cells than 45 or 90 day old rats ($F(2,72)=20.87$, $p<0.001$; Tukey's HSD, $p<0.05$).

ChAT-IR Cells: Caudate Nucleus

Figure 18. Photomicrographs of ChAT-positive cells in the male caudate nucleus. A: 21 day old control. B: 21 day old lesioned. C: 90 day old control. D: 90 day old lesioned.

Figure 19. Photomicrographs of AChE fiber density. Top four photographs: AChE fibers in the cortex of female rats. A: 21 day old control. B: 21 day old lesioned. C: 90 day old control. D: 90 day old lesioned. Bottom four photographs: AChE fibers in the hippocampus of female rats. E: 21 day old control. F: 21 day old lesioned. G: 90 day old control. H: 90 day old lesioned.

(see Figure 19 for photographic illustration). The decrease in AChE staining was still evident at 90 days and appeared to be equal for males and females.

Hippocampal Sympathetic Ingrowths

Analysis of variance on p75-positive hippocampal ingrowth length was performed using age (21, 45 or 90 days), gender (male or female) and surgery condition (vehicle control or 192 IgG-saporin) as factors.

Stratum Moleculare

The stratum moleculare was subdivided into upper and lower blades corresponding to the portions above and below the hilus of the dentate gyrus (as it appears in coronal sections) respectively. Average ingrowth length was 0.202 mm in the upper blade of stratum moleculare across groups in immunotoxin treated rats, while no ingrowths were observed in control rats at any age ($F(1,71)=7.10$, $p<0.02$). Ingrowths did not appear in lesioned rats before 45 days, at which time average length was 0.2 mm. By 90 days average length had increased to 0.42 mm, but the age by surgery condition interaction was not significant. No main effect of gender was found. See Table I for a list of group means. Average ingrowth length across groups in the lower blade of stratum moleculare of lesioned rats was 0.79 mm, which was significantly different from the average control length of 0.005 mm ($F(1,71)=16.78$, $p<0.001$). Post hoc analysis of a significant age by surgery condition interaction in the lower blade of stratum moleculare ($F(2,71)=4.42$, $p<0.02$) indicated that ingrowths did not appear before 45 days of age in lesioned rats, but average length of 45 and

Upper Blade of Stratum Moleculare				
		21 Days	45 Days	90 Days
Control	<i>Male</i>	0.000 ± 0.000	0.000 ● 0.000	0.000 ± 0.000
	<i>Female</i>	0.000 ± 0.000	0.000 ± 0.000	0.000 ● 0.000
192 IgG-Saporin	<i>Male</i>	0.000 ± 0.000	0.244 ± 0.173*	0.457 ± 0.383*
	<i>Female</i>	0.000 ± 0.000	0.156 ± 0.043*	0.382 ± 0.211*

Table 2. Length of ingrowths in the upper blade of stratum moleculare. Lengths are reported in millimeters, ± standard error of the mean. * indicates significant difference from control group, Tukey's HSD, $p < 0.05$.

Lower Blade of Stratum Moleculare				
		21 Days	45 Days	90 Days
Control	<i>Male</i>	0.005 ± 0.005	0.025 ± 0.020	0.000 ± 0.000
	<i>Female</i>	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
192 IgG-Saporin	<i>Male</i>	0.000 ± 0.000	1.334 ± 0.523*†	1.200 ± 0.821*†
	<i>Female</i>	0.000 ± 0.000	0.756 ± 0.274*†	1.454 ± 0.609*†

Table 1. Length of ingrowths in the lower blade of stratum moleculare. Lengths are reported in millimeters, $\pm$ standard error of the mean. * indicates significant difference from control group, Tukey's HSD, $p < 0.05$; † indicates significant difference from 21 day lesion group, Tukey's HSD, $p < 0.05$.

90 day old rats was not different (Tukey's HSD, $p < 0.05$). No main effect of gender on ingrowth length was seen in the lower blade of stratum moleculare, but a main effect of age was found ($F(2,71) = 4.45$, $p < 0.02$). Post-hoc analysis with Tukey's HSD indicated that lesioned rats showed a significant increase in ingrowth length of 440 times between 21 and 45 days and an increase in ingrowth length of 548 times between 21 days and 90 days ($p < 0.05$). See Table 2 for a list of group means.

Hilus of the Dentate Gyrus

A significant age by surgery condition interaction was found in the analysis of ingrowth length in the hilus of the dentate gyrus ($F(2,71) = 7.65$, $p < 0.005$). While overall average ingrowth length of 192 IgG-saporin treated rats in the hilus of the dentate gyrus was 1.21 mm, which was significantly different from the control average of 0.005 mm ($F(1,71) = 29.59$, $p < 0.001$), ingrowths in 192 IgG-saporin treated rats were not observed until 45 days of age (Tukey's HSD, $p < 0.05$). Average ingrowth length in lesioned rats at 45 days was 1.96 mm and at 90 days was 1.99 mm, which represented an increase in length of 393 times at 45 days as compared to 21 days and an increase of 332 times at 90 days as compared to 21 days (Tukey's HSD, $p < 0.05$). Average ingrowth length between 45 and 90 day old 192 IgG-saporin rats was not significantly different. No significant main effect of gender was found. Group means are listed in Table 3; see Figure 20 for photographic illustration.

Stratum Lucidum

A significant age by gender by surgery condition interaction was found in the analysis

Hilus of the Dentate Gyrus				
		21 Days	45 Days	90 Days
Control	<i>Male</i>	0.004 ± 0.004	0.000 ± 0.000	0.000 ± 0.000
	<i>Female</i>	0.014 ± 0.014	0.012 ± 0.012	0.000 ± 0.000
192 IgG-Saporin	<i>Male</i>	0.009 ± 0.009	2.168 ± 0.745*†	1.448 ● 0.768*†
	<i>Female</i>	0.000 ± 0.000	1.759 ± 0.542*†	1.913 ± 0.645*†

Table 3. Length of ingrowths in the hilar region of the dentate gyrus. Lengths are reported in millimeters, ± standard error of the mean. * indicates significant difference from control group, Tukey's HSD, $p < 0.05$; † indicates significant difference from 21 day lesion group, Tukey's HSD, $p < 0.05$.

Figure 20. Photomicrographs of hippocampal ingrowths in the dentate gyrus of male rats. A: 21 day old control, p75-positive. B: 21 day old lesioned, p75-positive. C: 45 day old control, p75-positive. D: 45 day old lesioned, p75-positive. E: 90 day old control, p75-positive. F: 90 day old lesioned, p75-positive. G: 90 day old control, DBH-positive. H: 90 day old lesioned, DBH-positive.

of ingrowth length in the stratum lucidum ($F(2,71)=3.62$, $p<0.04$). Average ingrowth length was different from control and from all other lesioned groups only in the 45 day old male lesioned rats; no ingrowths were detected in 90 day old male lesioned rats, 45 day old female lesioned rats, and ingrowth length in 90 day old female lesioned rats was not significantly different from control (Tukey's HSD, $p<0.05$). Similar to other subfields of the hippocampus, no ingrowths were detected in 21 day old lesioned rats. A significant main effect of surgery condition was found ($F(1,71)=4.43$, $p<0.04$), but main effects of age and gender were not significant. Group means are listed in Table 4.

Stratum Lucidum				
		21 Days	45 Days	90 Days
Control	<i>Male</i>	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
	<i>Female</i>	0.000 ± 0.000	0.018 ± 0.018	0.000 ± 0.000
192 IgG-Saporin	<i>Male</i>	0.000 ± 0.000	0.554 ± 0.344*†	0.000 ± 0.000
	<i>Female</i>	0.000 ± 0.000	0.007 ± 0.007	0.289 ± 0.217

Table 4. Length of ingrowths in stratum lucidum. Lengths are reported in millimeters, ± standard error of the mean. * indicates significant difference from control group, Tukey's HSD, $p < 0.05$; † indicates significant difference from all other lesion groups, Tukey's HSD, $p < 0.05$.

DISCUSSION

Post-Surgical Survival

192 IgG-saporin was found to cause death in a higher number of male (11) than female (5) rat pups. Female rats have been shown to express higher levels of p75 mRNA in the basal forebrain than males, presumably indicating more p75-positive cells during the first two weeks of life (Kornack et al., 1991). Accordingly they would then be expected to exhibit increased sensitivity to 192 IgG-saporin administration. Surprisingly though, this was not the case. In fact, if rats who did not survive post-surgically were those maximally affected by the toxin, then it would seem that male pups were more sensitive to this type of lesion. However it may be that the 600 ng dose of 192 IgG-saporin used here was saturating in both sexes. A dose-response curve would have to be generated before this could be determined. Leanza et al. (1996b) examined the effects of increasing doses of 192 IgG-saporin in neonates but unfortunately this group did not evaluate sex differences.

Weight

Similar to other reports on neonates (Pappas, unpublished observations; Robertson, Gallardo, Claytor, Ha, Ku, Yu, Lauterborn, Wiley, Yu, Gall & Leslie, 1998), 192 IgG-saporin administration resulted in significantly reduced body weights of both male and female rats at all ages. The most radical difference occurred at 21 days of age, which is suggestive of malnutrition at least during the preweaning period. For example, the dam may have neglected

lesioned pups if they appeared sickly compared to controls. Alternatively, lesioned pups may not have been as capable of competing with intact siblings for maternal milk. The effect of the toxin on body weight was less pronounced at later ages, perhaps reflecting the fact that after weaning, competition for food resources became unnecessary. Despite the incidence of decreased body weight, all lesioned pups who survived the surgical procedure appeared active and healthy.

Cell Counts

Neonatal i.c.v. administration of 192 IgG-saporin caused severe reductions in p75-positive cells in the medial septum (90% decrease), vertical and horizontal limbs of the diagonal band (90% and 86% decrease respectively), ventral pallidum (85% decrease), globus pallidus (82% decrease) and nucleus basalis magnocellularis (90% decrease). The magnitude of reductions seen here is greater than that previously reported in neonatal lesion studies from this and other laboratories (Leanza, Nikkhah, Nilsson, Wiley, Bjorklund, 1996a; Leanza et al., 1996b; Pappas et al., 1996). This difference probably reflects the higher dose of immunotoxin used in this study (i.e. 600 ng as opposed to 400 ng). However direct comparison of the present results to neonatal studies using nonspecific lesion methods such as AF64A (Brake & Pappas, 1994), excitotoxins (Sengstock et al., 1992) or electrolytic lesions (Hohmann et al., 1988) is problematic. These types of lesions are produced by ablating all cells in the cholinergic nucleus of interest. Thus histological inspection is usually cursory and often consists only of a verification of the size and location of the lesion.

Typically i.c.v. administration of 4 to 5 µg of 192 IgG-saporin to the adult rat results

in greater than 95% loss of p75-positive neurons in the basal forebrain (Wiley et al., 1991; Nilsson, Leanza, Rosenblad, Lappi, Wiley & Bjorklund, 1992; Leanza et al., 1995). Total loss of p75-positive cells has also been observed (Wiley, Berbos, Deckwerth, Johnson & Lappi, 1995). Nevertheless, 192 IgG-saporin administration to the neonate, as observed here and in other studies, rarely eliminates all p75-positive cell bodies from any given nucleus (Leanza et al., 1996a and 1996b; Pappas et al., 1996). There are several possible reasons for this. For example, although 192 IgG-saporin was administered at a time of peak p75 mRNA expression in the basal forebrain (Koh & Loy, 1989; Koh & Higgins, 1991), some cholinergic cells may not have been expressing the p75 receptor at the time of surgery and were thus spared. Alternatively, perhaps some cells were expressing low levels of the p75 receptor, which would result in fewer available sites at which access to the cell could occur, thus compromising the toxin's efficiency. Another possible explanation could be that the dose of toxin used here (600 ng) was simply not sufficient to produce complete destruction of all p75-bearing neurons. Conversely, this dose may have been very effective at killing p75-bearing cells but then other cholinergic cells which normally do not express the p75 receptor may have expressed this phenotype as a compensatory response.

It may not be possible to achieve a more complete lesion in the neonate. It is well documented that doses of 800 ng or more of 192 IgG-saporin are lethal to neonates, whereas doses of 400 ng are not (Leanza et al., 1996b; Pappas et al., 1996 and unpublished observations). It is not known why higher doses of the toxin cause death in the neonate. No obvious signs of nonspecific tissue damage such as gliosis or distortion of the ventricles are observed following 192 IgG-saporin administration in either neonates (Leanza et al., 1996b;

Pappas et al., 1996) or adults (Wenk et al., 1994; Leanza et al., 1995). Since the only obvious effect of 192 IgG-saporin is destruction of p75-bearing neurons, and since the 600 ng dose used here lies between the sublethal and lethal doses observed for neonates, it is conceivable that animals in this study who failed to thrive post-surgically were those maximally affected by the toxin (i.e. p75-bearing cells were completely eliminated in these rats).

The reductions in p75-positive cells in the basal forebrain nuclei of immunotoxin-injected rats were paralleled by somewhat lesser reductions in ChAT-positive cell numbers. The greatest decreases were seen in the medial septum (80% decrease) and vertical and horizontal limbs of the diagonal band (85% and 78% respectively). Decreases in ChAT-positive cells in the nucleus basalis area were substantially less than that for p75-positive cells: 29% decline in the ventral pallidum, 57% decline in the globus pallidus and 73% decline in the nucleus basalis magnocellularis. The most parsimonious explanation for this is that not all cholinergic cells in the basal forebrain express the p75 receptor. It has been reported that the nucleus basalis area has a relatively low proportion of cholinergic cells which co-express p75 (approximately 60%), as compared to areas such as the medial septum and diagonal band, of which more than 90% of cholinergic cells are also immunoreactive for p75 (Batchelor, Armstrong, Blaker & Gage, 1989; Woolf, Gould & Butcher, 1989).

Since ChAT-positive cells in the caudate have been shown to express the p75 receptor during the first week of life (Koh & Loy, 1989), these cells were counted to determine whether or not a striatal lesion was incurred. Similar to a recently published neonatal lesion study (Robertson et al., 1998), 192 IgG-saporin did in fact reduce ChAT-positive cells in the caudate by 22%. The lateral portions of the caudate were the most affected. These reductions

were not nearly as striking as those found in the medial septum and diagonal band areas and probably reflect the fact that by postnatal day 7, p75 expression in the caudate has already begun to decline (Koh & Loy, 1989). This is consistent with the fact that no adult lesion studies have reported changes in ChAT-positive cells in the caudate (Book, Wiley & Schweitzer, 1992; Berger-Sweeney et al., 1994; Wiley et al., 1995).

Unfortunately no assessment of ChAT activity was made either in the terminal fields of the basal forebrain (i.e. cortex and hippocampus) or in the caudate. Previously published results from this laboratory indicate that i.c.v. administration of 400 ng of 192 IgG-saporin to 7 day old rats causes reductions in ChAT activity on the order of 52% and 84% in the cortex and hippocampus respectively, and has no effect in the caudate when assessed at 80 days of age (Pappas et al., 1996). While brain sections from that study were not stained for ChAT protein, the reductions in p75-positive cell numbers in the basal forebrain were substantially less than what was seen here. It could be expected therefore, that ChAT activity in the cortex and hippocampus of rats in this study lesioned with 600 ng of 192 IgG-saporin would be reduced by at least as much as that noted in the Pappas et al. study. Consistent with this, AChE fiber density in both the cortex and hippocampus of lesioned rats was severely reduced as compared to controls. Therefore, it is reasonable to conclude that the 192 IgG-saporin injected rats in the current study suffered a profound reduction of cholinergic neurotransmission in the hippocampus and a severe but probably lesser reduction in the cortex.

It has been reported that the majority of cells in the septohippocampal pathway are non-cholinergic (Mesulam et al., 1983; Amaral & Kurz, 1985). Specifically, many of these

cells have been found to contain the inhibitory neurotransmitter γ -aminobutyric acid (GABA) (Freund & Antal, 1988; Kiss, Patel, Baimbridge & Freund, 1990a; Kiss, Patel & Freund, 1990b). The calcium-binding protein parvalbumin is expressed exclusively on GABAergic cells in the medial septum (Freund, 1988; Linke, Schwegler & Boldyreva, 1994). Thus parvalbumin-positive cell counts have been used in both adults (e.g., Berger-Sweeney et al., 1994; Leanza et al., 1995; Robner et al., 1995) and neonates (e.g., Leanza et al., 1996b) to determine whether 192 IgG-saporin had any nonspecific effect on GABAergic cells. As expected, none of the previously cited studies have reported reductions in parvalbumin-positive cell numbers in the medial septum. Thus the discovery here of reduced parvalbumin-positive septal cell counts following 192 IgG-saporin lesions was very surprising. However a 1996 paper by Winsky and Kuznicki has shed some light on these contradictory results. This group has demonstrated that some commercially available antibodies to calcium-binding proteins such as parvalbumin preferentially recognize epitopes exposed during specific calcium-induced conformations of these proteins in both fixed and unfixed tissue. Furthermore, the parvalbumin antibody tested in the Winsky and Kuznicki study was the same antibody used here. Consequently, parvalbumin cell counts cannot be considered an accurate method of determining nonspecific lesion effects on GABAergic cells. This may explain the high degree of variability in parvalbumin-immunoreactive cells in the current study. A more accurate method would be to assess levels of glutamic acid decarboxylase either by immunohistochemistry or *in situ* hybridization. The one study which did assess nonspecific effects of the toxin on GABAergic cells using *in situ* hybridization for glutamic acid decarboxylase mRNA reported no change in mRNA levels following postnatal day 2 i.c.v.

lesions (Robertson et al., 1998).

Sex differences in both p75- and ChAT- positive cell numbers were observed in several basal forebrain nuclei. Specifically, males had 40% and 25% more p75-positive cells in the medial septum and vertical limb of the diagonal band respectively, and 19% more ChAT-positive cells in the nucleus basalis magnocellularis than females. Sex differences were consistent across age groups, with the exception of the vertical limb of the diagonal band, where the gap between males and females had narrowed by 90 days of age (i.e. 11% as opposed to 25%). These sex differences could be easily explained if males had larger brains than females. In fact, sexual dimorphism in brain size has been reported in terms of a greater maximum cortical length and width in adult male rats (reviewed in Juraska, 1991). If male cortex has a greater volume than female cortex, then it could be expected that other regions may also display sex-dependent variations in size.

In addition to sex differences, older rats had significantly more p75-positive cells in the vertical limb of the diagonal band, and more ChAT-positive cells in the horizontal limb of the diagonal band, the ventral pallidum and the caudate. Once again, these differences could be easily explained considering that older rats have larger brains, and thus larger brain nuclei, than younger rats. However it is interesting to note that 21 day old rats had more p75-positive and ChAT-positive cells in the nucleus basalis magnocellularis, and more ChAT-positive cells in the globus pallidus than either 45 or 90 day old rats. Coyle and Yamamura (1976) have shown that the cholinergic system does not reach a mature pattern of activity until at least 4 weeks post-partum. Perhaps higher numbers of cholinergic cells in 21 day old rats reflect a subset of neurons present during development which become superfluous in a

mature system and thus do not survive into adulthood.

While the main findings of this study, namely that 192 IgG-saporin lesions produce substantial cholinergic cell loss in the basal forebrain, are not in dispute, the absence of a standardized, acceptable methodology for performing cell counts makes this type of data difficult to interpret and compare across studies. Traditionally, estimates of cell numbers have been generated by determining the numerical cell density in one or more brain slices containing the nucleus of interest, and then comparing these densities across experimental groups. Indeed, the data in this study was generated using this technique. However this method is based on the assumption that the areas and shapes of the regions being compared are the same, and that within these regions the neurons being counted are similar in size, orientation and distribution. An estimate of the total number of neurons in a given nucleus may be more relevant to studies of this nature, since it could be compared unambiguously across experimental groups and between studies. Stereological analysis produces unbiased estimates of total cell numbers provided that sampling and counting of cells are performed according to published mathematical protocols (West, 1994). It would therefore be worthwhile to perform stereological analysis to confirm the results regarding sex and age differences obtained in this study.

Hippocampal Sympathetic Ingrowths

Both p75- and DBH-positive ingrowths were observed in the dentate gyrus and stratum lucidum of lesioned rats by 45 days of age. These ingrowths resembled the type of sympathetic sprouting reported following medial septal lesions (reviewed in Crutcher, 1987).

The highest incidence of ingrowths occurred in the hilus of the dentate gyrus where average ingrowth length was 1.21 mm, followed by the lower blade of stratum moleculare at 0.79 mm, the upper blade of stratum moleculare at 0.202 mm and stratum lucidum (CA3) at 0.142 mm. Ingrowths were never observed in control rats or 21 day old immunotoxin-treated rats. While ingrowths in the dentate gyrus were observed to lengthen over time, length was not statistically different between the 45 and 90 day age groups. However unpublished observations from this laboratory indicate that by approximately 2 years of age, male rats who had received neonatal 192 IgG-saporin lesions exhibit a dense plexus of aberrant sympathetic axons throughout the dentate gyrus and stratum lucidum.

Milner and Loy (1980) examined the interaction between gender and age at the time of lesion in fimbria/fornix transected rats. They found that when lesions were performed at two weeks post-partum or in adulthood, sympathetic sprouting was greater in females than in males. Lesions at postnatal day 3 produced equivalent sprouting responses. The absence of a detectable gender difference in amount of ingrowths into the dentate gyrus of lesioned neonates in this study is consistent with the findings of Milner and Loy. Surprisingly though, the emergence of ingrowths in the stratum lucidum was gender dependent. Specifically, only 45 day old male and 90 day old female rats exhibited ingrowths in this region. However 90 day old female ingrowth length was not yet statistically different from control. On the surface, these results would seem to indicate that sympathetic ingrowth into the stratum lucidum occurs earlier in males than in females. However the fact that no ingrowths were observed in males at the 90 day time point appears to negate this interpretation. It is possible that these growths could have retracted for some reason, such as a lack of sufficient NGF for

maintenance. This seems implausible since unpublished observations from this laboratory indicate that by approximately 2 years of age, lesioned males exhibit considerable sprouting in this area. For this reason, and because considerable variability of ingrowth length within groups was found here, the results of this study which are suggestive of sex differences for ingrowths should probably be dismissed as spurious. An examination of ingrowths at more closely spaced time intervals over a longer total time period, and perhaps from more subjects, would resolve this issue.

The only other report of the occurrence of hippocampal ingrowths following 192 IgG-saporin lesions comes from this laboratory (Pappas et al., 1996). This raises the obvious question of why no other laboratory, using 192 IgG-saporin in either neonates or adults, has reported this finding. One possibility is that different strains of rats exhibit different degrees and rates of hippocampal sprouting, but this is unlikely since many 192 IgG-saporin lesion studies have used the same strain (Sprague-Dawley) as was used here (Wiley et al., 1991; Leanza et al., 1995; Robner et al., 1995; Leanza et al., 1996b; Robertson et al., 1998).

The sprouting response following fimbria transection has been shown to occur at a slower rate in adult than in infant rats (Milner & Loy, 1980). However it is unlikely that the failure to observe ingrowths following i.c.v. injections of 192 IgG-saporin in adult rats is due to the fact that investigators have not waited long enough to observe them. A more plausible explanation derives from the observation by Wiley et al. (1995) that 4 to 8 μ g of 192 IgG-saporin administered systemically to adults produces partial lesions of the superior cervical ganglion. Thus it seems plausible that i.c.v. administration of the toxin could destroy central p75 receptor-bearing sympathetic terminals in adults, thereby abolishing the sprouting

response. Sympathetic terminals in neonates may have been spared if they were not yet expressing the p75 receptor at the time of surgery, or alternatively sympathetic axons may not yet have sufficiently extended into the brain to be exposed to the toxin. Evidence suggests that sympathetic innervation of the brain vasculature develops over the first few postnatal weeks (Tsai, Tew & Shipley, 1989; Handa, Nojyo, Tamamaki & Kubota, 1993). Other neonatal lesion studies may not have observed ingrowths in the hippocampus simply because other researchers have not looked for or noticed this phenomenon.

Elevated Plus Maze

In a previous study from this laboratory (Pappas et al., 1996), neonatal 192 IgG-saporin lesioned rats, when tested at 100 days of age, were found to enter fewer open arms, and fewer arms overall, than controls in the elevated plus maze. The latter finding suggested that these rats were more timid than their vehicle injected littermates. Conversely, in the present study immunotoxin treatment did not reduce either open or total arm entries. In fact, lesioned females spent more time on the open arms than either lesioned males, control males or control females. This would indicate that in females, 192 IgG-saporin actually reduced timidity. The reason for the inconsistency between this study and previously published data from this laboratory is unclear. However after weaning, rats tested in this study were group housed (2 per cage) whereas rats in the 1996 study were singly-housed. It is possible that social housing attenuated timidity in rats tested in this study. In view of the fact that there are no other published reports of the effects of 192 IgG-saporin lesions on rat behavior in the elevated plus maze, the effects of the lesion on timidity remain to be resolved.

Morris Water Maze

There are reports of impaired spatial navigation in the Morris water maze following both i.c.v. and intraparenchymal 192 IgG-saporin injections in the nucleus basalis magnocellularis in adult rats (Berger-Sweeney et al., 1994; Leanza et al., 1995; Walsh et al., 1995). In contrast, unimpaired spatial navigation using the same injection methods has also been reported (Bannon et al., 1996; Baxter, Bucci, Sobel, Williams, Gorman & Gallagher, 1996; Dorman et al., 1996). One explanation for these disparate results could be that ChAT activity in the basal forebrain must be reduced below a certain threshold before manifestation of behavioral deficits occurs. Unfortunately it is difficult to compare the behavioral effects of lesions made with 192 IgG-saporin across studies since there is a lack of uniformity in dependent variables (i.e. ChAT activity, ChAT- and p75-positive cell counts, high affinity choline uptake, etc.) used to assess the extent of these lesions.

Neonatal i.c.v. 192 IgG-saporin had only a very mild effect on performance in the Morris water maze in the present study. Lesioned rats showed significantly longer platform latencies only at session 1; at all subsequent sessions, they were not different from controls. The difference at session 1 was not due to motor impairment since the lesioned rats had slightly faster swim speeds than controls. Neither did it reflect a learning impairment since, besides failing to differ at later sessions, lesioned rats were not different from controls at the probe trial. In fact, all groups of rats showed a preference for the target quadrant. Other studies also report an absence of spatial navigation deficits in the Morris water maze (Leanza et al., 1996b; Pappas et al., 1996). Arguably, this could be due to the ability of surviving cells to upregulate their cholinergic activity (Lapchack, Jenden & Hefti, 1991), causing functional

recovery. However this seems unlikely since the lesions produced here were quite substantial.

Females generally had longer platform latencies than males during hidden platform testing. However no sex differences were seen at the probe trial, indicating that females had successfully learned platform location. This is consistent with the results of a study which found that females in estrous had longer platform latencies than either diestrous females or males during the acquisition phase of the Morris water maze (Frye, 1995).

Except for the first test session, platform location latencies of 45 day old rats were longer than those of 90 day old rats. This could indicate that 45 day old rats failed to learn the maze as quickly as their older counterparts. Alternatively, the longer latencies could be explained by differences in swim speed, since 90 day old rats swam significantly faster than the 45 day group. Consistent with this latter interpretation, no differences between the age groups were seen at the probe trial, indicating that 45 day old rats had in fact successfully learned platform location.

Concluding Remarks and Suggestions for Future Study

Neonatal i.c.v. administration of 192 IgG-saporin profoundly reduced cholinergic cell body counts in the basal forebrain and cholinergic fiber density in the hippocampus and cortex. These deficits persisted for up to 3 months and are most likely permanent. Behaviorally, 192 IgG-saporin reduced timidity in female but not male rats. In addition, the toxin produced only marginal effects on Morris water maze performance which most likely do not reflect impaired spatial learning/memory. These behavioral effects were stable over time, indicating that 192 IgG-saporin-induced deficits in the neonate do not ameliorate during

maturation.

Sympathetic axons did not invade the hippocampi of lesioned rats until at least 2 weeks after surgery. By 5 weeks post-lesion sympathetic axons had extended into all subfields of the dentate gyrus, and by 12 weeks post-lesion they were well established in this area. However the temporal kinetics of sympathetic ingrowth to the stratum lucidum remain unresolved. Future studies could clarify this by assessing ingrowth over longer time periods, with shorter intervals at which histological assessment is made. An evaluation of hippocampal NGF mRNA and protein levels in lesioned and unlesioned animals would test the “passive accumulation” hypothesis of ingrowth development, namely that accumulation of NGF in the hippocampus is due to a loss of cholinergic afferents which would normally take up this neurotrophin. Furthermore, it has yet to be clearly demonstrated that these ingrowths arise from the superior cervical ganglion. Injection of retrograde tracing agents into hippocampal subfields demonstrating ingrowths would resolve the issue of their origin. Additionally, this investigation could be combined with a study of the distribution of anterograde tracer applied to the superior cervical ganglion in order to assess the effects of ablation of the ganglion on established ingrowths. Moreover, it would be instructive to examine the effects of i.c.v. administration of 192 IgG-saporin on sympathetic terminals in the adult rat brain. This would help clarify the reasons why ingrowths are not encountered in adult rats treated with 192 IgG-saporin.

With the exception of the elevated plus maze results and post-surgical survival rates, 192 IgG-saporin treatment did not differentially affect the sexes. However an assessment of ChAT activity is necessary before definitive conclusions about the impact of these lesions on

the sexes can be made. The sex differences which were observed across groups in numbers of cholinergic cells in the basal forebrain remain to be substantiated using unbiased stereological methods.

This study was undertaken in part to evaluate the validity of using neonatal 192 IgG-saporin lesions as an animal model of Rett syndrome. The absence of profound behavioral effects, most notably on the Morris water maze, and the failure to find marked sex-dependent effects of the lesion suggests that this technique, performed in isolation, does not create an adequate animal model of this disorder. It should be noted however, that while forebrain cholinergic dysfunction is characteristic of Rett syndrome, other biological correlates include anomalous cortical dendritic morphology and alterations in dopaminergic neurotransmission. Although one recent study has reported reduced dendritic branching of cortical pyramidal neurons one week after postnatal day 2 192 IgG-saporin lesions (Robertson et al., 1998), this effect could have been transiently produced by malnutrition. Thus the impact of 192 IgG-saporin on cortical dendritic morphology remains unclear. Future studies should examine the long term effects of this type of lesion on dendritic morphology and microtubule-associated protein levels. Furthermore, a selective neonatal cholinergic lesion in combination with a neonatal dopaminergic lesion may more closely approximate the Rett brain, thus providing a more viable model of this disorder.

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APPENDIX A.1

ANOVA Summary Table for Body Weight

Source	df	F	p
Age (A)	2	1146.90	0.000
Gender (G)	1	375.43	0.000
Surgery (S)	1	13.78	0.000
A x G	2	146.45	0.000
A x S	2	0.67	0.512
G x S	1	0.41	0.521
A x G x S	2	0.22	0.802
Error	153	MS error = 690.96	

APPENDIX A.2

ANOVA Summary Tables for Percentage of Total Entries Made in the Closed and Open Arms of the Elevated Plus Maze

Source	df	F	p
<i>% Closed Arm Entries</i>			
Age (A)	2	14.44	0.000
Gender (G)	1	1.34	0.249
Surgery (S)	1	1.52	0.219
A x G	2	2.50	0.085
A x S	2	1.50	0.226
G x S	1	0.00	0.987
A x G x S	2	0.36	0.695
Error	156	MS error = 334.28	
<i>% Open Arm Entries</i>			
Age (A)	2	14.07	0.000
Gender (G)	1	1.02	0.313
Surgery (S)	1	1.91	0.169
A x G	2	2.21	0.113
A x S	2	1.69	0.187
G x S	1	0.00	0.950
A x G x S	2	0.51	0.601
Error	156	MS error = 334.51	

APPENDIX A.3

ANOVA Summary Tables for Percentage of Total Time Spent in Closed and Open Arms in the Elevated Plus Maze

Source	df	F	p
<i>% Time in Closed Arms</i>			
Age (A)	2	18.36	0.000
Gender (G)	1	0.28	0.599
Surgery (S)	1	5.36	0.022
A x G	2	2.93	0.056
A x S	2	1.23	0.295
G x S	1	0.79	0.376
A x G x S	2	0.13	0.876
Error	156	MS error = 315.74	
<i>% Time in Open Arms</i>			
Age (A)	2	7.15	0.001
Gender (G)	1	6.66	0.011
Surgery (S)	1	5.03	0.026
A x G	2	2.28	0.106
A x S	2	0.71	0.494
G x S	1	5.71	0.018
A x G x S	2	0.73	0.481
Error	156	MS error = 158.72	

APPENDIX A.4

ANOVA Summary Table for Platform Location Latency in the Morris Water Maze

Source	df	F	p
Age (A)	1	10.64	0.001
Gender (G)	1	7.50	0.007
Surgery (S)	1	4.11	0.045
A x G	1	2.12	0.149
A x S	1	2.48	0.118
G x S	1	1.10	0.298
A x G x S	1	0.81	0.371
Error	104	MS error = 194.06	
Session	4	193.54	0.000
A x Session	4	0.25	0.907
G x Session	4	1.22	0.302
S x Session	4	1.89	0.111
A x G x Session	4	1.65	0.160
A x S x Session	4	0.57	0.682
G x S x Session	4	2.15	0.073
A x G x S x Session	4	1.64	0.164
Error	416	MS error = 105.81	

APPENDIX A.5

ANOVA Summary Table for Probe Trial Quadrant Bias in the Morris Water Maze

Source	df	F	p
Age (A)	1	0.95	0.332
Gender (G)	1	0.91	0.342
Surgery (S)	1	0.73	0.394
A x G	1	1.03	0.313
A x S	1	0.92	0.340
G x S	1	0.80	0.372
A x G x S	1	0.84	0.361
Error	106	MS error = 0.22	
Quadrant	3	255.20	0.000
A x Quadrant	3	1.43	0.233
G x Quadrant	3	2.90	0.035
S x Quadrant	3	0.95	0.415
A x G x Quadrant	3	0.05	0.986
A x S x Quadrant	3	1.50	0.215
G x S x Quadrant	3	0.89	0.446
A x G x S x Quadrant	3	0.36	0.783
Error	318	MS error = 80.07	

APPENDIX A.6

ANOVA Summary Table for Swim Speed in the Morris Water Maze

Source	df	F	p
Age (A)	1	20.69	0.000
Gender (G)	1	3.26	0.074
Surgery (S)	1	4.31	0.040
A x G	1	0.97	0.327
A x S	1	0.04	0.840
G x S	1	0.04	0.846
A x G x S	1	0.19	0.660
Error	106	MS error = 21.34	

APPENDIX A.7

ANOVA Summary Tables for p75- and ChAT-Positive Cell Counts in the Medial Septum

Source	df	F	p
<i>p75-Positive Cell Counts</i>			
Age (A)	2	0.92	0.402
Gender (G)	1	5.88	0.018
Surgery (S)	1	136.61	0.000
A x G	2	0.01	0.995
A x S	2	0.04	0.958
G x S	1	3.04	0.086
A x G x S	2	0.11	0.893
Error	70	MS error = 136.62	
<i>ChAT-Positive Cell Counts</i>			
Age (A)	2	1.31	0.276
Gender (G)	1	0.60	0.441
Surgery (S)	1	94.04	0.000
A x G	2	1.59	0.211
A x S	2	1.33	0.272
G x S	1	0.00	0.995
A x G x S	2	1.05	0.355
Error	70	MS error = 282.84	

APPENDIX A.8

ANOVA Summary Table for Parvalbumin-Positive Cell Counts in the Medial Septum

Source	df	F	p
Age (A)	2	21.57	0.000
Gender (G)	1	0.91	0.343
Surgery (S)	1	4.73	0.033
A x G	2	1.38	0.259
A x S	2	1.47	0.238
G x S	1	0.07	0.786
A x G x S	2	0.26	0.769
Error	64	MS error = 67.59	

APPENDIX A.9

ANOVA Summary Tables for p75- and ChAT-Positive Cell Counts in the Ventral Pallidum

Source	df	F	p
<i>p75-Positive Cell Counts</i>			
Age (A)	2	0.26	0.773
Gender (G)	1	0.14	0.711
Surgery (S)	1	206.86	0.000
A x G	2	2.51	0.088
A x S	2	0.51	0.605
G x S	1	1.07	0.305
A x G x S	2	0.95	0.390
Error	70	MS error = 16.13	
<i>ChAT-Positive Cell Counts</i>			
Age (A)	2	4.01	0.022
Gender (G)	1	3.77	0.056
Surgery (S)	1	41.09	0.000
A x G	2	1.43	0.247
A x S	2	1.76	0.180
G x S	1	0.03	0.856
A x G x S	2	0.42	0.659
Error	70	MS error = 20.36	

APPENDIX A.10

ANOVA Summary Tables for p75- and ChAT-Positive Cell Counts in the Globus Pallidus

Source	df	F	p
<i>p75-Positive Cell Counts</i>			
Age (A)	2	1.18	0.312
Gender (G)	1	0.72	0.399
Surgery (S)	1	274.53	0.000
A x G	2	0.72	0.492
A x S	2	0.70	0.499
G x S	1	0.50	0.481
A x G x S	2	0.72	0.491
Error	72	MS error = 24.61	
<i>ChAT-Positive Cell Counts</i>			
Age (A)	2	13.23	0.000
Gender (G)	1	1.77	0.187
Surgery (S)	1	116.47	0.000
A x G	2	0.23	0.794
A x S	2	3.81	0.027
G x S	1	0.02	0.881
A x G x S	2	1.84	0.167
Error	72	MS error = 35.97	

APPENDIX A.11

ANOVA Summary Tables for p75- and ChAT-Positive Cell Counts in the Nucleus Basalis Magnocellularis

Source	df	F	p
<i>p75-Positive Cell Counts</i>			
Age (A)	2	5.68	0.005
Gender (G)	1	0.32	0.576
Surgery (S)	1	693.36	0.000
A x G	2	0.01	0.989
A x S	2	10.03	0.000
G x S	1	0.04	0.846
A x G x S	2	0.74	0.481
Error	72	MS error = 74.78	
<i>ChAT-Positive Cell Counts</i>			
Age (A)	2	4.55	0.014
Gender (G)	1	4.53	0.037
Surgery (S)	1	203.32	0.000
A x G	2	0.17	0.842
A x S	2	2.47	0.092
G x S	1	1.69	0.198
A x G x S	2	1.16	0.320
Error	72	MS error = 249.66	

APPENDIX A.12

ANOVA Summary Table for ChAT-Positive Cell Counts in the Caudate

Source	df	F	p
Age (A)	2	20.87	0.000
Gender (G)	1	0.02	0.884
Surgery (S)	1	19.99	0.000
A x G	2	0.58	0.563
A x S	2	0.25	0.777
G x S	1	1.57	0.214
A x G x S	2	0.83	0.440
Error	72	MS error = 2530.52	

APPENDIX A.13

ANOVA Summary Tables for p75-Positive Ingrowth Length in the Stratum Moleculare

Source	df	F	p
<i>Lower Blade of Stratum Moleculare</i>			
Age (A)	2	4.45	0.015
Gender (G)	1	0.09	0.759
Surgery (S)	1	16.78	0.000
A x G	2	0.44	0.647
A x S	2	4.42	0.015
G x S	1	0.07	0.798
A x G x S	2	0.39	0.681
Error	71	MS error = 0.76	
<i>Upper Blade of Stratum Moleculare</i>			
Age (A)	2	2.42	0.096
Gender (G)	1	0.12	0.728
Surgery (S)	1	7.10	0.010
A x G	2	0.03	0.969
A x S	2	2.42	0.096
G x S	1	0.12	0.728
A x G x S	2	0.03	0.969
Error	71	MS error = 0.12	

APPENDIX A.14

ANOVA Summary Tables for p75-Positive Ingrowth Length in the Hilus of the Dentate Gyrus and Stratum Lucidum

Source	df	F	p
<i>Hilus of the Dentate Gyrus</i>			
Age (A)	2	7.57	0.001
Gender (G)	1	0.00	0.959
Surgery (S)	1	29.59	0.000
A x G	2	0.31	0.734
A x S	2	7.65	0.001
G x S	1	0.00	0.985
A x G x S	2	0.33	0.721
Error	71	MS error = 1.03	
<i>Stratum Lucidum</i>			
Age (A)	2	1.63	0.202
Gender (G)	1	0.37	0.547
Surgery (S)	1	4.43	0.039
A x G	2	3.29	0.043
A x S	2	1.43	0.245
G x S	1	0.49	0.487
A x G x S	2	3.62	0.032
Error	71	MS error = 0.09	

APPENDIX B

List of Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
ChAT	choline acetyltransferase
DBH	dopamine β -hydroxylase
GABA	γ -aminobutyric acid
HSD	Honestly Significant Difference
i.c.v.	intracerebroventricular
NGF	nerve growth factor
p75	low affinity neurotrophin receptor
PB	phosphate buffer
PBS	phosphate buffered saline
TH	tyrosine hydroxylase

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