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Toward an Understanding of Alzheimer's Disease: The Effects of B-Amyloid(1-42) and Ibotenic Acid on the Retention of a Spatial learning Task in Rats F:ollowing Multiple Injections into the Hippocampus

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Toward an Understanding of Alzheimer's Disease: The Effects of ß-Amyloid(1-42) and Ibotenic Acid on the Retention of a Spatial learning Task in Rats Following Multiple Injections into the Hippocampus

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Running head: MULTIPLE INJECTIONS OF ßA(1-42) + IBO

Abstract

Neuropathologically, Alzheimer's disease (AD) is characterized by neuritic plaques and neurofibrillary tangles. Evidence has suggested that a protein called ß-amyloid (ßA) is a major component of the neuritic plaques and may play a role in the neurodegeneration seen in AD. The cellular mechanisms by which ßA induces neurotoxicity, however, are still unclear. Recent evidence suggests that the aggregational state of ßA may be relevant to its neurotoxicity. Whether portions of the ßA protein or the entire sequence produces neurotoxicity in neurons, however, remains a controversy. Still another controversy is whether ßA is directly neurotoxic to neurons or whether it increases the vulnerability of neurons. Recent evidence reported by Dornan, Kang, McCampbell and Kang, that injections of ßA(25-35) with a low dose of ibotenic acid into the hippocampus did disrupt the acquisition of spatial learning in the rat, supports the vulnerability hypothesis. They suggest that the synergistic effect between ßA and ibotenic acid may have produced the neurotoxic effect. In light of recent evidence (McCampbell, Peterson and Tinkler, unpublished) that injections of ßA(1-42) alone did not disrupt the retention of a spatial learning task, in this study we assessed the increased vulnerability

hypothesis by co-injecting ßA(1-42) with a subthreshold dose of ibotenic acid into the hippocampus of male rats. Another problem related to ßA's neurotoxicity may concern the extent of hippocampal damage it produces. Therefore, we assessed the effects of multiple injections of ßA(1-42) and ibotenic acid into the hippocampus of male rats. Although preliminary, the results of this study conclude that co-injections of ßA(1-42) and ibotenic acid do not disrupt the retention of a spatial learning task.

Alzheimer's Disease (AD) was first described by psychiatrist, Alois Alzheimer, in 1907 (9). Today, AD is the most common of the neurodegenerative diseases of aging (9). In the aging population of the world, AD represents a major health problem for which there is at present no effective treatment. Epidemiological studies report that currently there are over 4 million people suffering from AD in the United States alone. James Goldman and Luden Cote (8) estimate that AD accounts for about 70% of all age related cases of dementia. It afflicts an estimated 5-11 % of the population over 65 and more than 47 % over the age of 85 (8).

Alzheimer's disease is clinically characterized by progressive impairments in memory, language, visuo-spatial skills and behavior (5). Typically, the neuropathology of AD is characterized by extracellular neuritic plaques and intracellular neurofibrillary tangles (13,19,17). Although a certain amount of neuritic plaques and neurofibrillary tangles can be found in normal aged people, they are not as dense or severe as seen in victims of AD. The cerebral cortex and hippocampus appear to be selectively targeted and are the primary sites of this

neuropathology.

Recently, several studies have reported that a 39-42 amino acid long peptide, called ß-amyloid (ßA) is a major component of the neuritic plaques and may play a role in the neuronal degeneration of AD. This peptide is called ßA because of its partial ß-pleated structure (5).

The amyloid peptide is derived from a larger amyloid transmembrane glycoprotein precursor (ßAPP) that exists in several forms (15,18,25,32). Two forms contain Kunitz-type protease inhibitor (KPI) while two forms lack this domain. Mature ßAPP consists of a long N-terminal (which lies outside the cell), a transmembrane portion (a segment that spans the membrane) and an intracellular C-terminal portion. The ßA peptide resides in the transmembrane portion of the peptide (part of it lies within the membrane and part of it lies in the extracellular portion of the membrane).

The ßAPP gene is localized to chromosome 21 (27). Interestingly, Down's syndrome patients also have mutations of chromosome 21 and develop amyloid pathology (27). These findings have created increasing interest in the role of amyloid in the pathology of AD.

To date two different enzymatic processing pathways of ßAPP have been described. They are α and ß secretase pathways (18). If enzymatic cleavage occurs along the α-secretase pathway the protein is cleaved within the ßA fragment (preventing the formation of intact ßA) and normal secreted ßAPP (APP_s) results. APP_s can contain several functional domains including the KPI region, which is described as being a serine protease inhibitor. This suggests that a normal function of secreted ßAPP could be the regulation of protease activity. Other functional domains may stimulate cell proliferation, or regulate Ca^{2+} and neuroprotection.

The fact that the intact ßA fragment is generated and deposited in the brains of healthy aged humans, has encouraged researchers to investigate an alternative processing route that leaves the ßA region intact. In this second secretory pathway, involving ß-secretase, ßAPP is cleaved at the amino terminus (beginning) of the ßA sequence (18). This means that intact ßA is released into the extracellular fluid.

The implication of these two pathways (α and ß-secretase) is that not only does APP_s have normal functions in healthy brains but the ßA fragment is also generated in healthy humans. Therefore, high levels of ßA in the cerebral spinal fluid (CSF) is not a straightforward indicator of AD. Realizing, however that the

alternative proteolytic pathway (ß-secretase) is markedly increased in AD, it may serve as a risk factor in the development of the disease.

Whether ßA is directly neurotoxic, or produces neurotoxicity via an indirect mechanism , however, remains somewhat controversial. For example, ßA fragments have been reported to be neurotoxic to hippocampal neurons *in vitro* (in cell culture)(4,14,24). Accumulating evidence suggests that ßA's neurotoxic properties are presumably localized to amino acid residues 25-35 (12,26). As a result, it has been suggested that the accumulation of extracellular amyloid deposits may play a role in the neuronal degeneration that occurs in AD.

Studies that have examined the effects of ßA *in vivo* (in live animals) have resulted in apparent contradictions. Some studies, *in vivo*, have reported direct neurotoxicity of ßA (11,17). For example, Kowall, Beal, Busciglio, Duffy and Yankner (1991) recently reported that intracerebral unilateral injections of 20 fmol of ßA(1-40) into the hippocampus of the adult rat brains caused a significant neuronal degeneration in the CA1 layer of the hippocampus 0.5 - 1.0mm from the injection site. Other studies, however, report that ßA is not directly neurotoxic *in vivo* (1,28,23). Stein-Behrens, Adams, Yen and Sapolsky recently reported that

injections of ßA(25-35) in the hippocampus showed no evidence of neurotoxicity in the neurons (28).

One possible explanation for these conflicting reports may be related to the self-aggregational properties of ßA. Recently Cottman, Pike and Copani suggested that some of the discrepancies in the experiments may be due to the aggregational state of ßA (4). ßA peptide can exist in both soluble and insoluble forms, but toxicity appears to reside in insoluble aggregates, whereas soluble ßA may have little direct neurotoxicity. Cottman reports that ßA protein (*in vivo*) exists primarily in an insoluble aggregated state within neuritic plaques. It has the property of spontaneous self-assembly into large, insoluble aggregates. Cottman also reported that *in vitro* only aggregated ßA is associated with toxicity in developing hippocampus cultures. These findings suggest that in experiments done *in vivo* ßA may be aggregating inside the injection needle, never entering the animals system. This may explain why some *in vivo* studies report that ßA is not neurotoxic to neurons. If ßA, therefore, is contributing to the progressive neurodegeneration of AD, the aggregated state of the protein may very well be relevant to its neurotoxicity.

The sequence of ßA (either portions of the peptide or the entire sequence) that produces neurotoxicity in neurons, however, is yet to be elucidated. Several studies have reported that the neurotoxic properties of ßA are localized to the amino acid residues 25-35 or 1-40 (7,12,26,29,31). In light of recent evidence suggesting that the soluble and insoluble states of ßA may play a role in its toxicity, a newly purified portion of the peptide (Abbott Laboratories, Abbott Park, Illinois) consisting of residues 1-42 has been under investigation. Presumably, ßA(1-42) has more hydrophobic regions on the peptide, making it more insoluble. It is readily dissolved in dimethylsulfoxide (DMSO). Intriguingly, this may solve some of the controversies described earlier with *in vivo* studies. If the aggregation of ßA can be prevented until the solution enters the animal's tissue, by dissolving the peptide in DMSO it will have an increased chance of exiting the needle. Recent *in vitro* studies also report that aggregated ßA(1-42) is neurotoxic to immature rat hippocampal neurons (21,22). For this reason, the present study used ßA(1-42) to examine the effects of the amyloid peptide.

Currently, little is known about the behavioral effects that ßA injections may produce. Some investigators, however, have demonstrated that while ßA was not,

by itself neurotoxic to neurons in rats, mouse and human cortical neurons, it indirectly caused neurotoxicity. According to these studies, ßA is rendering neurons more vulnerable to outside insults such as excitotoxins or to glucose deprivation (3,6,10,16). For example, Dornan, Kang, McCambell and Kang (6) reported that bilateral injections of ßA(25-35) injections did not produce neurotoxicity in rat hippocampal neurons. Co-injections of ßA(25-35) with a subthreshold dose of ibotenic acid (an excitotoxin that causes Ca^{2+} influx), however, did produce lesions along with focal deposits in the hippocampus of rats. This ßA/ibotenic acid injection also disrupted the acquisition of spatial learning in the rat. This strongly suggests that ßA is exerting its neurotoxic effects not directly, but indirectly via an unknown mechanism. In that study, Dornan et al. suggest that the neurotoxic effect on hippocampal neurons and the subsequent disruption of the acquisition of spatial learning was a result of the increased vulnerability of neurons. They further suggest that the neurotoxicity may be produced by the synergistic effect of ßA(25-35) with ibotenic acid.

In light of the above findings and recent evidence reported at the 5th annual Illinois Wesleyan Student Research Conference, that bilateral injections of ßA(1-

42) alone does not produce a disruption of the retention of a spatial learning task, in the present study we further assessed the increased vulnerability hypothesis by co-injecting ßA(1-42) with a subthreshold dose of ibotenic acid into the hippocampus of male rats.

Another problem related to ßA's neurotoxicity may concern the extent of hippocampal damage it produces. Therefore, in this study we also assessed the effects of multiple injections of ßA(1-42) and ibotenic acid into the hippocampus of male rats.

Method

Animals and Groups

A total of 29 male Long Evans rats were used in this study. They were housed individually on a 12L:12D cycle and maintained at 80-85% of free body weight. They had free access to water except during behavioral testing.

All animals were pre-tested on a partially baited 8-arm radial arm maze until the criterion of eating for at least three consecutive sessions was met. Seven animals did not meet this criterion and were not used in the study. Immediately following the screening test the remaining animals were randomly placed into three groups

for surgery. Each animal was anesthetized with sodium pentobarbital (40mg/kg) and received four bilateral injections throughout the hippocampus. A peptide corresponding to the first 42 amino acids of ßA [ßA(1-42)] was synthesized, purified and dissolved in dimethylsulfoxide (DMSO). This vehicle was used in all control injections as well. The following injections were administered in a 1 μL volume via a 1 μL Hamilton syringe: Group 1, ßA(1-42) 7 nmol/injection per side + 4 nmol/injection per side of ibotenic acid (IBO); Group 2, scrambled ßA peptide 7 nmol/injection per side + 4 nmol/injection per side of IBO; Group 3, IBO 4 nmol/injection per side. In order to prevent back flow and minimize tissue damage, the injection took place over approximately 3 minutes and the syringe was left in place after the injection for another 3 minutes.

Surgery

After the animals were anesthetized, they were placed into a stereotaxic apparatus. A stereotaxic apparatus is a device containing a holder that fixes the animal's head in a standard position and a carrier that moves a cannula (hollow tube) or syringe in all three axes of space. By empirically determining coordinates using a stereotaxic atlas, and experimenter may locate and insert a cannula or

syringe to a specific part of the brain without serious damage to the overlying tissue. As can be seen from Table 1, the stereotaxic coordinates for this experiment were empirically determined using the atlas of Paxinos and Watson (20).

Insert Table 1 about here

Apparatus and Behavioral Testing

In order to asses the retention of spatial learning in the rats, we tested them on a partially baited 8-arm radial arm maze (RAM). The RAM consists of a center platform about two and a half feet across with eight(six inch wide, two and a half feet long) arms radiating from it. At the end of each arm there will be a white painted plastic cup to hold reinforcement for the animals. Prior to actual testing, all animals were given at least 5 days where the reinforcer (Froot Loops) was liberally scattered on the RAM and the animal allowed to explore (habituation). Following this phase, each subject was assigned to one of three maze orientations and a random set of 5 arms which served as the baited set. This orientation remained the same throughout the entire experiment. All behavioral testing was

conducted blind (the experimenters did not know the condition of the rat). The session began when the experimenter placed the subject on the center of the platform. The animal was then allowed to choose among the arms until it either successfully completed the test (obtains all 5 rewards) or until 10 minute had elapsed. If the subject did not make a partial entry (approximately 2/3 of the way down each arm is a piece of tape, if the subject crosses this line it will be considered a partial entry) within the first 5 minutes the session was terminated.

The following parameters were reported at each session: the number of arms revisited (total errors); correct errors, repeated entry into baited arms; incorrect errors , repeated entry into non-baited arms; reference memory, entry into arms that are never baited. Other behaviors of the subjects were also be reported such as, urination and defecation. All scores were be summed and averaged over every 7 day block.

Results

The present study used an analysis of variance (ANOVA) test to qualitatively examine the groups. Specifically, the ANOVA was a split plot mixed design with blocks as the repeated measure and injection condition as the between group

measure. Multiple ANOVA's were performed on total errors, correct errors, incorrect errors, reference memory errors and percent correct choice. Qualitative examination of multiple injections of ßA(1-42) + ibotenic acid reveal no significant effects when compared to controls. Interestingly, the animals in all three groups not only retained the learning from the pre-surgery task, but also improved by the end of the fourth block (Figures 1-5). All three groups had a higher percentage of correct choices by the fourth block (Figure 1) and all error parameters that were recorded were drastically reduced by the fourth block (Figures 2-5).

Insert Figures 1-5 about here

Discussion

The results of the present study indicate that multiple injections of ßA(1-42) with a subthreshold dose of ibotenic acid does not significantly disrupt the retention of a spatial learning task in male rats. In contrast to recent reports (7,29), this study indicates that the neurotoxicity of ßA may not reside in the amino acid residues 1-42. Intriguingly, in the study done by Dornan et al. (6) co-injections of

ßA(25-35) + IBO did produce a deficit in a spatial learning task. It is interesting to note, however, that Dornan et al. assessed the effects of the ßA injections on the acquisition of spatial learning while the present study investigated the retention of the task. The acquisition of learning is the initial learning done by the animals while the retention of learning refers to what the animal has already learned.

In interpreting the data there are several factors to be considered. First of all, the study lacked two control groups. Dimethylsulfoxide (DMSO) was used as a vehicle for all of the conditions. Therefore, one group should have been injected with DMSO alone to show that it has no neurotoxic effects by itself. The second control group that should have been added was a group injected with scrambled peptide. This would reveal any effect that a peptide, in general, would have on the animal's behavior. Due to the high mortality rate resulting from multiple injections (about 30%) and to the limited number of animals available, these control groups were eliminated from the study.

Also related to the high mortality was the small statistical samples: Group 1, n=5; Group 2, n=4; Group 3, n=4. Small statistical samples may lead to misinterpreted data. They can either magnify random trends or "hide" behavioral

effects that may be significant.

At present histological examinations have not been performed. Histological assessment can verify injection sites as well as provide information as to hippocampal damage. Therefore, the results of this study must be considered preliminary and acknowledged with caution.

Finally, evidence has suggested that the sensitivity of the radial arm maze to measure the behavioral effects of hippocampal damage is controversial (2). According to Neal Cohen (2), the hippocampal system plays a critical role in mediating declarative memory. Declarative memory is a memory system that consists of a relational form of representation and has the property of flexibility. For example, when a person finds their way from one point to another by using a map, they are presumably using declarative memory. In contrast, a form of memory that operates independent of the hippocampal system, is procedural memory (2). Procedural memory supports a fundamentally inflexible form of representation. For example, riding a bike or driving a car would be procedural memory. If the radial arm maze task is used as a behavioral measure to assess hippocampal damage, then its efficiency is determined by how well it can tap

declarative memory.

While the rat is running the maze, it must learn to minimize the number of arms that it enters due to the time constraint in which it is placed. Therefore, the animal must identify arms that contain the reinforcer while keeping track of arms that have already been visited. The rat may accomplish this by using spatial representations of extraneous cues surrounding the maze. These abilities may be dependent on declarative memory. It is possible, however, that the animals develop a "strategy" to complete the maze. They may enter one arm and then proceed to complete the maze in systemic circular fashion. This type of strategy is related to procedural memory. Therefore, if the animal runs the maze using a "strategy" it may decrease the reliability of the radial arm maze to effectively measure the behavioral effects of hippocampal damage.

While the results of the present study did not produce any significant results, the extent of hippocampal damage that is required to elicit the behavioral effects of ßA is still unclear. Therefore, further investigation using multiple injections of various sequences of ßA may be required to better understand the role of ßA in the pathogenesis of AD. As others have suggested ßA may be involved in promoting

the vulnerability of otherwise heathy neurons to excitotoxic damage (3,6,10,16). In contrast to the results of this study, ßA may exert its neurotoxic effect of hippocampal neurons indirectly via a Ca^{2+} dependent mechanism. In a recent study done by Weiss, Pike and Cotman (29), the Ca^{2+} channel blocker, nimodopine, attenuated ßA toxicity (residues 1-40 and 1-42) to cortical neurons in culture. They also reported that glutamate receptor antagonists, such as MK-801, had no effect of ßA neurotoxicity, This suggests that voltage-sensitive Ca^{2+} channel blockers may be required to attenuate ßA injury. Therefore, further research on ßA may include investigating the behavioral effects of nimodopine following injections of ßA as well as the interaction of stress hormones on the ßAPP induced neurotoxicity.

These studies may allow for a better understanding of Alzheimer's disease and the role of ßA. Critical to this work, however, is the development of an animal model of AD that mimics the profound impairment in memory characteristics found in human AD patients. It is with this knowledge that new approaches to a treatment for the disease will be possible.

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Table 1
Hippocampal Injection Coordinates

	Direction		
Injection #	AP	ML	DV
1	4.6	2.3	3.3
2	4.6	3.3	3.3
3	5.2	4.6	5.0
4	5.2	4.6	5.5

Note. The table shows the hippocampal injection coordinates empirically determined using the atlas of Paxinos and Watson. AP= anterior posterior, ML= medial lateral and DV= dorsal ventral. The injections were bilateral giving each rat a total of eight injections.

Figure Caption

Figure 1. Group differences in the mean percent correct choices over blocks 2-3 (1 block= 7 sessions).

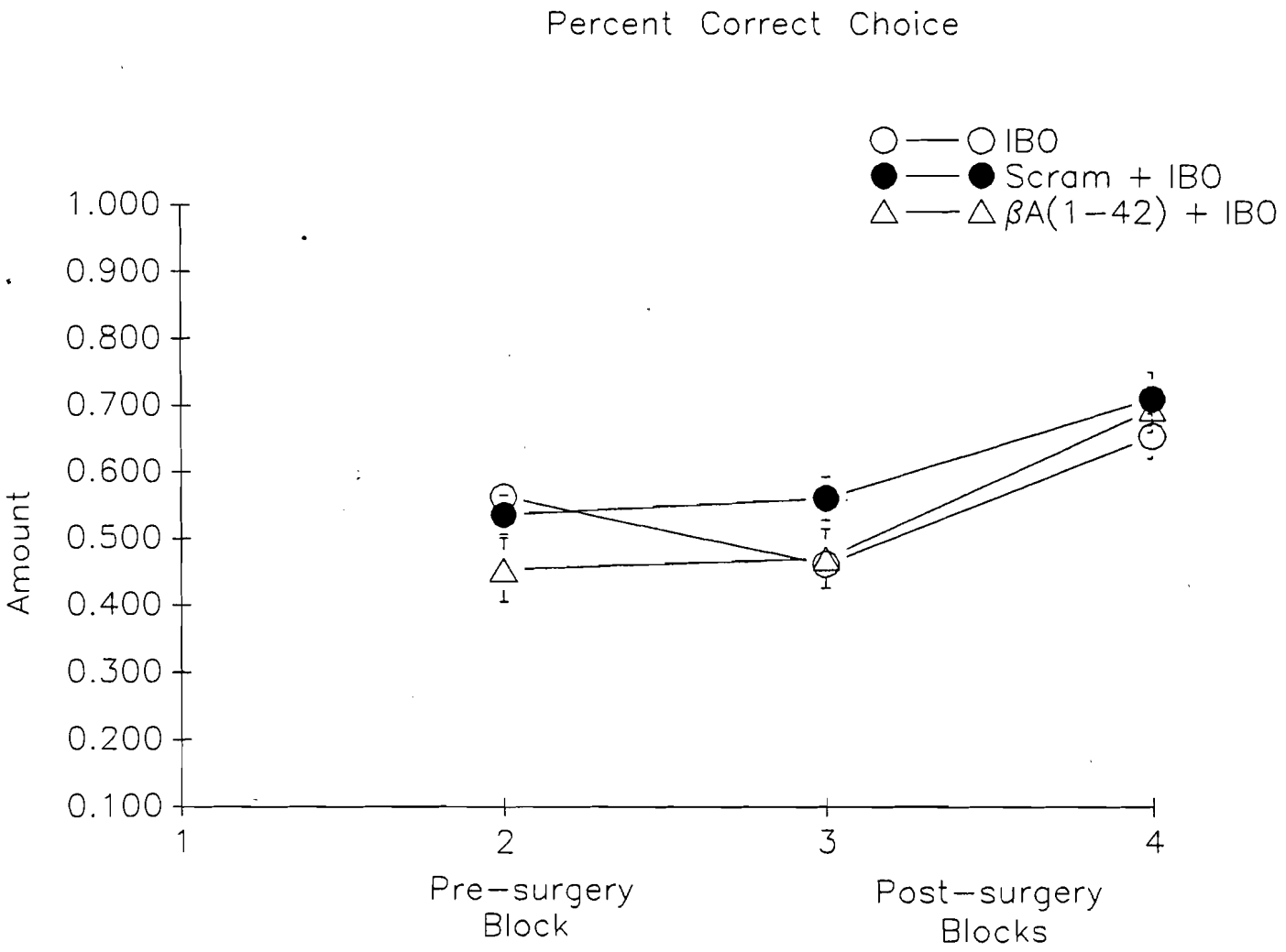

Percent Correct Choice
IBO
Scram + IBO
βA(1−42) + IBO
Amount
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
1
2
3
4
Pre−surgery Block
Post−surgery Blocks

Figure Caption

Figure 2. Group differences in the mean number of correct errors committed over the blocks (1 block= 7 sessions). Correct errors are entry into arms that the rat has already obtained food in the same session. Vertical lines indicate the standard errors.

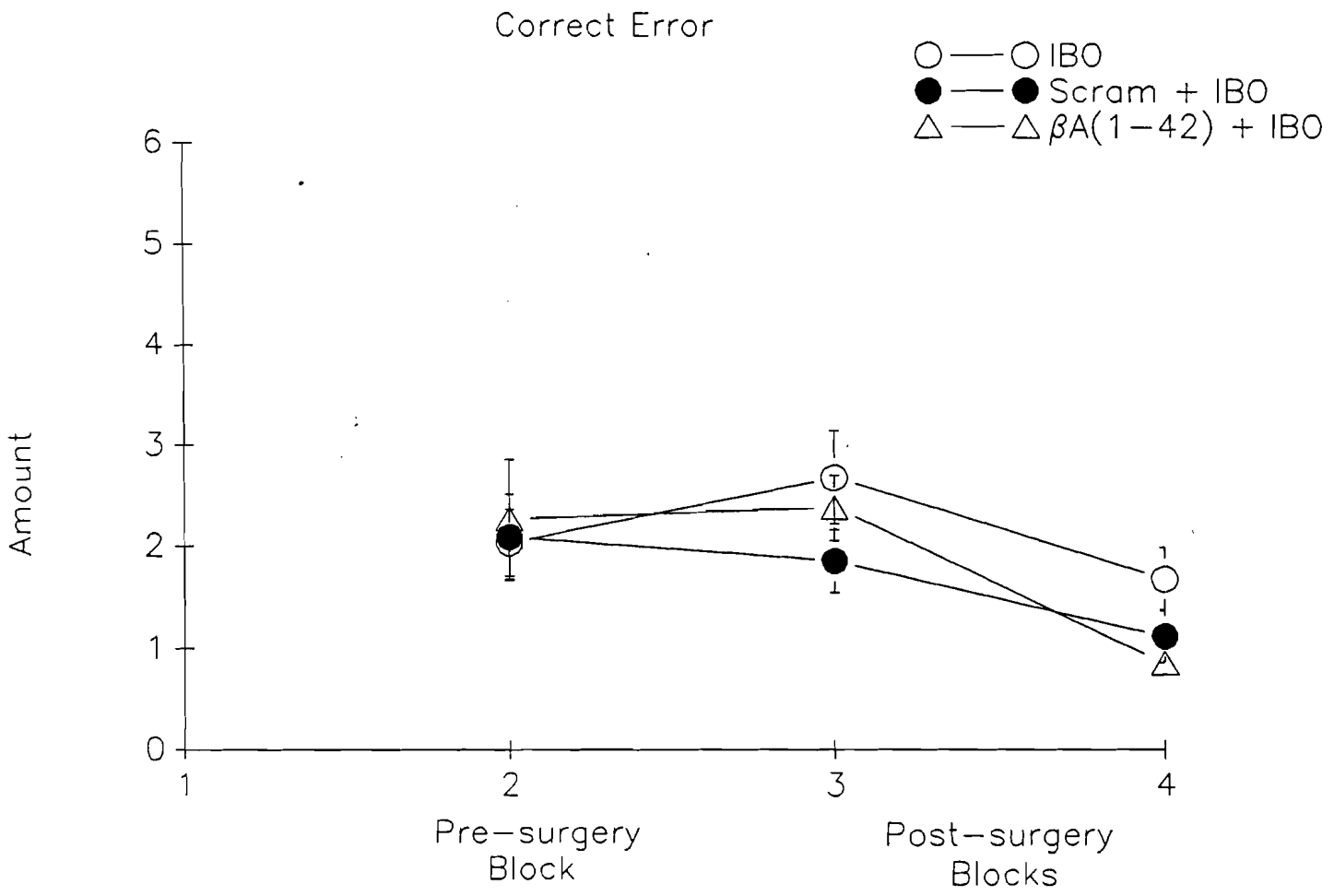

Correct Error
IBO
Scram + IBO
βA(1−42) + IBO
Amount
6
5
4
3
2
1
0
1
2
3
4
Pre−surgery Block
Post−surgery Blocks

Figure Caption

Figure 3. Group differences in the mean number of incorrect errors committed over the blocks (1 block+ 7 sessions). Incorrect errors are repeated entry into arms of the maze that are never baited. Vertical lines indicate the standard errors.

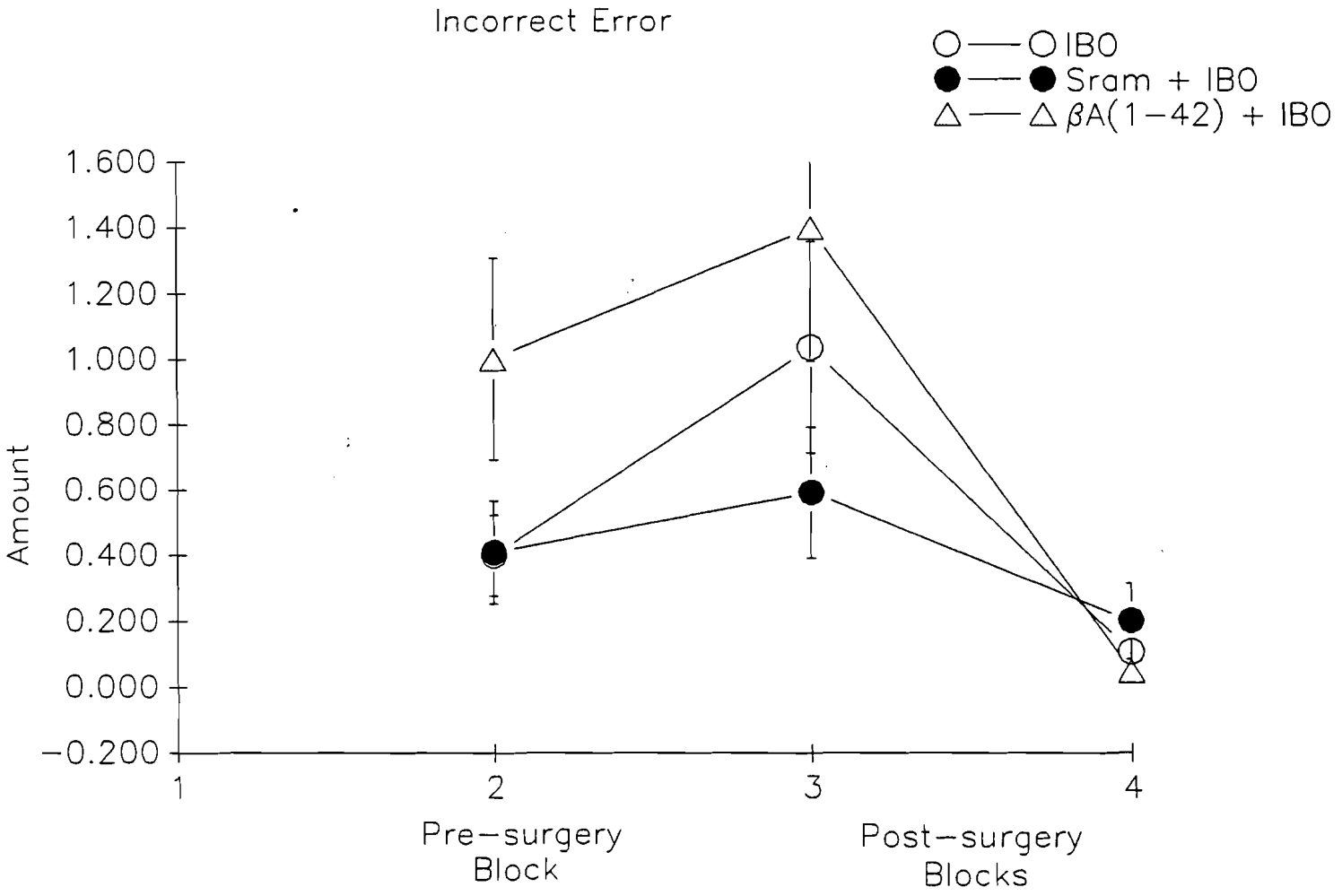

Incorrect Error
IBO
Sram + IBO
βA(1−42) + IBO
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
−0.200
Amount
1
2
3
4
Pre−surgery Block
Post−surgery Blocks

Figure Caption

Figure 4. Group differences in the mean number of reference memory errors made over the blocks (1 block+ 7 sessions). A reference memory error is an entry into an arm of the maze that is never baited. Vertical lines indicate the standard errors.

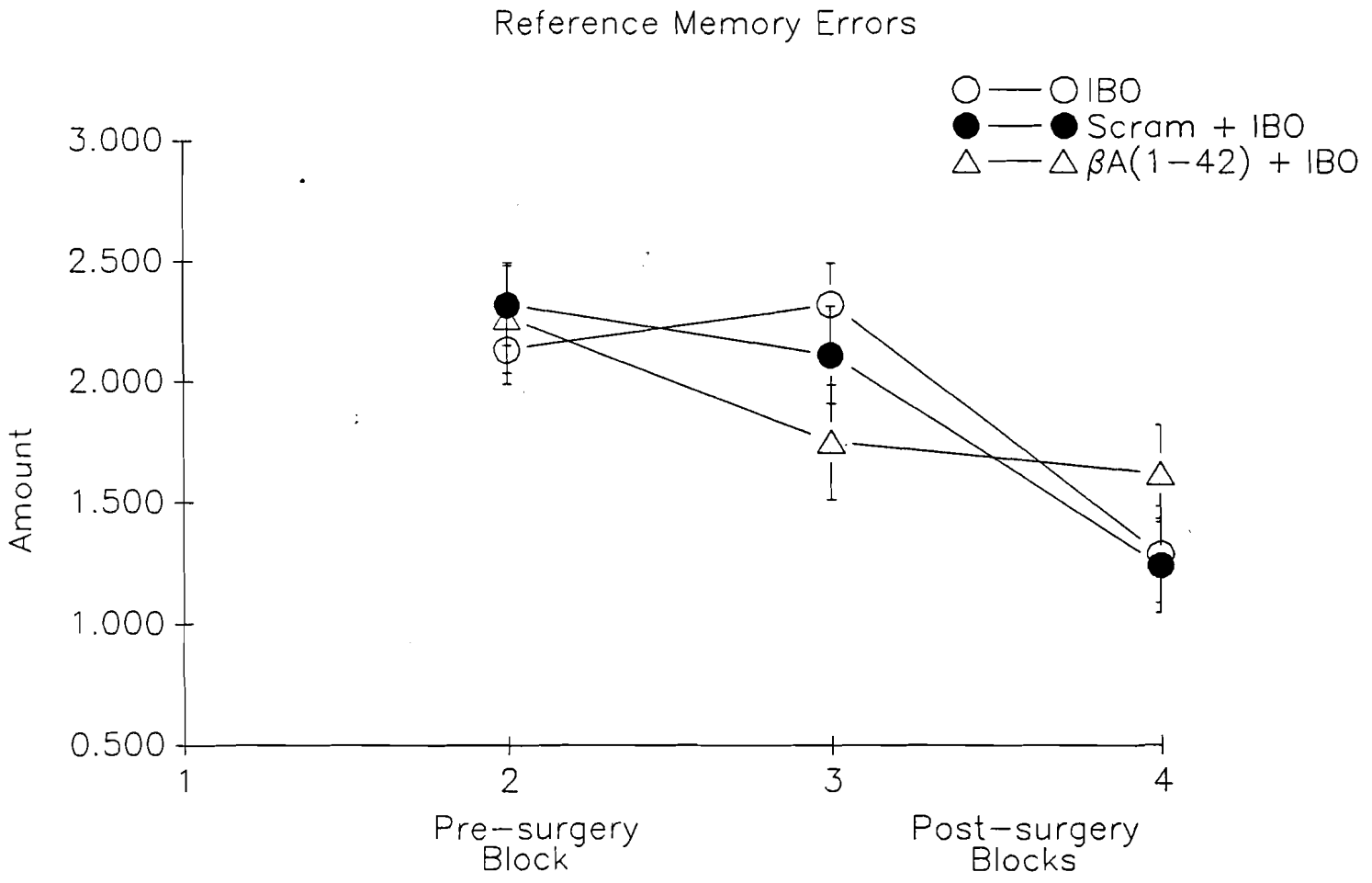
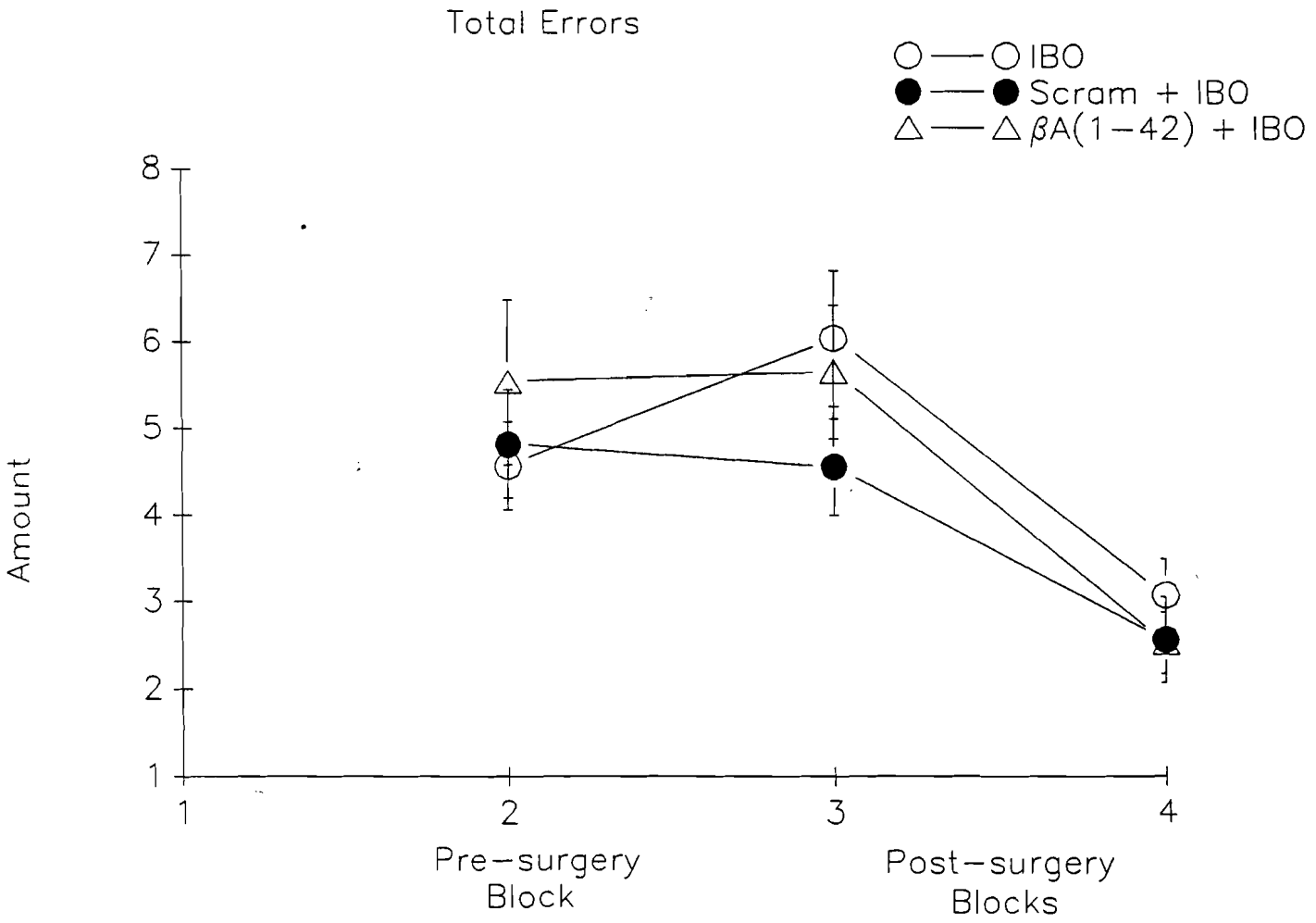

Reference Memory Errors
IBO
Scram + IBO
βA(1−42) + IBO
3.000
2.500
2.000
1.500
1.000
0.500
Amount
1
2
3
4
Pre−surgery Block
Post−surgery Blocks

Figure Caption

Figure 5. Group differences in the mean number of total errors committed over the blocks (1 block + 7 sessions). Total errors include correct, incorrect and reference memory errors. Vertical lines indicate the standard errors.


Total Errors
IBO
Scram + IBO
βA(1−42) + IBO
Amount
8
7
6
5
4
3
2
1
1
2
3
4
Pre−surgery Block
Post−surgery Blocks