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About this Issue

Statement of Purpose

The Rhodes Journal of Biological Science is a student-edited publication which recognizes the scientific achievements of Rhodes students. Volume XXV marks the fifth year since the journal was brought back into regular publication by Mark Stratton and Dr. David Kesler in 2006. Founded as a scholarly forum for student research and scientific ideas, the journal aims to maintain and stimulate the tradition of independent study among Rhodes College students. We hope that in reading the journal, other students will be encouraged to pursue scientific investigations and research.

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From Harlow to solitary confinement: the debilitating effect of social isolation on primates

Guy Handley
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Human and primates share several evolutionary links that stem from their social nature. Sociality drives the evolution of a large social brain and allows primates to overcome the ecological problems that restrict this increased brain function. The driving force in this evolutionary path ranges from hypotheses that support deception to others which support cooperation between conspecifics, or members of the same species. At any rate, it is generally accepted that sociality and the evolution of a large social brain are intertwined and constantly exert selection pressures on each other. Animals observed in social developmental settings have performed significantly better than those raised in non-social developmental settings, pointing to sociality as a vital part of normal development. Observation of non-human primates under prolonged periods of social isolation reveals extremely debilitating losses of social behaviors. This can parallel the human penal institution of solitary confinement. The testimony and observed symptoms of humans who have undergone a long period of solitary confinement elucidates the several similarities to non-human primates in similar tested situations. Solitary confinement as a punitive prescription may need to be re-evaluated for the aims it seeks to achieve.

Introduction

The origin of primate sociality and its effects on organisms has been an area of particular interest to scientists. Studies of learning patterns and social structure in non-human primates can impact viewpoints on comparable human examples. There are several selection pressures driving an increased brain size. Most studies have centered on sociality, but others propose that body size, basal metabolic rate, and life history restrict brain evolution in primates with sociality being the solution to these problems (Dunbar, R.I.M. & Shultz, S. 2007). As brain size and sociality increased, primates exhibited several new cognitive abilities. A need arose to be able to determine social structures and coordinate operations to ensure survival (Barrett, L. & Hanzi, P. 2005). Some primates developed abilities to transfer knowledge to one another with novel observations, and even "traditions" that were specific to communities (Drea, C. & Wallen, K. 1999; Whiten, A. & Schaik, C. 2007). While observations have illuminated parallels to human analogues, humans still retain specific cognitive abilities separating themselves from other primate species. Developmental studies show that primates reared in isolation exhibit debilitating consequences from the experience (Harlow, H., et al., 1965). Social interaction seems necessary for nearly all sophisticated functions of primate life. These studies can be applied in order to gain a stronger understanding of human reliance on social interactions. Humans detained in isolation, through punitive periods of solitary confinement, are shown to exhibit a variety of mental debilitations as well (Robertson, R., 1998). These social and mental debilitations also could translate into long-term social problems that inhibit reintegration.

Primate Brain Evolution

The evolution of the primate brain has drawn connections to primate sociality. Many hypotheses have given reasons for an enlarged brain. One, the 'Machiavellian Intelligence Hypothesis' (MIH), states that because primates lived in permanent social communities that competed against one another for resources, natural selection worked to push evolution towards primates that could "out-wit" others (Barrett, L. & Henzi, P., 2005). As primates struggled to out-compete each other for resources, they invented novel strategies in order to secure more for themselves. The MIH assumes that a community exists in, "a zero-sum game in which manipulating others for personal gain was achieved at a cost to the manipulated individuals" (Barrett, L. & Henzi, P., 2005). While this hypothesis may be able to explain interactions at a localized level, the MIH might fail at a systemic level where primate communities must coordinate activities in order to survive. The updated 'Social brain hypothesis' contends that the complexities of social existence (alliances, foraging, and decision making) require an extensive cognitive network (Dunbar, R.I.M. & Shultz, S., 2007). A developed brain removes barriers, allowing primates to organize and engage in more complex social behaviors, seemingly linking the two concepts. Different selection pressures in the sexes have been observed; females are pressured towards social integration and males by male-male competition and fighting (Dunbar, R.I.M., 2007). Some claim the 'Social brain hypothesis' divorces primate brain size from ecological constraints, perhaps because the original analyses of sociality in primates were organized as alternatives to ecological hypotheses (Dunbar, R.I.M. & Shultz, S., 2007). The hypothesis helps explain how communities overcame

ecological problems through communal foraging, reduction of predation risks, and taking care of young, yet it does not recognize other ecological factors that could act against a developed social brain. Animals with large brains have "higher average metabolic rates, larger bodies, longer life spans, and longer juvenile periods;" all of which can pose their own ecological problems (Dunbar, R.I.M. & Shultz, S., 2007). Size of the neocortex, thought to be critical in sociality, correlated positively with length of juvenile periods; however, caloric intake only correlated positively with total brain size, not neocortex size, suggesting it was only a constraint on primate brain evolution (Dunbar, R.I.M. & Shultz, S., 2007). In order overcome these problems, primates may organize communities in order to ensure longer life-spans and a greater caloric intake. This affords a longer juvenile period in order to allow the accrual of social patterns.

Primate Cognition

The MIH proposes that primates act in such a way to intentionally deceive other individuals (Dunbar, R.I.M. & Shultz, S., 2007). While this seems to necessitate a high level of cognitive function, it has been shown that primates do not have the capacity to understand the intentions of other conspecifics. In a study where high-ranking macaques of a coalition were removed, the remaining members exhibited continued aggression despite their lack of support, resulting in losses in social rank (Barrett, L. & Henzi, P., 2005). It is perhaps premature to assign such complex behaviors usually reserved for humans to non-human primates. In the past what has been interpreted as higher levels of cognition can be attributed to having developed multiple methods of addressing the same short-term problem. Animals seeking to avoid aggression can either act by, "hiding from agressors, using 'protected threats' [or] alarm-calling as a distraction," yet none of these lend credence to the claim that non-human primates can anticipate the actions of others (Barrett, L. & Henzi, P., 2005). If anything, the nature of non-human primate cognition is to find solutions to short-term problems through conditioned means rather than evaluating some long-term benefit. Non-human primate cognition may be better addressed at a communal level. Baboon groups determine which direction to travel by a very peculiar method. Several 'initiators' independently choose a direction in which they take a few paces and sit facing away from the group, while the overall movement of the community is determined by which 'initiator' the majority of the community gathers behind (Barrett, L. & Henzi, P., 2005). Focusing on individual cognition masks this social cognition derived from conspecific interaction. These interactions require in depth social bonding, bonding which individuals must foster over time through actions such as grooming (Barrett, L. & Henzi, P., 2005). Time spent grooming is often a function used to measure the strength of the social bonds in a community (McComb, K. & Semple, S., 2005). Human beings can employ language; however, non-human primates have not yet shown indisputable evidence of a language. Rather than language, many non-human primates may use grooming in order to allow for social relationships (McComb, K. & Semple, S., 2005). Grooming can build social bonds that allow individuals to communicate and recognize one another. This fact in turn would select for a large social brain in order to organize these various relationships. Despite the lack of language, non-human primates vocal repertoire size correlates positively with group size as well as time spent engaging in social bonding (McComb, K. & Semple, S., 2005). This may have ultimately led to evolution of human linguistic capabilities to enhance this function.

Primate Social Learning

Sociality in primates provides innovative approaches to several problems. In addition, learning especially appears to be a very social phenomenon. In a study where Rhesus Macaques were raised in two groups, one with a canine companion and one with an inanimate object, the ones raised with canines displayed significantly higher abilities to solve simple problems (Capitanio, J. & Mason, W., 2000). Some component of a social environment provides cues that enhance primates' ability to manipulate their surroundings. The study found that the approach to problems by the macaques varied based on the environment in which they were raised. Canine-raised primates showed a greater responsiveness to tasks, developed more novel solutions, and were far more active with their environment (Capitanio, J. & Mason, W., 2000). This interaction with another social creature may have assisted macaque development by pushing them to be more observant and attentive to their surroundings. Interestingly, the canine-raised primates inhibited their more direct urges when approaching simple tasks to obtain a reward, such as forgoing direct force and looking for tactical methods (Capitanio, J. & Mason, W., 2000). Wild-born primates show a better ability to solve problems than their lab-raised counterparts, suggesting that some component of social living is conducive to learning. Neurobiologists have observed the same neurons in primate brains fire whether the actor is engaging in a specific action or observing another performing the same action (Barrett, L. & Henzi, P., 2005). Something resonates in the primate brain, allowing an individual to perform the action of another simply after viewing it. Strikingly many primate communities can develop unique approaches, or traditions, to the same problem, and this can be passed down through generations and ultimately become a sort of "culture" (Whiten, A. & Schaik, C.P., 2007). Perhaps it is simply exposure to other conspecifics that allow an individual to develop a "repertoire" of responses to actions, with the most successful individuals determining the most effective responses the most rapidly. Dominant individuals in a colony appeared to outperform subordinates in "discriminate learning" tasks (Drea, C. & Wallen, K., 1999). This could support the idea that dominant individuals in a colony have a greater capacity to learn and perform. However, the study showed that the subordinate individuals fully comprehended these learning sessions and performed equally well as dominant individuals immediately after the two groups were separated (Drea, C. & Wallen, K., 1999). This rejects the claim that dominant individuals have a greater capacity to learn. Even in some situations subordinate individuals have shown the ability to out-wit dominant

individuals in order to obtain a valuable resource and in social settings will often "play dumb" in order to avoid aggression from dominant individuals (Capitanio, J. & Mason, W., 2000; Drea, C. & Wallen, K., 1999). Non-human primate learning seems to be dominated by observance and repetition of actions. An accrual of responses to the same problem allows an individual to find success; moreover, individuals raised with conspecifics are more likely to adapt novel solutions to problems, expanding their "repertoire." Optimal learning occurs when the most successful communities have developed the most adapted solutions to particular problems.

Social Isolation and Social Impairment

Several studies have shown that nearly every aspect of primate life involves sociality, but the effects of isolation on primates can be debilitating. Evolution, cognitive function, and learning depend heavily on communal living and interactions. When non-human primates are removed from their normal social environments, especially during early development, the consequences can be disastrous. One study found that primates raised from birth with no social interactions immediately seized up in emotional shock upon return to social environments (Harlow, H., et al., 1965). Primates isolated during juvenile periods fail to achieve some form of social maturity. The social capacities of the primates studied plummeted as the isolation period increased, with one year of isolation "almost obliterate[ing] the animals socially" (Harlow, H., et al., 1965). Primates require contact with conspecifics in order to develop normally. Another study suggested that the primates lack a "social contact acceptability" normally provided by maternal care, a property that was somewhat restored with warm inanimate surrogate cloths (Harlow, H. & Suomi, S., 1971). Something providing a contact for developing primates to clutch seems to restore an individual social acceptance, marked by reduced emotional shock. As suggested earlier, when the object is an animate organism, even a non-conspecific, the social functions of these animals are further restored. When observing canine-raised macaques and inanimate raised macaques, both isolated from others, the previous study found the canine-raised group organized more rapidly into a far more cohesive social community when united as compared to the other group (Capitanio, J. & Mason, W., 2000). Primates may require this social interaction during development in order to accept themselves as a member of the larger community, allowing the development of standard social behavior. Primates reared in isolation exhibited fear towards socially raised adults, while those who were isolated after a period of social interaction became hyper-aggressive, perhaps because they did not receive "complex social interaction" provided by conspecifics (Harlow, H., et al., 1965; Harlow, H. & Suomi, S., 1971). In the next section, this will correlate with human behavior after periods of social isolation, through solitary confinement. Humans released into social contexts after solitary confinement similarly have a documented history of exhibiting hyper-aggression. After isolations of no more than six months, non-human primates showed somewhat normal, though clumsy, play with their isolation-reared conspecifics (Harlow, H., et al., 1965; Harlow, H. & Suomi, S., 1971). This unrefined behavior lends more support to the idea that primate social learning involves an observance and capturing of the actions of conspecifics during development. It has been shown that somewhat normal social behavior can be restored between isolated (less than 3 month periods) primates and control primates, with the "optimal" period of reintroduction being 3-4 months of age for both isolates and controls (Harlow, H. & Suomi, S., 1971; Harlow, H., et al., 1965). If the control animals have not reached an age where they express unwarranted aggression towards the isolates, they may actually provide some support in restoring the normal behavior. It appears primates receive "social contact acceptability" from maternal care and "complex social stimulation" from conspecifics that are both required in order for them to fully develop socially (Harlow, H. & Suomi, S., 1971). Non-human primates require social interaction with conspecifics before at least one year of development, if they are to have any chance of normal social interaction. If isolation occurs after development for prolonged periods, it can lead to hyper-aggression and a failure to integrate socially as well.

Human Analogues and Solitary Confinement

Humans and non-human primates exhibit many genetic similarities and the evolutionary pressures that affect non-human primates may also influence human counterparts. Humans live in a social world in which language has expanded the "grooming" culture observed in non-human primates, offering an even higher level of cognition and social interaction (Barrett, L. & Henzi, P., 2005). Humans engage in dialogue and company every day, yet when this interaction is taken away the individual can suffer. In non-human primate models a study isolated two of thirteen gene loci that when homozygous led to significantly less affiliation, through grooming rates and greater received aggression (Charpentier, M.J.E. & Prugnolle, F., 2007). These loci correlated to phenotypes for alcohol dependence and myeloid disorders in humans (Charpentier, M.J.E. & Prugnolle, F., 2007). The same loci in non-human primate models that led to lower affiliation, also codes for social phenotypes that can lower fitness in humans. Even more startling are the parallels between the punitive practice of solitary confinement and social isolation in previously explored non-human primate models. Solitary confinement has been implemented by prisons and judicial systems for a range of crimes. This form of punishment involves near-total social isolation for extended periods of time. Inmates placed into solitary confinement after being part of a social community display hyper-aggression towards others rather than fear (Harlow, H. et al., 1965; Robertson, R., 1998). The same results were seen in the previously presented study for non-human primates. It seems extended periods without "complex social interaction" can also greatly alter human social interactions. Similar to the non-human primates which displayed little or no social behavior after a year of isolation, inmates have often described being in public places as "traumatic" and only find consolation in, "a small cell-like consultation room" (Harlow, H. et al., 1965;

Robertson, R., 1998). After release from confinement, humans curiously seem to only find some level of comfort in isolated situations. One individual who was kidnapped abroad and held hostage in solitary confinement recalls that as he continued to be kept in confinement he though, "'The mind is a blank. Jesus, I always thought I was smart. Where are all the things I learned, the books I read, the poems I memorized? There's nothing there, just a formless, gray-black misery. My mind's gone dead. God, help me'" (Gawande, A., 2009). He seems to be detailing a sort of brain atrophy initiated by total social isolation. Another account details a prisoner who initially believed solitary confinement would be an easy experience but soon experienced hallucinations, unnatural rage, and panic attacks (Gawande, A., 2009). Solitary confinement is an extremely dehumanizing experience that parallels several of the primate models presented earlier. Isolation from social contact may cause a mental atrophy because it precludes the sociality of primates. As isolation continues, debilitating effects may occur in the frontal lobe, a primary enhancer of cognitive function (Dunbar, R.I.M. & Shultz, S., 2007). Solitary confinement as a punishment may cause irreparable damage to inmates, rendering them unable to successfully reintegrate with their fellow humans like their primate counterparts.

Conclusion

It appears that sociality evolved as a core feature of primate species, linking individuals to a group and provides an impetus for brain evolution. Some have argued this comes from a desire to deceive and outsmart fellow conspecifics while others argue that sociality evolved from positive cooperative interactions such as foraging, hunting, and caring for young. The ecological problems posed from a large social brain require social solutions, linking sociality and brain evolution and constantly pressuring primate species to adapt, learn, and improve on their ancestors' methods. Primates have particularly long juvenile periods that allow them to learn multiple approaches to problems, approaches which enhance their chances of survival. In social settings they can even "play dumb" because of social hierarchy despite nearly equal learning capacities. Social actions such as grooming create social cohesion, perhaps at some point humans developed language from this. Conditions of total social isolation cause disastrous effects on well-being for primates. If they are isolated from birth, they can become extremely fearful of any social contact while later isolated may elicit the opposite; hyper-aggression. The human analogue of solitary confinement provides similar results of hyper-aggressive ex-felons that have an extremely difficult time reintegrating. Perhaps the institution of solitary confinement needs to be reconsidered as a punishment. Is the aim of this punitive treatment to potentially render a felon unable of reintegrating into society? There have been several studies into the effects of solitary confinement on humans as well as the effects of social isolation on non-human primates; however, future studies could look to expand and investigate the ultimate evolutionary reasons behind these effects.

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The Effects of Olfactory Enrichment on Captive Jaguar (*Panthera onca*) Behavior at the Memphis Zoo

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Endangered and threatened species are frequently preserved through zoo and conservation programs. Felids, including the jaguar (Panthera onca), require enrichment to promote active behavior to maintain fitness while in captivity. Recent studies have shown a behavioral response to olfactory stimulation through the introduction of novel scents such as perfumes and spices. The Memphis Zoo used olfactory enrichment to stimulate activity of their two female jaguars, Maya and Inca over a period of three weeks during the fall of 2009. However, there has been limited research on the effects of this enrichment on felid behavior. The purpose of our study was to evaluate changes in behavioral and spatial activity with the introduction of novel scents. During 60-minute control and enrichment periods, jaguars were monitored through 5-minute interval group scans, recording behavior and location. Following exposure to olfactory enrichment, a continuous behavior scan was also conducted on behaviors associated with enrichment (sniffing, rubbing, licking and scratching). The duration of association with each stimulus was also recorded. We found that overall, the jaguars spent more time sleeping and resting during control periods, using only a few areas within their exhibit. During enrichment periods, sleeping and resting behavior frequency decreased and other behaviors, particularly those associated with enrichment, increased. The jaguars also used more areas of their enclosure when enrichment items were present. Scents such as perfumes received a greater frequency of enrichment behavior. The duration of time spent at a stimulus was greatest for perfumes and deodorant. In conclusion, jaguars presented with scents exhibited increased active behavior and habitat use, showing the possible effectiveness of olfactory enrichment.

Introduction

Jaguars (*Panthera onca*) are the largest felids in the Americas. They range from southern Arizona and New Mexico south toward northern Argentina and northeastern Brazil. Body size ranges from approximately 1.57 to 2.41 m from the head to the end of their body (Seymour 1989). These cats may either be tan to pale yellow, dark reddish brown, or black, with brown or black spots. Jaguars are solitary creatures that may inhabit a variety of different areas including rain forests and dense woodlands. They prefer to be near water and are very skilled swimmers (Seymour 1989).

Jaguars are considered "near threatened" by the IUCN due to habitat loss, fragmentation and poaching of prey species (Caso et al. 2008). Many populations remain stable but jaguars are threatened throughout most of their range due to hunting, persecution, and habitat destruction (Baker et al. 2002). Deforestation and food depletion has lead jaguars to prey upon livestock and pets in order to survive. This has created hostile feelings towards the jaguars from ranchers and local residents, leading to the killing of jaguars (Nowell & Jackson 1996).

Captive jaguars lack the sensory stimulus provided to them in the wild through hunting and social behaviors. Jaguars are also a nocturnal species; however, multiple studies have shown activity during the day, including hunting behavior (Baker et al. 2002). Overall, it has been shown that these cats are active for around 50-60% of the day in the wild (Nowell & Jackson 1996).

Environmental enrichment is necessary to help maintain the behavioral health of captive animals (Wells 2009). Recent studies have shown the beneficial influence of olfactory stimulation on large felids, including black-footed cats, leopards, and jaguars (Wells & Egli 2004, Wells 2009, Rosandher 2005). Types of olfactory stimuli include exposure to animal scents, such as feces or meat, as well as a wide variety of spices. In an unpublished study by Rosandher, captive snow leopards (*Uncia uncia*) were exposed to the following scents: lavender, lemon balm, cumin, cinnamon, and catnip. His study showed an odor specific increase in active behavior of the leopards following olfactory stimulation.

Our study focused on olfactory enrichment for captive jaguars at the Memphis Zoo. Therefore, the goal of our study was to address how olfactory enrichment affects the behavior of captive jaguars (*Panthera onca*). Our first hypothesis was that olfactory enrichment would increase jaguars' activity levels. We predicted that following olfactory stimulation, there would be a decrease in resting behavior and an increase in enrichment behavior. Our second hypothesis was that olfactory enrichment would change the animals' use of the exhibit. We predicted that when exposed to olfactory stimulation, jaguars would use more areas of their exhibit.

Methods

Study Animals

Our study was conducted at the Memphis Zoo during the fall of 2009 on the two female captive black jaguars (*Panthera onca*), Maya and Inca. The sisters were bottle-raised at the Memphis Zoo after being transported from the Montgomery Zoo in Alabama when the jaguars were six-weeks old. They have been at the Memphis Zoo for more than six years. Their enclosure at the Zoo includes space for pacing, and jumping and climbing on both rock and log substrates (Diagram 1). Inca has white fur in her ears, making her identifiable from Maya. The jaguars regularly received enrichment items prior to the start of this study. These items included exposure to a variety of scents ranging from spices to deodorant, as well as exposure to meat and fish, starting at 8:30 am on scheduled enrichment days. Scents and meat were placed around the jaguar exhibit prior to the release of jaguars into their habitat. In addition, the jaguars were provided with a "boomer" ball, which is one example of an enrichment item.

Experimental Design

Our study was conducted in two parts over the course of three weekends. On Saturdays we collected the Enrichment portion of our data. We observed the behaviors of the felids during a 60-minute period in the morning, directly following exposure to olfactory stimulus. Each day, scents were placed within the environment and both the scent and location were recorded and given an identifiable series of letters (Table 1). Scents and location varied based upon keeper preference. Upon exposure to their environment, one student observed the felids' behaviors using Continuous Behavior Sampling. The duration, location, and behavior were recorded for every behavior exhibited by both individuals. The other student conducted 5-minute interval Group Scans of the felids, recording location within the exhibit, and behaviors being exhibited. The location of individuals was collected through location mapping of the exhibit.

On Sundays, we collected data as part of our Control (no enrichment). We observed the behaviors of the felids during a 60-minute period in the morning, on days without olfactory enrichment stimuli. This study was done through 5-minute interval Group Scans of the felids, recording location within the exhibit, and behaviors being exhibited. One student kept time and recorded animals' locations, while the other student recorded observed behaviors. The location of individuals was collected through location mapping of the exhibit (Figure 2).

Activity budgets were then created for both jaguars combined behaviors exhibited during 5-minute interval group scans during both control and enrichment periods by analyzing the ratio of each behavior based upon all behaviors observed. We compared the activity budgets during the enrichment periods to the control periods using the data from the group scan. The location of both individuals was observed at each 5-minute interval and the average number of locations used per session was graphed (Figure 2) for both control and enrichment weeks. The observational behaviors recorded in the "Enrichment" portion of the experiment were graphed to compare frequency of activity with each stimulus (Figure 3), as well as duration spent at each scent in seconds (Figure 4). Behaviors in both studies were identified and recorded using the following ethogram based on an ethogram by in Weller and Bennett (2001).

In order to record location, we created a mapping system of the habitat (Diagram 1). The codes for each area correspond with a location, which could then be used to compare time spent at each area within the enclosure.

Ethogram

Behavior	*Code*	*Description*
Resting	RE	Laying/Sitting down, head up, eyes open; Sitting down, eyes closed; Laying down, head down, eyes closed; Standing without moving
Traveling	TR	Moving around enclosure
Pacing	PA	Repetitive movement around enclosure
Scratching	SC*	Scratching self or object in habitat, including scent enrichment
Bathing	BA	Licking self or conspecific to clean
Licking	LK*	Licking the area of scent enrichment
Sniffing	SN*	Sniffing the area of scent enrichment
Rubbing	RU*	Rubbing face along the area of scent enrichment
Body Movement	BM	Tail Flicking, Head Movement, Ear Movement, Tongue out, etc.
Feeding	FE	Eating/Drinking or Playing with food
Visitor Interaction	VI	Response to the presence of zoo visitors in front of exhibit such as staring, vocalization or movement
Other	O	Other behavior not described in Ethogram

* behaviors exhibited when olfactory stimuli were present

Key

Location	*Code*
Ground	G
Upper Rock	UR
Cave 1	C1
Cave 2	C2
Cave 3	C3
Rock	R
Tree	T
Log 1	L1
Log 2	L2
Log 3	L3
Log 4	L4
Log 5	L5
Log 6	L6
Log 7	L7
Log 8	L8
Log 9	L9

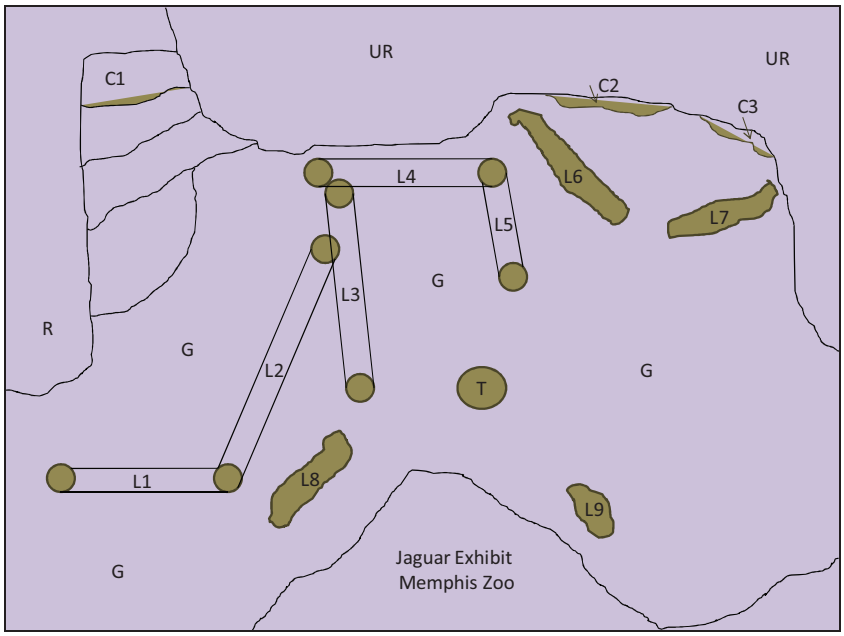

Diagram 1. Diagram of area locations within *Panthera onca* exhibit at the Memphis Zoo

Stimulus Category	*Olfactory Stimulus*	*Code*
Spice	Cinnamon	CI
	Pumpkin Pie Spice	PP
	Allspice	AL
Vanilla	Vanilla	VA
Basil & Bay	Bay Leaves	BL
	Basil Leaves	BA
Doe Urine	Doe Urine	DU
Right Guard	Right Guard Original Sport	RG
Perfume	Salvatore Farragamo	SF
	Confess	C
	Beautiful	B

Table 1. Legend of olfactory stimuli presented to Maya and Inca.

Results

Group Scans

During the control period, the jaguars displayed more frequent Resting behavior (Figure 1). During the enrichment period however, the range of behaviors exhibited increased, specifically through enrichment behaviors such as Sniffing and Rubbing. The frequency of Resting behavior also decreased.

During the control period, Inca and Maya used fewer locations within their habitat than during enrichment periods (Fig. 2).

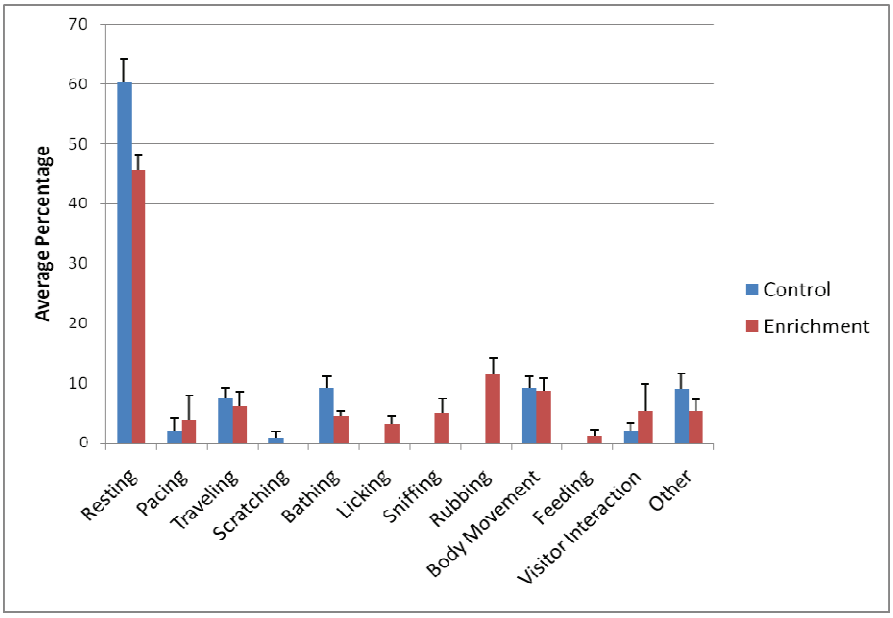

Fig. 1 Graph of average behavioral activity budgets for Maya and Inca during enrichment and control weeks.

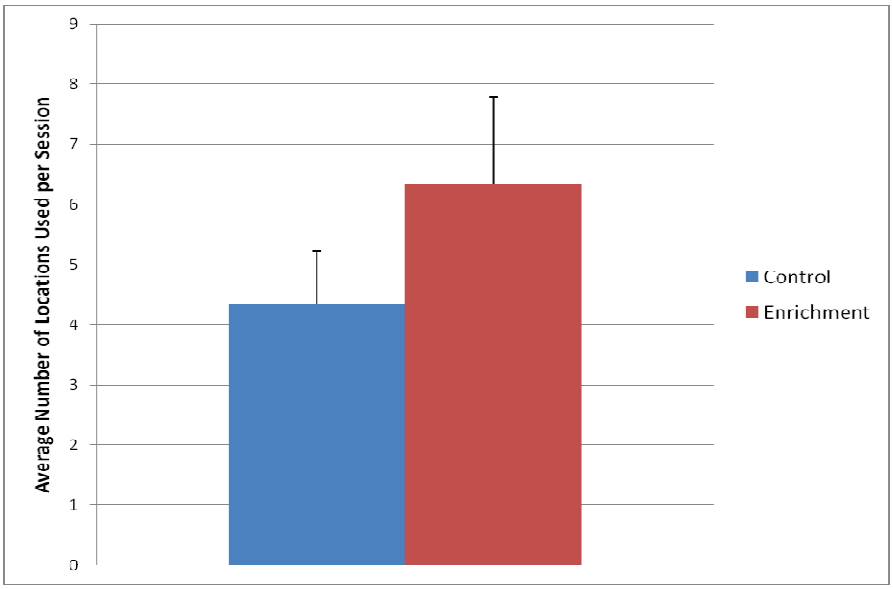

Fig. 2 Graph of average number of locations used by Maya and Inca during enrichment and control weeks.

Continuous Behavior Sampling

After continuous behavior data collection following enrichment exposure, the behaviors associated with olfactory stimulation were: Rubbing (RU), Sniffing (SN), Licking (LI), and Scratching (SC). The greatest number of behaviors exhibited (frequency) was at stimuli Right Guard and various perfumes (Figure 3). The jaguars also frequently spent time at the spices. Enrichment behaviors were exhibited less at scents such as Vanilla, Doe Urine, Basil and Bay Leaves.

The majority of time spent exhibiting enrichment behaviors (duration in seconds) was at Right Guard and the perfumes (Figure 4). The greatest amount of time spent exhibiting enrichment behaviors was at perfume stimuli (267.5 s). The least amount of time was at stimuli Doe Urine (1 s).

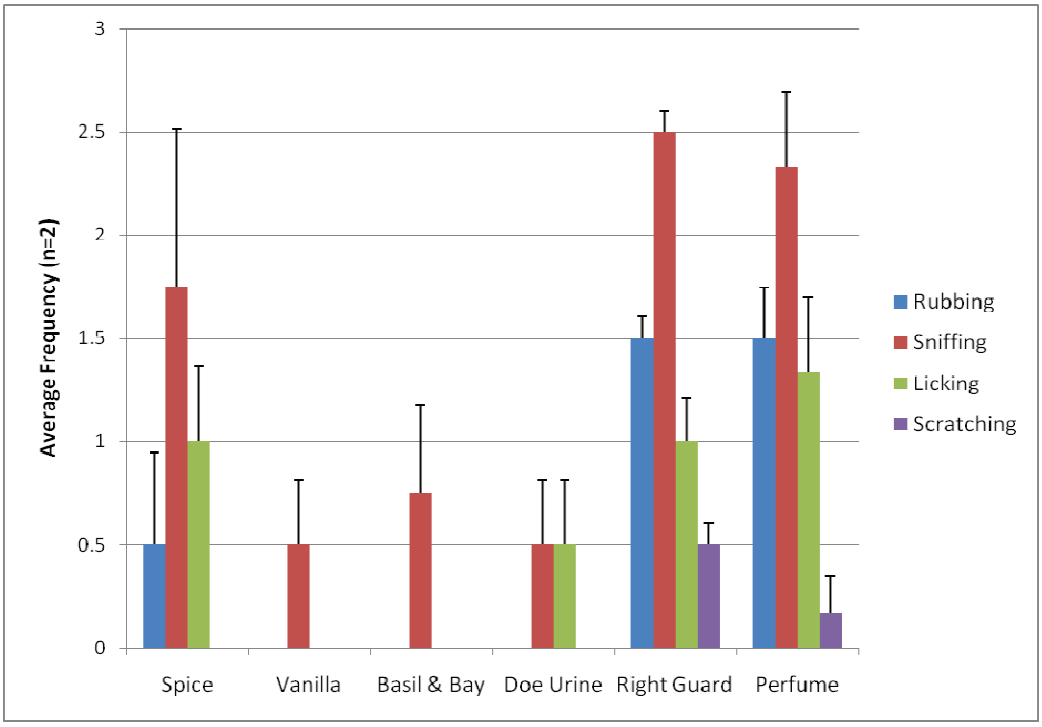

Fig. 3 Graph of enrichment behaviors exhibited in association with specific scents by Maya and Inca during olfactory enrichment. Values are the average of both individuals.

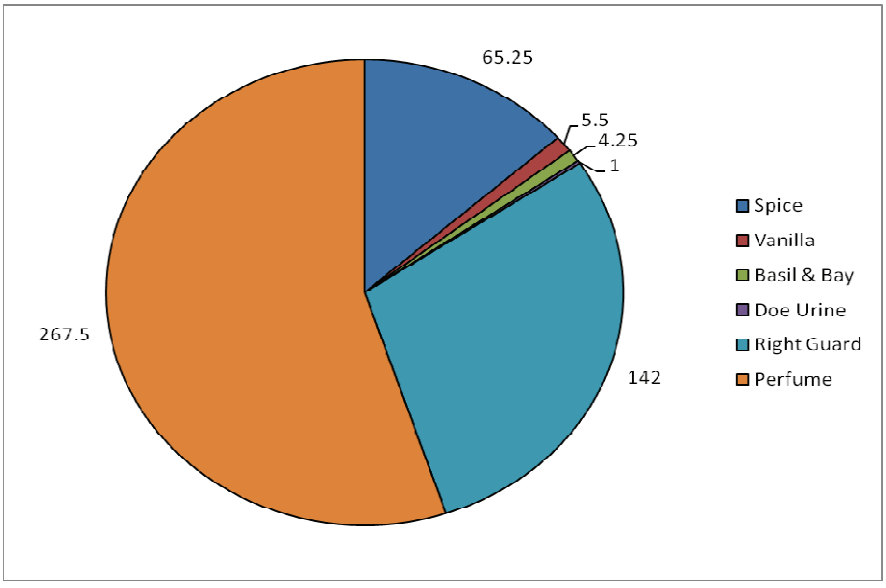

Fig. 4 Duration (in seconds) spent at a specific scent by Maya and Inca during olfactory enrichment.

Discussion

Enrichment Behavior

During control periods, jaguars exhibited greater amounts of time Resting. They also spent less time performing other behaviors, such as Pacing, Rubbing and Sniffing. During enrichment periods, felids were observed with a wider range of behaviors including specifically those associated with stimuli, such as Rubbing and Sniffing. When exposed to olfactory enrichment, jaguars exhibited specific behaviors (Sniffing, Rubbing, Licking, and Scratching) that were not exhibited during control periods. This supports the study by Rosandher (2005) in which snow leopards responded to olfactory stimuli and did not interact with controls in the same way, making olfactory enrichment a more effective method of promoting activity in captive felids. By exposing captive felids to novel scents, they exhibit non-stereotypical behaviors, promoting their overall fitness. However, these results are species and individual specific and may differ due to other environmental factors (Clark 2005). Captive felid enrichment must therefore be analyzed on a case-by-case basis to determine full effectiveness.

The average number of locations used by individuals during control periods was less than the number used during enrichment periods. The promotion of movement and use of areas not typically visited points to the success of olfactory stimulation in encouraging spatial use of captive habitat. Through olfactory enrichment, it is therefore possible to influence the spatial behavior of captive jaguars and encourage habitat use. This may be useful in attracting felids to areas better for viewing by zoo visitors or to promote safe transportation of animals.

There are five goals which environmental enrichment hopes to accomplish: to increase behavioral diversity, to reduce the frequency of abnormal behavior, to increase the range or number of natural behavior patterns, to increase positive utilization of the environment, and to increase the ability to cope with challenges in a more normal way (Young 2003). The olfactory stimulation of this study increased behavioral diversity (through enrichment behaviors such as Rubbing and Sniffing) and positive utilization of environment (through promotion of time spent in various areas of the exhibit). Rosandher (2005) found that olfactory stimulation increased behavioral diversity and increased natural behavior patterns in snow leopards. Overall, we found that olfactory stimulation is an effective enrichment program for captive jaguars.

Olfactory Stimulation

When exposed to olfactory stimulation, jaguars exhibited four specific behaviors associated with novel scents: Sniffing, Rubbing, Licking and Scratching. These behaviors differed from normal behavior exhibited during control periods and were a response to olfactory stimuli. Stimulation increased behavioral diversity through the use of novel scents. The frequency and type of behaviors exhibited with different scents varied with scent type. Deodorants and perfumes received the greatest range of behaviors. Deodorants, perfumes, and spices received the greatest frequency of behavior exhibited at stimuli. Vanilla, Doe Urine, and Bay Leaves and Basil received much less frequent or no behavior. However, other studies have found that the presence of fecal and/or urine samples from prey species effectively promotes activity through enrichment (Clark & Mitchell 2005).

As described by Wells, stimulating odors, such as Cinnamon may promote mental stimulation and overall wellbeing in captive animals (Wells 2009). The increased behavior associated with these stimulating scents in this study agrees with Wells' findings. The introduction of perfumes and deodorants has not been fully studied but seems to agree with scent stimulation promoting active behavior. Differences of reactions across a number of perfumes and colognes elicited various responses in cheetahs, tigers, and leopards including cheek rubbing (Thomas 2005). Non-stimulating scents such as Bay leaves did not promote such increased levels of activity. Further studies have describes calming scents such as lavender and chamomile in influencing felid behavior (Wells 2009). Through manipulation of these scents, it may therefore be possible to alter behavior in captive felids to promote fitness through increased activity due to stimulating scent exposure or the reduction of stress through calming scent exposure. Further research is necessary to investigate these effects.

Duration of time spent exhibiting enrichment behaviors at specific scents also varied. Jaguars spent the greatest amount of time at novel scents of deodorant and perfumes. Spices accounted for almost all of the other time spent exhibiting behavior. Rosandher (2005) also describes a preference for scents such as Cinnamon and Allspice by snow leopards. Other scents had limited or no time spent exhibiting behavior. This is congruent with the conclusions drawn concerning olfactory stimuli above. Overall, captive jaguars responded to stimulating novel scents in this study, with the majority of time spent at deodorants and perfumes.

Conclusion

Jaguars are top predators and considered a keystone species because of their impact on the populations of other animals in the ecosystem (Seymour 1989). While jaguars have been studied in the wild (Seymour 1989), compared with other large cat species, jaguars are still one of the most un-researched due to the difficulty associated with studying a species that has a low population density and obscure habits in its natural environment (Mallapur 1999). Environmental pressures such as deforestation and habitat destruction have led to a large decline in jaguar populations worldwide. In order to promote non-invasive research on felids in the wild, olfactory stimulation may be used to attract animals to hair traps, allowing scientists to collect data with minimal stress to the animal (Thomas 2005).

The information gleaned from these studies can help to promote conservation programs. An increasing number of these felids exist in zoos and a more comprehensive study of jaguar behavior could improve conservation efforts by zoos in maintaining this species in the future. By studying the behavioral effects of specific forms of olfactory enrichment, we hope to improve captive jaguar enrichment programs at the Memphis Zoo and perhaps educate other zoos around the

world. We are currently continuing our evaluation of the effects of olfactory enrichment on one of the captive jaguars, Maya, at the Memphis Zoo. We will explore the differences in behavior based upon the removal of conspecific interaction as the other jaguar, Inca, was moved from the Memphis Zoo following the conclusion of this study.

Acknowledgements

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Proteomic Comparison of Spleen Leukocyte Nuclei from Non-Obese Diabetic and Control Strains of Mice

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A better understanding of cell receptor signaling and its involvement in target and effector tissues can shed spotlight the pathogenic processes associated with type 1 diabetes mellitus at the molecular level. The goal of this research was to discover spleen leukocyte nuclear proteins that are differentially expressed between Non-Obese Diabetic (NOD) mouse model and the non-diabetogenic C57B1/6 and Non-Obese Resistant (NOR) control strains and probe the cellular signaling mechanism that characterizes the development of type 1 diabetes. Five mice from each strain were sacrificed at 3 weeks of age (this period precedes diabetic pathogenesis). The spleen leukocyte nuclear fraction was isolated, and 2-dimensional gel electrophoresis was performed on the samples. The gels were then stained with a ProQ Diamond phosphoprotein specific stain and a Krypton total protein stain. The data was subjected to a one way analysis of variance (ANOVA) followed by hierarchical clustering to identify proteins and phosphorylated proteins that were differentially expressed between the three strains. Results from the protein staining indicated that 212 proteins were differentially expressed between NOD and C57 (control) mice, and 118 phosphorylated proteins were differentially expressed between the NOD and C57 (control) mice.

Introduction

The human genome consists of approximately 25,000 genes, but the proteome consists of four to five times that number of proteins. Furthermore, this number can change depending on the cellular environment. The dependence of the proteome on the cellular environment shows the complexity of biological systems and hints at the potential uses of proteomic analysis in examining pathological disease processes, such as the onset of type 1 diabetes mellitus, at the molecular level. Type 1 diabetes mellitus is an autoimmune disorder in which leukocytes progressively destroy pancreatic beta islet cells, eventually leading to a cessation of insulin production by the islets and consequent insulin dependence. We hypothesize that a proteomic study of the pancreatic Islets of Langerhans and the attacking leukocytes may uncover proteins that are involved in the mechanisms that lead to the destruction of the insulin-producing beta cells. We searched for proteins that were differentially expressed between the Non-Obese Diabetic (NOD) mouse, which spontaneously develops type 1 diabetes mellitus after the fifth week of life, and the C57BI/6 and Non-Obese Resistant (NOR) control strains. The studies were conducted at a very young age before the immune system begins to attack the beta cells. Previous studies conducted in this lab have targeted protein expression levels in the Islets of Langerhans and have investigated whole leukocytes, isolated from the mouse spleen. In the current study, we focus specifically on the proteomic identity of leukocyte nuclei. By purifying the nuclear fraction, a more comprehensive analysis of signaling pathways can be conducted because a higher concentration of nuclear proteins can be loaded onto the gels. Identification of differentially expressed total and phosphorylated proteins will be helpful in determining causative factors of type 1 diabetes mellitus and may dictate new methods of treatment.

Methods

Leukocyte Isolation

Spleens were removed from mice sacrificed at 3 weeks of age and were placed in a small petri dish filled with 10 mL of Hank's Balanced Salt Solution (HBSS) from Gibco BRL (Rockville, MD, USA). The spleens were then transferred onto a metal mesh and were ground through the mesh using the plunger of a syringe. HBSS was flushed over the mesh to obtain maximum yield. The cells were placed in a 15 mL tube and were centrifuged for 5 minutes at 1000 rpm's. The supernatant was removed, and the pellet was resuspended. 3.5 mL of HBSS was added to the tube. The fraction was then underlaid with 3.5 mL of Lympholite-M (Density separation medium) from Cedarlane (Westbury, NY, USA) using a 3cc syringe and a 21G 1.5 needle. The tube was then centrifuged (400 g, 30 minutes, 20º C). After centrifugation, the leukocytes from the interface were transferred using a siliconized pasteur pipette to a new 15 mL tube, and HBSS was added to a volume of 15 mL. The tube was centrifuged for 5 minutes at 1000 rpm's, the supernatant was removed, and the pellet was resuspended. 5 mL of ACK lysing buffer was added to the tube, and the mixture was allowed to incubate for 5 minutes with occasional shaking. After the

incubation, the contents were centrifuged (5 minutes, 1000 rpm's). The supernatant was removed, and the pellet was resuspended. The cells were then washed twice with 15 mL of HBSS. Depending on the pellet size, between 3 and 5 mL of HBSS was added to the tube prior to the second wash, and 10 µL was extracted from the center of the tube and transferred to a hemocytometer for cell counting. The sample was separated into aliquots of 50 million cells each.

Nuclear Extraction

The Pierce Nuclear and Cytoplasmic extraction kit NE-PER (Pierce, cat# 78833) was used to separate the nuclear fraction from the isolated spleen leukocytes. 200 µL of ice-cold CERI + 2 µL protease inhibitor cocktail was added to the cell pellet, and the tube was then vortexed and incubated. 11 µL of ice-cold CER II was added, and the tube was then centrifuged (16,000 g, 5 minutes, 4º C). The supernatant (cytoplasmic extract) fraction was transferred to another tube and put on ice. 100 µL of ice-cold NER + 1 µL protease inhibitor cocktail was added to the cell pellet containing the nuclei, and the tube was then vortexed and put on ice. The tube was then centrifuged (16,000 g, 10 minutes, 4º C), and the supernatant (nuclear extract) fraction was transferred to a 1.5 mL tube and put on ice. Subsequently, this tube was stored at -80º C until first dimension was performed.

2-D PAGE

To prepare the samples for the first dimension, 25 µL of IPG ampholyte buffer from Amersham Pharmacia Biotech (Piscataway, NJ, USA), corresponding to the desired pH range, was added to 3 mL of rehydration buffer. 450 µL of the rehydration buffer solution was then added to each sample. The samples were placed in a sonication bath for 10 minutes, next on a shaker for 30 minutes, and thereafter were centrifuged at 12,000 g for 20 minutes at room temperature. 370 µL of each sample was added to a precast IPG strip (180 x 3 x 0.5 mm) with a linear 4-7 pH gradient (Amersham), and the strips were placed in an IPGphor isoelectric focusing system (Amersham). The strips were allowed to equilibrate for 12 hours and then underwent an isoelectric focus at 20º C under the following conditions: 100 V for 2 hours, 500 V for one hour, 1000 V for one hour, and 8000 V up to 90,000 Vhr. The strips were then removed and stored at -80º C until the second dimension. To prepare for second dimension gel electrophoresis (SDS-PAGE), a stock equilibration solution was first prepared containing 36 g urea, 2 g SDS, 25 mL of 1.5 M Tris, 20 ml glycerol, and distilled water to reach 100 mL. The stock solution was split into two 50 mL aliquots. 1 g dithiothreitol was added to the first sample while 1.25 g iodoacetamide and a trace of bromophenol blue were added to the second solution. The strips were first incubated at room temperature in the dithiothreitol solution with gentle agitation for 10 minutes. Following this, the strips were transferred to a fresh tray and were incubated in the iodoacetamide solution for 10 minutes with gentle shaking. 5 µL of molecular marker was added to 2 mm squares of filter paper, and a square was placed on the negative end of each strip to serve as a molecular weight marker. The strips were applied onto pre-cast SDS-PAGE gels and were fixed in place with 3-5 mL of 1% hot agarose. The gels were loaded into a Protean Plus Dodeca Cell electrophoresis apparatus from Bio-Rad (Hercules, CA, USA) containing 25 L of running buffer (75.69 g Tris, 360.34 g glycine, 25 g SDS, and 25 L distilled water) that was cooled to 15º C by a refrigerated circulator and were then run in the second dimension at a constant voltage of 200 V for 430 minutes.

Gel Staining

After the 2D preparation, each gel was washed fast with distilled water. The gels were next placed in a 50% methanol, 10% acetic acid fixation solution on a shaker for 30 minutes in the dark. The fixation was repeated a second time and left overnight on the shaker in a dark environment. The following day, each gel was washed 3 times for 15 minutes in the dark on a shaker. The gels were incubated in the dark for 1.5 hours in the ProQ Diamond stain, and were then destained three times for 30 minutes in the dark on a shaker. The gels were washed with water twice for 5 minutes in the dark on a shaker, and an image was acquired using an FX Molecular Imager laser scanner (Bio-Rad) with 532 nm laser excitation and 555 nm bandpass emission filter. 100 mL of Krypton protein stain was then diluted with 900 mL of distilled water, and the gels were allowed to incubate overnight in the solution in a dark environment on a shaker. The next day, the gels were destained with a 5% acetic acid solution for 5 minutes on a shaker in the dark and were then washed twice for 30 minutes under the same conditions. A second image was captured with a laser scanner with an excitation 488 nm and 555 nm bandpass emission filter to detect all proteins.

Data Analysis

Protein spot analysis was performed using the Progenesis Same Spots software program version 2.0. The program aligned, processed, and analyzed the 15 images and generated a list of protein spots that were ranked by spot intensity for each sample. The spots that were identified as matches between the gels were visually inspected to check for errors. The expression data was then imported into the GeneSpring software program, and a one-way ANOVA analysis was performed to identify proteins that were expressed at different levels between the three mouse strains. The results were grouped by hierarchical clustering and presented in a gene tree that was colored according to the protein expression levels (red for relatively high expression and blue for relatively low expression).

Results

We isolated nuclei from 5 independent spleen leukocyte samples from each of the 3 strains (NOD, NOR and C57Bl/6). The proteomes of these 15 samples were analyzed by 2D-gel electrophoresis followed by staining with ProQ Diamond phosphoprotein stain and Krypton total protein stain. After the gels were scanned, the images were uploaded into Progenesis software, which aligned and analyzed the images to determine the total number of different spots and their relative intensity within each strain. For the Krypton strain, the program determined that there were a total of 530 different protein spots detected among the 15 gels, while the phosphostained gels had a total of 420 proteins detected (Figure 1). These identified spots were visually inspected for errors when matching spots from different gels. Spot intensity data was then imported into the GeneSpring software, and a one-way ANOVA was performed on this data to determine spots that had levels of expression that were significantly different ($p<0.05$) between the three strains (Table 1.)

Table 1.

Stain	Total Number of Spots	Differentially Expressed Spots
Krypton Stain	530	212
ProQ Diamond Stain	420	118

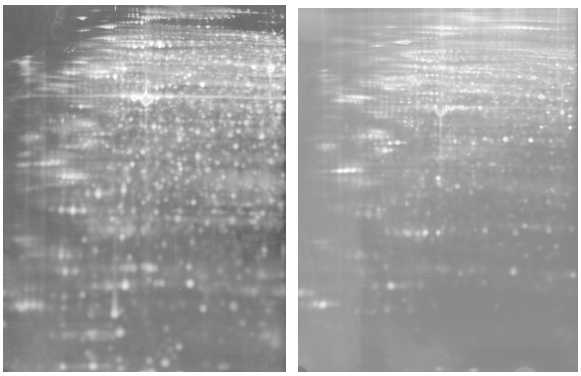

Figure 1. Example of a 2D gel that has been sequentially stained, first with Krypton Stain (left picture) to detect total proteins and then with a ProQ Diamond Stain (right picture) to detect phosphorylated proteins present within mouse leukocyte nuclei.

expression levels within each strain. The results were displayed in a gene tree, examples of which are depicted in Figure 2 and Figure 3. Of the 212 differentially expressed total proteins (Krypton stain), 41 had relatively lower expression in the NOD mice. The remaining proteins were seen to have different expression levels between the NOD and C57 mice but were similar in expression among NOD and the NOR control strain and were therefore not of interest. Among the 118 differentially expressed phosphorylated proteins, 30 had relatively lower expression in the NOD mice while 8 had higher relative expression compared to the controls. The remaining proteins seen in the gene tree had different levels of expression between the NOD and NOR mice but were similar between the NOD mice and the C57 control. These proteins were therefore not of interest in our study. The spots of interest will be cut from the gels and be trypsinized to prepare them for MALDI-TOF MS analysis. This identification of differentially expressed proteins is in progress.

Discussion

Proteomics is a much more complex study of biological systems than genomics primarily because both the amount and posttranslational modifications of particular proteins within the cell can change depending on the cellular environment. The method of proteomic analysis proved to be successful in our study. This technique measured the amount and phosphorylation of proteins that were differentially expressed between experimental and control strains of mice. Once our proteins are identified, the results can be compared with molecular networks from previous studies to identify signaling mechanisms causing abnormal biological activity of the NOD spleen leukocytes. We hypothesize that differentially expressed nuclear protein markers may suggest a particular cellular target that is altered during the early development of type 1 diabetes mellitus. With the knowledge of specific proteins' 3-D structures, drug researchers can design drugs that interfere with the action of those specific proteins. This and further studies are a necessary step in the future development of better treatments for those afflicted with or genetically predisposed to acquiring type 1 diabetes mellitus.

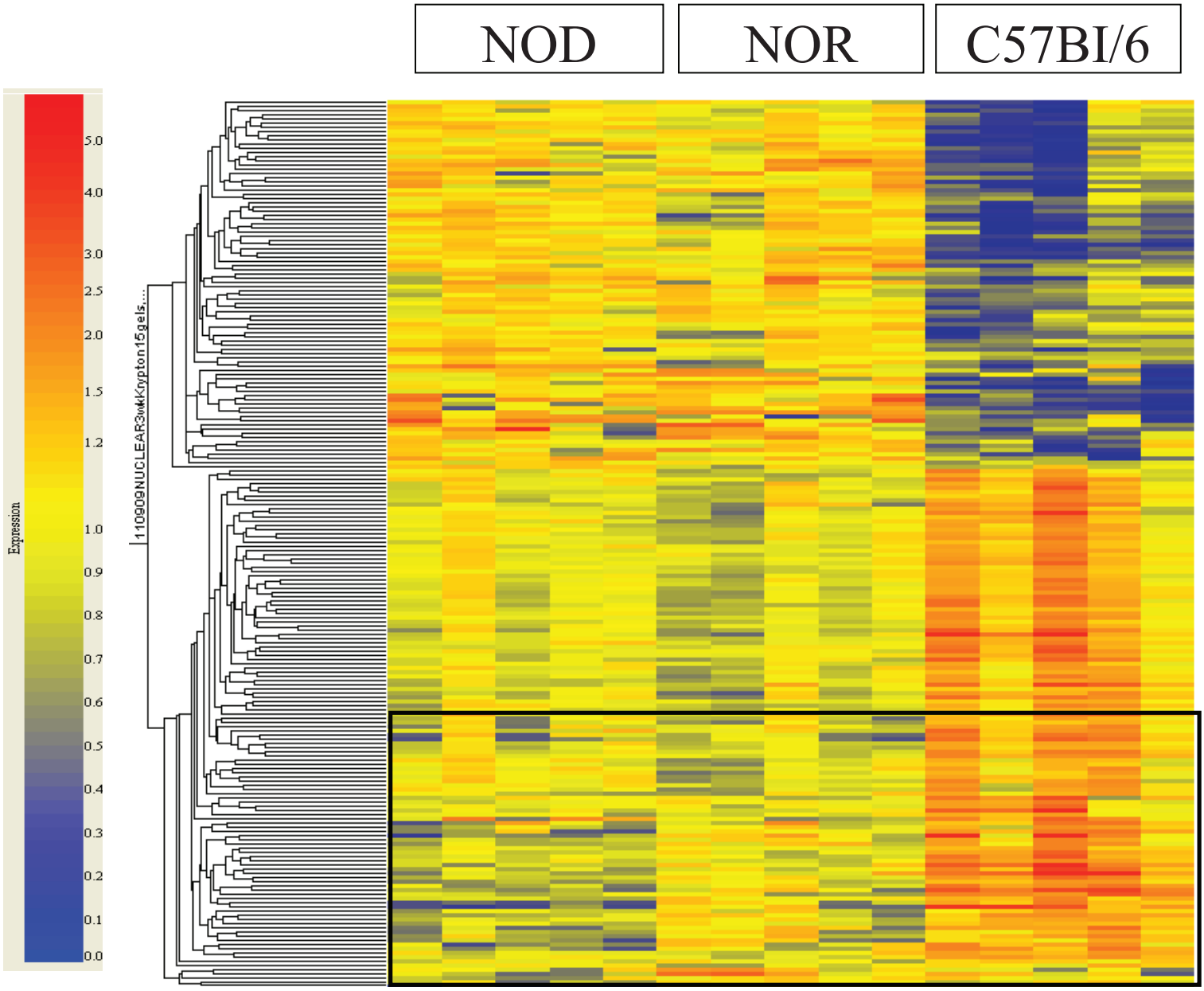

Fig. 2 Hierarchical cluster of the 212 differentially expressed proteins on the Krypton stained gels. The boxed region indicates that 41 of the proteins show lower expression in the NOD strain when compared with the C57BI/6 control strain.

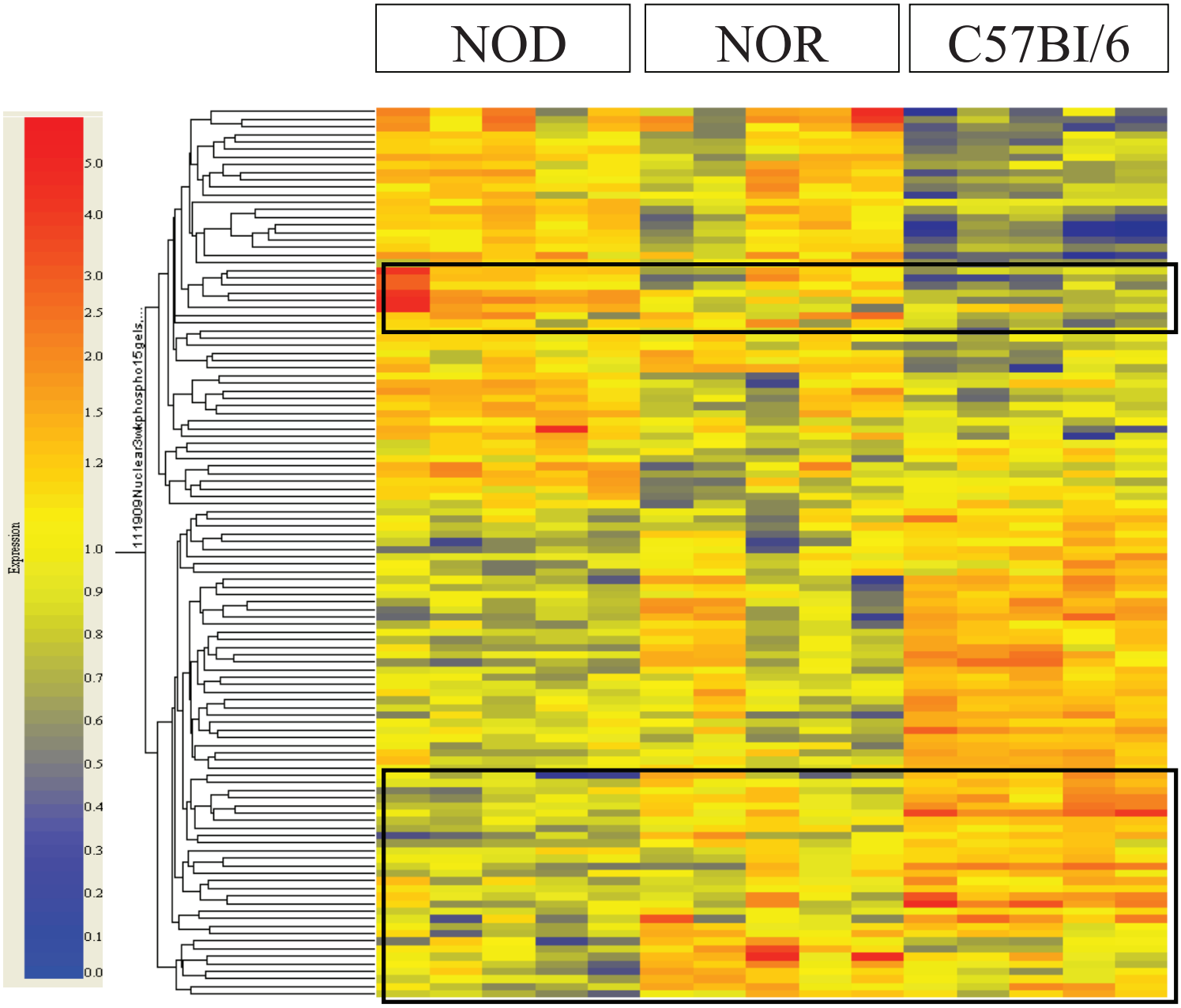

Fig. 3. Hierarchical cluster of the 118 differentially expressed proteins on the ProQ Diamond phosphostained gels. The top boxed region indicates that 8 of the proteins show higher expression in the NOD strain when compared with the C57BI/6 control strain. The bottom boxed region indicates that 30 of the proteins show lower expression in the NOD strain when compared with the C57BI/6 control strain.

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The Neuroprotective Effects of Exercise in an Immobilization Chronic Stress Model in the Mouse Hippocampus

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A number of environmental factors have been indicated to increase vulnerability to neurodegenerative disorders, and one possible environmental factor is chronic stress, which triggers the release of glucocorticoids in the brain. Glucocorticoids are known toxic factors that may cause cell death (apoptosis) in the hippocampus. Exercise has been shown to increase the secretion of factors that may help to protect the brain against the toxic effects of glucocorticoids. Therefore, we hypothesize that chronic restraint stress will increase neurodegeneration in the brain as measured by apoptosis and astrogliosis. In addition, exercise will decrease the expression of these markers and will protect against neurodegeneration. To test this, female mice were randomly assigned to standard housing (SH) or placed in cages equipped with running wheels (Exercise). Half of the mice from each group were exposed to chronic restraint stress for two hours each day for two weeks prior to sacrifice. To analyze the expression of toxic effects, sections of the hippocampus will be labeled for markers of apoptosis (Bax) and glial activation (GFAP) using immunohistochemistry techniques. We predict that chronic restraint stress will induce toxic effects in the hippocampus and that exercise will protect against these effects. However, our hypotheses were not supported and the Bax staining was inconsistent, so no definite conclusions can be drawn from this data. Our only significant finding was that in the no stress conditions exercise increased astrocyte expression as compared to standard housing.

Introduction

Because American society revolves around fast food, sweetened sodas, large portion sizes, and sedentary activities, obesity has become an epidemic (Brannon & Feist, 2007). Since the 1950's what constitutes "normal" body weight consistently increased, and since the early 1980's adult obesity increased by 50% with currently 35% of all American adults being obese and another 29.8% being overweight (Brannon & Feist, 2007). One cultural trend that contributes heavily to America's increasing waistlines is the overall decrease in physical activity. Instead of blue-collar jobs involving manual labor, more Americans work white-collar jobs behind computers or in cubicles; budget cuts cause physical education programs to disappear from schools; personal automobiles replace walking or riding a bike as a mode of transportation; and parents who fear for their children's safety discourage them from playing outside, so instead children remain inside in front of a television or computer screen. As Americans remove exercise from their daily lives, they endanger not only their bodily health by increasing their risk for a variety of diseases and pathological conditions, such as cardiovascular diseases, Type II diabetes, muscular atrophy, and obesity. They also endanger the health of their brains by increasing their risk for Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases (Booth & Lees, 2007). For centuries human survival was dependent on mobility (see Radak *et al*., 2008b for review), so the human body evolved to expect, even need, a certain level of physical activity. Hence, physical inactivity leads to impairment of physiological processes and reduces the whole body's resistance to the damaging effects of oxidative stress (see Radak *et al*., 2008a for review).

Oxygen is metabolized in all parts of the body, and although this process is necessary for life, it also comes at a cost. Oxygen consumption involves oxidative phosphorylation of different materials; this process produces superoxide and electronegative, unstable free radicals that tend to damage and inactivate other molecules by stripping them of electrons (see Kiraly & Kiraly, 2005 for review). Normally, superoxide and free radicals are neutralized by the body's natural stores of antioxidants; however, when oxidative agents reach such an overwhelming concentration that they cannot be neutralized by antioxidants present in the tissue, a condition known as oxidative stress occurs (see Madrigal *et al*., 2006 for review). The brain is particularly vulnerable to oxidative stress because: 1) the brain utilizes about one-fifth of all the oxygen consumed by the body, and greater oxygen consumption means greater production of free radicals and oxidants; 2) neuronal membranes are made of lipids enriched with easily oxidizable polyunsaturated fatty acids, 3) the brain has naturally low levels of antioxidant enzymes, and 4) the brain contains high concentrations of iron, which is a potent catalyst for oxidant formation (see Liu & Mori, 1999 for review). Oxidative stress is a natural, inescapable process, but

unfortunately the brain is especially vulnerable to the potentially damaging effects of this natural process.

Glucocorticoids are hormones secreted by the body that are responsible for many of the detrimental effects of oxidative stress. Glucocorticoids damage neurons by causing the accumulation of glutamate (Stein-Behrens *et al*., 1992; Moghaddam *et al*., 1994; Stein-Behrens *et al*., 1994), which binds to an N-methyl-D-aspartate neuronal receptor. Glutamate binding increases the number of open calcium ion channels, which greatly increases calcium influx (Choi, 1992; Hertz *et al*., 1999; Kandel & O'Dell, 1992; Mawatari *et al*., 1996. As the calcium ion concentration increases inside the neuron, the xanthine dehydrogenase/oxidase system is activated which produces free radicals (Mawatari *et al*., 1996). Not only do glucocorticoids contribute to the production of free radicals, they also decrease already low neuronal antioxidant capacity (see Kiraly & Kiraly, 2005 for review). Glucocorticoids can cause potent long-term damage because after they bind to their receptors, chaperone proteins transport them into the nucleus where they influence gene transcription. If gene transcription is altered over a period of time, the protein content of a neuron will change, affecting its function (see Joëls, 2008 for review).

Human bodies evolved the oxidative stress response as a way to deal with acute stressors, or any immediate threat, whether real or perceived, that disrupts homeostasis and requires a compensatory response (see Radley & Morrison, 2005 for review). For example, if a person suddenly meets a bear in the woods, his or her heart rate and breathing rate immediately increase, pupils dilate, and overall metabolism speeds up in order to provide the body with the energy needed to flee. The acute stress response is adaptive, but when multiple, often uncontrollable and unpredictable, stressors are presented consecutively with little time for recovery in between, the chronic stress response becomes maladaptive (see Joëls, 2008 for review). Modern society has achieved living conditions in which the acute stress response is not necessarily important for survival. Instead, people experience chronic stress, such as in response to daily problems experienced at work or with friends or family (see Sapolsky, 1998 for review).

Chronic stress experienced by most people today has been linked to maladaptive changes in the brain. For example, studies have shown that chronic stress leads to dendritic retraction, loss of synapses, and less postsynaptic density in the hippocampus; it also reduces neuronal proliferation, survival, migration, and differentiation (see Joëls, 2008 for review). Chronic stress also decreases levels of brain-derived neurotrophic factor (BDNF), a substance necessary for neuronal growth and survival (see Kiraly & Kiraly, 2005 for review). Moreover, chronic stress increases brain metabolism and oxygen consumption (Landfield & Eldridge, 1994), which increases the brain's oxidative burden and the potential for oxidative damage (see Liu & Mori, 1999 for review). Compounding this effect is the fact that chronic stress also decreases neurons' natural defense capacity (see Kiraly & Kiraly, 2005 for review). Finally, chronic stress increases the amount of damaging glucocorticoids in the brain (see Joëls, 2008 for review). There is evidence suggesting these hormones cause cytoskeleton destabilization, which effectively kills neurons by destroying their internal structure (see Radley & Morrison, 2005 for review). Overall, chronic stress exacerbates the damaging effects of oxidative stress, making the flow of information within the brain less efficient, making it harder to induce the basic mechanisms of memory, and preventing the normalization of activity after a stressful event, all of which can lead to cognitive impairment (see Joëls, 2008 for review).

Since recognizing the damaging effects of chronic stress and oxidative stress, researchers have begun looking for therapies to protect the brain against these threats, and one promising possibility is exercise. Studies suggest that exercise protects the brain from many different insults, facilitates learning and memory, dampens the effects of brain injury, and delays the onset of neurodegenerative disorders and slows symptom progression. Exercise has these benefits because it increases and makes more efficient the mechanisms that support neuronal plasticity, such as synaptic stabilization, neurogenesis, and vascular development and function (see Cotman *et al*., 2007 for review). Studies have found that mice allowed to run on an exercise wheel showed increased neurogenesis in the hippocampus and the survival of these newly formed neurons increased (see Kiraly & Kiraly, 2005 for review; van Pragg *et al*., 2005). In human studies, cardiovascular training before and after stroke reduced neuronal damage and improved recovery time (see Cotman *et al*., 2007 for review). Exercise can even slow generalized neuronal death due to aging and help protect areas of the brain associated with cognitive function from age-related atrophy (see Cotman *et al*., 2007 for review); for example, aged rats allowed to exercise for four weeks expressed new neurons with the same dendritic length, dendritic branching, and spine density as those in young rats (van Pragg *et al*., 2005). Even in human studies involving Alzheimer's disease, exercise reduced the number of cytotoxic amaloid-beta plaques in both the cortex and the hippocampus (see Kiraly & Kiraly for review, 2005; see Cotman *et al*., 2007 for review).

Since exercise is neuroprotective in such debilitating conditions as stroke and Alzheimer's disease, perhaps it would also protect neurons against the damaging effects of oxidative stress induced by chronic stress. Since examination of this research question would be unethical in humans, we chose instead to use mice immobilization as a model of human emotional stress. Previous studies have established that limb immobilization and tube immobilization stress in mice produces oxidative agents which result in oxidative damage (Radak *et al*., 2001b; Fontella *et al*., 2005). We chose to focus on the hippocampus specifically because of its high vulnerability to oxidative damage due to its low antioxidant stores (see Kiraly & Kiraly, 2005 for review) and high number of glucocorticoid receptors (see Radley & Morrison, 2005 for review). Moreover, the hippocampus is essential to the integration of information from all areas of the cortex and to the storage and retrieval of long-term memories. Because of this, atrophy, degeneration, or malfunction of the hippocampus is often implicated in disorders of memory, such as dementias and Alzheimer's disease (see Miller & O'Callaghan, 2005 for review).

In this experiment, damage to the hippocampus will be measured by the presence of astrocytes, which are glial cells that normally provide neurotrophic support to neurons and aid in the maintenance of neuronal integrity and function by connecting neurons to blood vessels (see Miller & O'Callaghan, 2005 for review). Because of their role in removing waste and damaged tissue, astrocytes usually increase in number in response to neuronal damage or injury (Anderson, 2004; see Miller & O'Callaghan, 2005 for review). However, an over abundance of astrocytes, as occurs in oxidative stress, can actually contribute to neuronal damage because the astrocytes begin to release neurotoxic factors, such as pro-inflammatory cytokines, reactive nitrogen species, proteases, reactive oxygen species, eicosanoids, and excitatory amino acids (see Gao *et al*., 2003 for review). Another measure used to assess hippocampal damage will be the presence of apoptotic neurons. It has been observed that characteristics of neuronal death due to oxidative stress are similar to characteristics of neuronal death caused by apoptosis (see Satoh *et al*., 1998 for review), suggesting that oxidative stress causes tissue damage by triggering neurons to begin apoptosis. Indeed, in a PC12 cell *in vitro* neuronal model, cells exposed to conditions conducive to oxidative stress showed marked degeneration and any observed cell death was triggered by apoptosis (Kubo *et al*., 1995; see Satoh *et al*., 1998 for review).

Therefore, we hypothesize that subjecting mice to immobilization stress will increase oxidative stress in the hippocampus as measured by increased astrocyte expression and increased apoptotic neuron number. Secondly, we expect exercise to provide protection against the harmful effects of oxidative stress as measured by a decrease both in the number of astrocytes and in the number of apoptotic neurons in animals allowed to exercise as compared to those living in standard housing conditions. If our hypotheses are supported by the data then this will add to the growing body of research suggesting the importance of physical activity. Americans are bombarded with information about how exercise is beneficial for the heart, weight maintenance, etc. but it must also be stressed that exercise is crucial for optimum brain function.

Methods

Animals

Thirty-two, eight-week-old, female mice (C57/B16J) were obtained from Jackson Laboratories (Bar Harbor, ME) and kept in a room at constant temperature (68 ± 2°C) and humidity with a light set to a 12-hour light/12-hour dark cycle (6AM-6PM). Two mice were placed in each cage and given standard mouse chow and water *ad libitum* while in home cages. Mice were monitored daily to ensure maintenance of proper living conditions. Six mice were randomly assigned to each experimental condition: standard housing (SH), standard housing plus stress (SH + S), exercise (Ex), and exercise plus stress (Ex + S). Mice in the SH and SH + S conditions were housed in a standard colony cage and mice in the Ex and Ex + S conditions were housed in standard breeding cages equipped with two running wheels for voluntary exercise. At all times the animals were maintained and handled according to guidelines approved by the Rhodes Institutional Animal Care and Use Committee and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Chronic Restraint Stress Paradigm

The mice were habituated into their new living conditions for one week. Stress was administered by placing the mice in Broome Restraint tubes (Harvard Apparatus; Holliston, MA) for two hours a day for fourteen consecutive days. Each day, during the time the other mice were being stressed, the mice not receiving stress were picked up in order to avoid confounding effects of handling stress.

Immunocytochemistry Techniques

Tissue Preparation

After the two weeks of experimental manipulation, mice were euthanized using tribromoethanol (250 mg/kg (i.p.)). After euthanization, the brains were transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS; pH 7.4). Whole brains were extracted and postfixed overnight at 4°C in the same fixative for preservation purposes. The next day the brain was processed for embedding in paraffin for sectioning. A microtome was used to make eight-micron-thick sections in the coronal plain which were mounted into SuperFrost Plus Slides (Fisher Scientific; Atlanta, GA).

GFAP Staining Technique

Stains that mark the presence of glial fibrillary acidic protein (GFAP) were used to visualize astrocytes. Once the hippocampus was clearly visible, every third slide was selected for staining. The slides were then deparaffinized in xylenes and rehydrated in graded alcohols before being rinsed and rehydrated in PBS-Tween (PBS-T; PBS containing 0.05% Tween 20). After removing the excess PBS-T around the tissue, the tissue was encircled using a PAP pen (Research Products International; Mt. Prospect, IL) and placed in PBS-T/Blocking Buffer (PBS-T/BB; 0.5% Bovine Serum Albumin, 0.3% Triton, in PBS) for 30 minutes at room temperature. After blocking, slides were incubated in the primary antibody (anti-GFAP; clone G-A-5; Sigma Chemical Company; St. Louis, MO) at a 1:1000 dilution in PBS-T/BB overnight at 4°C.

The next day the slides were washed in three changes of PBS-T for five minutes each. The secondary donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories; West Grove, PA) was added to the slides in a 1:250 dilution in PBS-T/BB and incubated in a humid chamber for two hours at room temperature. The slides were washed in PBS-T as before and visualized by peroxidase-mediated deposition of diaminobenzidine (DAB; DAB Immunoperoxidase Substrate kit; Vector Laboratories; Burlingame, CA) per manufacturer's instructions. The slides were washed in running tap water for 10 minutes and dehydrated though a series of graded alcohols and xylenes before being coverslipped using Permount.

Bax Staining Technique

Bax was used to detect apoptosis. The slides were deparaffinized and rehydrated though xylenes and graded alcohols and placed in tap water for 5 minutes. Antigen retrieval was performed by gently boiling the slides in a

10mM citrate buffer (0.1M citric acid, 0.1M sodium citrate, distilled water) for 15 minutes. The slides were allowed to cool to room temperature and were then rehydrated in PBS-T for 30 minutes. The tissue was encircled using a PAP pen as described above. Nonspecific binding was blocked by preincubating sections for 30 minutes at room temperature in blocking buffer [TNB (0.1M TrisHCL, 0.15M NaCl, 5% blocking buffer); pH 7.5; Renaissance TSA-Indirect kit, NEN Life Science Products; Boston, MA]. The primary anti-Bax antibody (clone 6A7; BD Biosciences; San Jose, CA) was diluted 1:250 in TNB and placed on the slides which were incubated overnight in a humid chamber at 4°C.

The slides were washed in PBS-T as before. The same secondary donkey anti-mouse antibody was diluted 1:250 in TNB and added to the slides before incubating for 2 hours in a humid chamber at room temperature. The slides were washed in PBS-T as previously described. In order to amplify the stain, the slides were incubated in biotinyl tyramide (BT; 1:50 dilution in Amplification Diluent from the Renaissance TSA-Indirect kit) for 10 minutes at room temperature. The slides were washed in PBS-T as before and incubated in a 1:100 dilution of streptavidin conjugated to HRP in TNB for 1 hour at room temperature. Again the slides were washed in PBS-T and visualized with DAB per instructions on the kit. The slides were next counterstained by placing the slides in hematoxylin for 15 seconds followed by 10 minutes in running tap water and 30 seconds in acid alcohol (3% acetic acid in 70% EtOH). The slides were then dehydrated through graded alcohols and xylenes and coverslipped using Permount.

Data Analysis

To quantitatively analyze the amount of astrocytes detected with GFAP staining, images of the hippocampus on the prepared slides were taken with a Leica microscope equipped with a digital camera. Five images of tissue sections containing CA 1-3 fields were randomly chosen from each animal in each condition. The images were coded so as to make the quantification process blind to the raters. Astrocytes in each image were counted by three independent reporters and their data compiled to achieve an average amount of astrocytes for each image. These averages were used to perform statistical analyses to determine if there were significant differences among the variables. Statistical Program for Social Sciences (SPSS) was used to perform a 2 x 2 factorial Analysis of Variance (ANOVA) and a one-way ANOVA with Tukey and LSD post-hoc analyses.

Images of the CA fields of the hippocampus were also taken of the prepared tissue stained with Bax. The slides photographed were chosen in the same way as GFAP imaging. These images were qualitatively analyzed for high, medium, or low levels of apoptotic nuclei marked by the expression of the Bax protein.

Results

Astrogliosis in the Hippocampus

One marker of degeneration in the hippocampus is enhanced glial expression. To assess glial recruitment, photomicrographs were taken of the CA fields of animals from each condition, and the number of GFAP labeled astrocytes was assessed (Figure 1). The numbers observed from three independent reporters were averaged for each condition and the marginal means (±SEM) were: SH = 36.80 (±5.02); SH + S = 46.40 (±3.55); Ex = 51.30 (±4.46); Ex + S = 39.80 (±4.24) (Figure 2). There were no significant main effects in either the housing or the stress conditions, [$F(1, 85) = 0.820$, $p = 0.368$; $F(1,85) = 0.045$, $p = 0.832$]. A factorial ANOVA revealed a significant housing by stress interaction [$F(1,85) = 5.87$, $p<.05$]. In order to further examine the interaction, the factorial ANOVA was followed up with a one-way ANOVA and post-hoc analyses using Fisher LSD and Tukey HSD for pair-wise comparisons. The second series of tests revealed that the only significant simple effect was between the SH condition and the Ex condition (ps<0.05). Mice in the Ex condition expressed significantly more astrocytes (M = 51.26) than mice in the SH condition (M = 36.80).

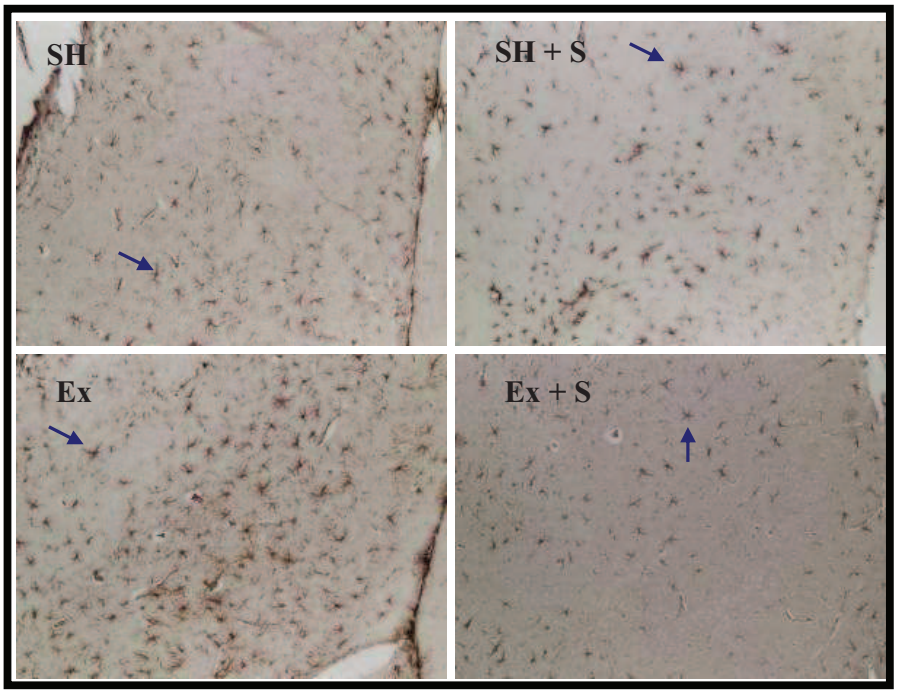

Fig. 1 Low power photomicrographs (10X) of GFAP labeled astrocytes in the CA fields of the hippocampus. The number of astrocytes in each image is representative of the average for each group and arrows point to an astrocyte with well defined cell soma and processes.

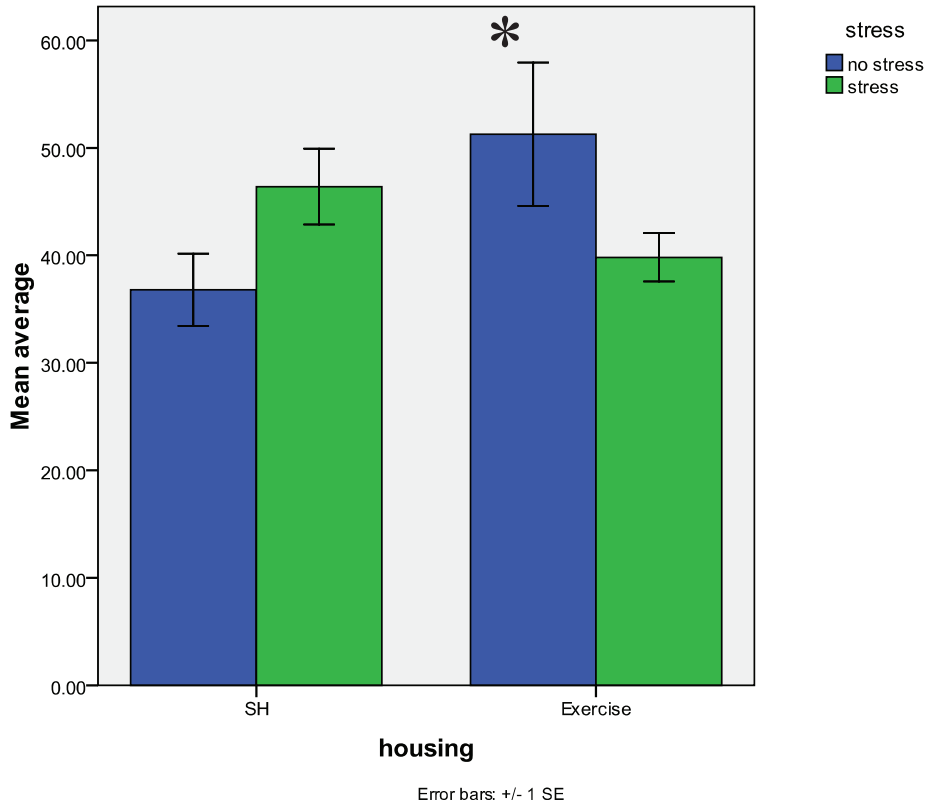

Fig. 2 A bar graph showing the average number of astrocytes expressed in the CA region of the hippocampus of mice in each experiemental condition. A factorial ANOVA revealed a significant housing by stress interaction. Bars represent means ± SEM; * represents $p<0.05$ as compared to standard housing.

Expression of the Apoptotic Marker Bax in the Hippocampus

Initial observation seemed to indicate that the Bax staining was optimized and appropriate; however, upon further analysis the staining proved to be inconsistent and therefore unquantifiable. Qualitative analysis was attempted but since the quality of the staining varied between slides from the same animal, the results were inconclusive. Further optimization is needed to obtain quantifiable results. Selected images are representative of slides with appropriate staining for Bax labeled neurons (Figures 3 and 4).

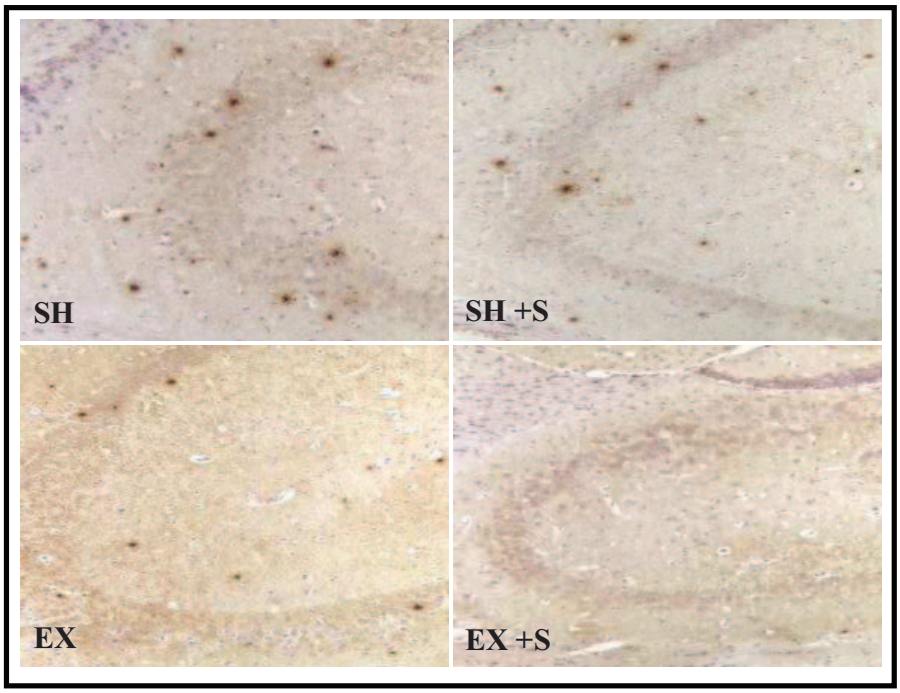

Figure 3: Low power photomicrograph (10X) of Bax labeled apoptotic nuclei in the CA fields of the hippocampus.

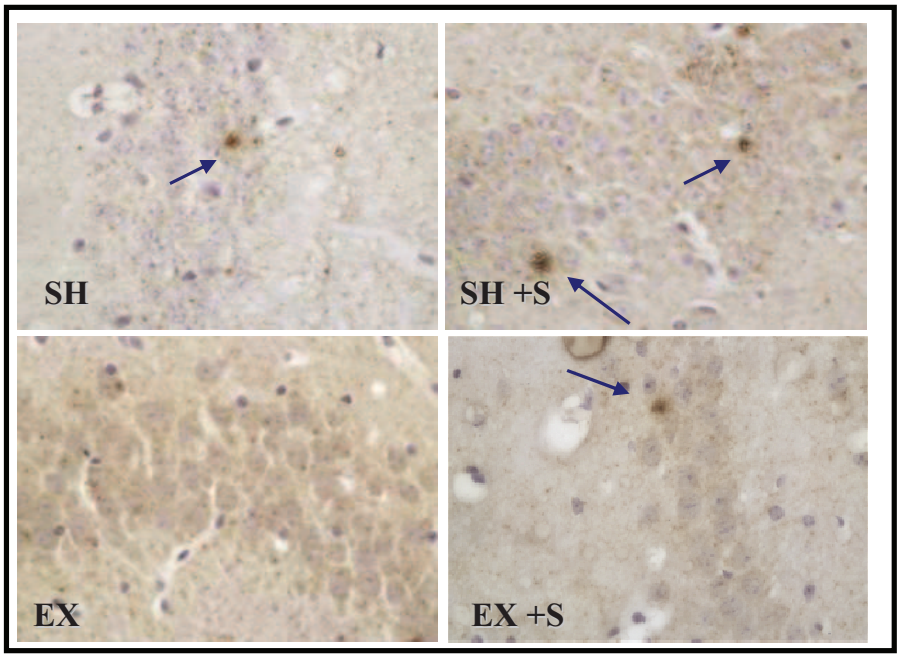

Fig. 4 High power photomicrograph (40X) of Bax labeled apoptotic neurons in the CA fields of the hippocampus. Arrows point to cells that phenotypically appear to be prominent apoptotic neurons.

Discussion

The data from this experiment did not support our original hypothesis that immobilization stress would increase oxidative stress, in that there was not a significant increase in astrocyte expression in either of the stress conditions as compared to the conditions that did not experience stress. Nor did the data support the hypothesis that exercise would protect against oxidative stress because there was no significant difference between the number of astrocytes in the exercise conditions as compared to the standard housing conditions.

Our only significant finding was that exercise increased astrocyte expression in animals that did not experience stress, which contradicted our hypothesis. One possible explanation for this finding is that exercise causes oxidative stress and the increase in astrocyte number is a result of astrogliosis in response to exercise-induced neuronal injury. Some previous research does suggest exercise is a stressor and causes the production of reactive oxygen species (Powers *et al*., 1999; Ji *et al*., 2006). Reactive oxygen species are involved in oxidative stress and can be harmful to tissue, so it is possible that the mice engaged in enough exercise to increase the levels of reactive oxygen species in their hippocampi. Since activated astrocytes help rescue injured neurons by eliminating toxic substances, releasing neurotrophins, and promoting tissue repair (see Gao *et al*., 2003 for review), more astrocytes were recruited to alleviate the damage caused by the oxidative challenge of exercise.

However, this explanation is probably not accurate because the reactive oxygen species created during exercise tend to only be harmful to tissue unaccustomed to this type of stressor (Powers *et al*., 1999; Ji *et al*., 2006; see Radak *et al*., 2008b for review). Mice in the exercise conditions of our experiment were placed in cages with exercise wheels and allowed to run for two weeks prior to the two-week experimental stress manipulation as well as run during the time of the stress. Thus, the mice had been exercising for almost a month, so the tissue can hardly be considered unaccustomed to the stress of exercise. Instead, these mice were regular exercisers and regular exercise has been shown to upregulate the body's defense mechanisms against oxidative stress (see Radak *et al*., 2008b for review). Indeed, the reactive oxygen species generated during regular exercise can actually serve as messengers that help change gene expression so as to increase the activity of antioxidant enzymes and promote more effective housekeeping by DNA repair enzymes and proteasome complexes (see Radak *et al*., 2008a, b for review).

A more likely explanation of the increase in astrocyte expression in the exercised animals is that additional astrocytes were recruited as increased metabolic support. Muscular activity during exercise increases heart rate, which increases blood flow to all areas of the body including the brain. As more blood is pumped though the brain, metabolism increases and more oxygen is taken up by the neurons (see Cotman & Berchtold, 2002 for review; Fabel *et al*., 2003; Radak *et al*., 2001a, 2006). In order to maintain the high level of metabolism, as well as the resulting growth of new neurons, expression of enzymes involved in glucose metabolism and growth of new blood vessels must be increased (see Cotman *et al*., 2007 for review). Trophic factors, such as BDNF, insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor, are increased and all work in concert to promote both neurogenesis and angiogenesis (see Cotman *et al*., 2007 for review).

Therefore, the observed increase in astrocyte number in the exercised animals must have been due to increased astrocyte recruitment to help maintain the exercise-induced metabolic changes. Astrocytes are a primary source of neurotrophic factors (see Gao *et al*., 2003 for review), without which the growth of new neurons would be severely hampered. Secondly, astrocytes are involved in the transportation of materials between blood vessels and neurons (Rosenzweig *et al*., 1972); thus, more astrocytes would be needed to meet the metabolic demands of not only new neurons but also established ones. Moreover, a study examining mice in an enriched environment, a part of which was exercise, observed more glial cells, one type of which is astrocytes, in the enriched environment animals as compared to mice in an impoverished environment (Rosenzweig *et al*., 1972). This suggests that astrocyte upregulation can happen in response to neuronal growth and angiogenesis. We suggest this is what happened in our animals and the increase in astrocyte number in the exercise group was due to an increased metabolic need, not due to oxidative stress.

Astrogliosis is only part of the complicated oxidative stress process. In order to get a better idea of the overall process, we chose to combine our astrocyte investigation with an examination of apoptotic neurons. The Bax protein staining used to mark apoptotic neurons would have shown us whether the increase in astrogliosis was associated with an increase in apoptosis, both of which are indicative of oxidative stress. Had expression of both astrocytes and the Bax protein increased in the hippocampus, then we could confidently conclude that astrogliosis was indicative of a response to oxidative stress.

The Bax staining, however, proved inconclusive and one possible reason is that we waited to harvest the tissue until twenty-four hours after the last stress, in hopes of observing long-term changes caused by chronic stress. This time period might have been too long, allowing Bax expression to normalize. A study performed by Krajewski *et al*. (1995) looked at Bax expression from thirty minutes to three hours after the last stress and found that Bax expression was markedly increased in the CA1 region of the hippocampus. However, this study not only used adult rats instead of mice but used a completely different model: generalized ischemia following induced transient cardiac arrest as opposed to chronic immobilization. In future studies investigating exercise and the immobilization stress model, researchers might look into harvesting the tissue at intervals beginning at thirty minutes after the last stress. At these intervals, increased Bax expression might be at its height and consistent staining could be obtained before protein expression normalizes, as it seems to do within twenty-four hours.

Oxidative stress is a complex, global phenomenon in the brain that involves more than just astrogliosis and apoptosis, so in the future researchers should look for different

markers of oxidative stress. One possible marker is nitric oxide (NO) because stress has been shown to generate high levels of NO in the brain (Olivenza *et al*., 2000; see Madrigal *et al*., 2006 for review). In rodent immobilization stress models, it was observed that enzymes that degrade NO were upregulated following the stress (Madrigal *et al*., 2002) and the longer an animal was stressed, the higher the expression of NO-degrading enzymes (Olivenza *et al*., 2000). NO is a gas produced in the brain that normally crosses cell membranes and, at moderate concentrations, has a similar function to neurotransmitters and can even serve as an antioxidant. However, at high concentrations, NO interacts with superoxide, another chemical increased during oxidative stress, to form peroxynitrite ($ONOO^-$), a very reactive, highly cytotoxic substance that can damage neuronal lipids, proteins and DNA (Beckman *et al*., 1990; Pacher *et al*., 2007). In future research, perhaps during Western blotting for which we harvested and prepared tissue this summer, one element to be looked for could be the concentrations of NO-degrading enzymes because increased NO-degrading enzyme concentrations would indicate oxidative stress.

Another indicator of oxidative stress that could be analyzed is the presence of glucocorticoids in the brain. Glucocorticoids are normally involved in the metabolism of sugars, proteins, and fats and are released after stressors because they play a role in the stress-induced fight-or-flight response (see Liu & Mori, 1999 for review). In response to stress, the hypothalamic-pituitary-axis in the brain signals the adrenal glands, which sit just above the kidneys, to secrete glucocorticoids. These hormones provide the body with a readily available energy supply by stimulating the release of energy substrates into the blood stream and inhibiting glucose uptake and fatty acid storage while also suppressing unessential anabolic processes, such as protein synthesis (Cannon, 1915; Baxter, 1976; see Munck *et al*., 1984 for review; see Joëls, 2008 for review).

Sustained high concentrations of glucocorticoids, such as is the case of chronic stress, can damage neurons, and hippocampal neurons are especially vulnerable because they contain a high number of glucocorticoid receptors (see Radley & Morrison, 2005 for review). When glucocorticoids bind to their receptors, chaperone proteins take them into the cell and into the cell's nucleus where they influence gene transcription. Over time, chronic exposure to elevated levels of glucocorticoids will change gene expression and thus change the protein content of the cell, which will eventually change the cell's function (see Joëls, 2008 for review). Furthermore, glucocorticoids increase the accumulation of glutamate (Stein-Behrens *et al*., 1992; Moghaddam *et al*., 1994; Stein-Behrens *et al*., 1994), which binds to its receptor, opening more calcium ion channels and letting massive amounts of calcium into the cell (Choi, 1992; Hertz *et al*., 1999; see Kandel & O'Dell, 1992 for review; Mawatari *et al*., 1996). Increased internal calcium concentration can initiate free radical and NO generation, mitochondria dysfunction, protein misfolding and cytoskeletal damage (Mawatari *et al*., 1996). Finally, increased glucocorticoids can decrease a neuron's antioxidant capacity (see Kiraly & Kiraly, 2005 for review), make neurons more vulnerable to damage from which they would normally be able to recover (Muhlen & Ockenfels, 1969; Sapolsky, 1985a; Sapolsky *et al*., 1986; Tombaugh *et al*., 1992; see Lupien & Meaney, 1998 for review), and cause the loss of synapses, dendritic atrophy, and a decrease in neurogenesis (Sapolsky *et al*., 1985a; Magariños & McEwen, 1995; Eriksson *et al*., 1998; Gould *et al*., 1998; see Miller & O'Callaghan, 2005 for review). Thus, it would be interesting to examine the levels of glucocorticoids in the brain following chronic stress to get a better understanding of how chronic stress relates to oxidative stress.

Another marker that would be worth examining is the amount of growth factors present in brain tissue following chronic stress and exercise. Previous studies have established that growth factors, especially BDNF, are downregulated in response to stress (see Kiraly & Kiraly, 2005 for review). However, exercise has been shown to increase growth factor levels or at least maintain them (see Cotman *et al*., 2007 for review). If our research continued to look specifically at the hippocampus, it would be beneficial to examine the levels of BDNF and IGF-1 because BDNF is important for hippocampal function, especially in maintaining synaptic plasticity that is essential for learning, and IGF-1 is important for neurogenesis, an essential part of memory formation (see Cotman *et al*., 2007 for review).

Overall, our hypothesis was not supported and the inconclusive Bax staining prevents us from drawing definite conclusions about the meaning of the observed increase in astrocyte expression in exercised animals. However, future studies in this lab could look for other markers of oxidative stress that would give a more complete picture of the consequences of immobilization stress. It is important to note that restraint stress is used as a model for human emotional stress (Fontella *et al*., 2005), so conclusions drawn from this research can be somewhat translated to the impact of emotional stress on humans. Indeed, studies examining repeated recreated in the animals the same types of behavioral abnormalities characteristic of human mental illnesses, making stress experiments with animals a reliable model for stress-related mental illness in humans (Ottenweller *et al*., 1989; Willner, 1997). Further supporting the link between our research and human experience is our focus on the detrimental effects of stress in the hippocampus, a brain structure highly conserved throughout all levels of animal brains, and results from research on the hippocampi of both animals and humans tend to be consistent (see Kiraly & Kiraly, 2005 for review).

Stress is an unavoidable part of the human experience and unfortunately it is also associated with neurodegenerative diseases such as Huntington's disease, Parkinson's disease, Alzheimer's disease, and other forms of dementia (see Madrigal *et al*., 2006 for review). These diseases are especially terrifying because they involve the loss of one's bodily functions, one's memories, and one's personality, all of which are characteristics that make us undeniably "human." Currently, there is no cure for neurodegenerative diseases, only treatments that slow symptoms or alleviate suffering. Thus, it is worth looking for something that might offer protection or perhaps prevent the development of such catastrophic diseases. Because of exercise's success with delaying the onset and slowing symptom progression of

neurodegenerative diseases, it is scary to think of the implications of eliminating exercise as part of one's daily routine. More sedentary lifestyles lead to impairment of physiological functioning and reduce the entire body's ability to combat oxidative stress (see Radak *et al*., 2008a for review), making a person more vulnerable to stress-related, neurodegenerative diseases. As Americans eliminate exercise, they are cutting out one of the most reliable forms of neuronal protection and as a result the incidence of stress related neurodegenerative diseases is increasing. If this trend continues, we may see younger people develop these diseases and deteriorate more rapidly.

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The Role of Vasopressin and Oxytocin in Romantic Attachment

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Human males and females tend to form long-term, selective, mostly monogamous, social attachments that are often described as romantic love. What accounts for this monogamous tendency is the subject of on-going discussion. Because of their role in bonding between parents and offspring, researchers have begun investigating the role of the neurohormones vasopressin and oxytocin in non-familial, romantic bonding. Most research has been done using a monogamous prairie vole (Microtus ochrogaster) model, and these studies suggest that the pattern of oxytocin and vasopressin receptors differ between monogamous and nonmonogamous voles. Moreover, in monogamous species, activity of oxytocin and vasopressin influence areas of the brain associated with the dopamine reward pathway, making social behavior and affiliation with one's partner rewarding. Additionally, these hormones, especially oxytocin, help solidify social memories of one's partner. The combined effect of oxytocin and vasopressin facilitate the formation of strong social attachment between two individuals and often leads to, if not sexual monogamy, then at least monogamous social affiliation.

Social attachments are important in all areas of the human experience from parent-child relationships to work relationships. Societies and personal well-being are built upon the human ability to form meaningful social attachments, and a deficit in this ability often causes psychological impairment. Indeed, difficulty forming social attachments and impaired social functioning are key characteristics of some mental disorders, such as schizophrenia (Linkowski, Geenen, Kerkhofs, Mendlewicz, & Legros, 1984) and autism (Hammock & Young, 2006), and the loss of significant social bonds exacerbates depressive symptoms (Eisenberger & Lieberman, 2004).

One of the most complex manifestations of human social bonding is what is often referred to as "romantic love," a phenomenon experienced by humans of every culture. Human love has been separated into three categories: lust, attraction, and attachment (Fisher, Aron, Mashek, Li, & Brown, 2002). Lust is also called one's sex drive or libido and refers to cravings for sexual gratification. These feelings are mostly mediated by estrogens and androgens (Fisher et al., 2002). Attraction is sometimes called "obsessive love, passionate love, being in love, or infatuation" (Fisher et al., 2002). It is characterized by increased energy and focus on one's preferred partner and is often accompanied by feelings of exhilaration, intrusive thoughts about one's partner, and a craving for emotional connection (Fisher et al., 2002). Attraction is influenced by elevated levels of dopamine and norepinephrine and decreased levels of serotonin (Wang, Yu, Cascio, Liu, Gingrich, & Insel, 1999; Bartels & Zeki, 2000). The third type of love, and the type that is the focus of this paper, involves adult male-female attachment. This attachment is a strong social bond usually mediated by the neurohormones oxytocin and vasopressin (Carter, DeVries, & Getz, 1995) and is characterized by both partners experiencing feelings of calm, security, social comfort, and emotional union (Fisher et al., 2002).

After the formation of a strong social attachment between two individuals, it is generally accepted that they will remain monogamous, sharing an excusive sexual and social relationship. Most people claim to be, if not exclusively monogamous, then at least serially monogamous, but current trends of casual sexual relationships and the prominence of pornography, prostitution, and divorce would suggest otherwise. In the United States divorce rates for a first marriage are as high as 41% to 50% and climb even higher for second (60-67%) and third (73-74%) marriages (Bramlett & Mosher, 2002). Although stereotypes label men as the typically unfaithful partner, 14-22% of women admit to having had sexual contact with another person during a supposedly monogamous relationship, and it is estimated that 3-4% of births are the result of an illicit affair (Bramlett & Mosher, 2002). Examining these statistics makes one question whether humans really are naturally monogamous.

Some researchers suggest genetic diversity of the human population provides evidence that strict monogamy was never the norm for humans (Dupanloup, Pereira, Bertorelle, Calafell, Prata, Amorim, et al., 2003). Monogamy may have only become common in the last 5,000-10,000 years when cultures transitioned from hunter-gatherer lifestyles to settled households (Dupanloup et al., 2003). Anthropologists also suggest that if ancestral human relationships resembled monogamy, then it was more a pattern of serial monogamy with couples only associating for about four years, just long enough to raise a child past infancy. After four years, parents would separate and eventually form new families, perpetuating the cycle (Fisher et al., 2002). Neural mechanisms that support long-term male-female attachment may have begun developing during this period primarily to

motivate individuals to stay together long enough to complete their parental duties (Fisher, 1998).

One possible neural mechanism involves the neurohormones vasopressin and oxytocin, which are similar to the nanopeptides implicated in the reproductive behavior of amphibians and reptiles (Moore & Zoeller, 1979). Oxytocin and vasopressin are evolutionary recent, however, and are expressed almost exclusively in mammals (Archer, 1974). They are both synthesized in magnocellular neurons near the hypothalamus and then stored in and secreted by the posterior lobe of the pituitary gland (Goodman, 2003).

Oxytocin and vasopressin are structurally similar with only two of their nine amino acids differing at the three and eight positions, which results in different functions within the body (Archer, 1974). Vasopressin is also known as antidiuretic hormone because it promotes water reabsorption within the kidneys (Goodman, 2003). In high amounts, vasopressin raises blood pressure by causing smooth muscle contractions, resulting in vasoconstriction (Goodman, 2003). Oxytocin also plays a role in smooth muscle contraction and is released during uterine contractions following orgasm and at the beginning of labor and is released during milk ejaculation when breast feeding (Young & Wang, 2004). Oxytocin release is associated with a decrease in the production of stress hormones and a lowering of blood pressure (Grewen, Girdler, Amico, & Light, 2005)

Oxytocin and vasopressin were first linked to social bonding because of their role in parental behavior. In fact, it is suggested that social bonding between animals uses a mechanism co-opted from that by which maternal bonds are formed (Curtis & Wang, 2003). Further supporting this hypothesis is that prairie voles, a monogamous rodent species, display more maternal care than nonmonogamous meadow voles (Numan & Sheehan, 1997), suggesting a positive correlation between maternal care and monogamous tendencies. Moreover, central administration of oxytocin enhances maternal behavior in both rodents and sheep, and hormones secreted during pregnancy and lactation increase expression of oxytocin mRNA (Brooks, Lund, Stumpf, & Pedersen, 1990; Broad, Kendrick, Sirinathsinghji, & Keverne, 1993). Oxytocin release during childbirth facilitates the shift from infant avoidance to nurturing behavior in female rats (Wang, Zhou, Hulihan, & Insel, 1995) and sheep (DaCosta, Guevara-Guzman, Ohkura, Goode, & Kendrick, 1996). In humans, studies have shown that increases in oxytocin levels during pregnancy correspond with higher self-reported bonding to the unborn fetus (Levine, Zagoory-Sharon, Feldman, & Weller, 2007). Because oxytocin and vasopressin play a role in maternal bonding, researchers began to investigate whether these neurohormones also affect other types of bonding, especially non-familial bonding.

Vasopressin and oxytocin do seem to influence social bonding; for example, chronic intracerebroventricular infusion of oxytocin increases social contact in male rats (Witt, Winslow, & Insel, 1992), and vasopressin seems to play a direct role in expression of territorial aggression in male hamsters (Ferris, Albers, Wesolowski, & Goldman, 1984; Ferris & Potegal,1988), a behavior associated with guarding one's mate. In humans, increases in oxytocin are associated with higher levels of trust, especially in social situations. An intranasal oxytocin spray made people with a social phobia feel less anxious and more sociable, and those without a social phobia more willing to take social risks (Kosfeld, Heinrichs, Zak, Fischbacher, & Fehr, 2005). A similar nasal spray also increased ratings of trustworthiness and attractiveness of pictures of male and female faces in raters of both sexes (Theodoridou, Rowe, Penton-Voak, & Rogers, 2009). Thus, oxytocin and vasopressin seem to be important in mediating socially salient behaviors and connections between individuals.

Although oxytocin and vasopressin are involved in male and female pair bonding, there do seem to be differences in the effects of these chemicals between the sexes (Curtis & Wang, 2003). Intracerebroventricularly administered doses of oxytocin shown to be effective in establishing a female partner preference are ineffective in males (Insel & Hulihan, 1995). Likewise, similarly administered doses of vasopressin shown to be effective in establishing a male partner preference are ineffective in females (Insel & Hulihan, 1995). Therefore, it is generally accepted that oxytocin influences female pair bonding while vasopressin has a greater influence on male behavior.

Oxytocin facilitates social bonding in females and the effects are mediated by estrogen and progesterone (Johnson, Ball, Coirini, Harbaugh, McEwen, & Insel, 1989). The role of oxytocin has been examined in the formation of female partner preference, and it was observed that administering oxytocin to the lateral ventricles of a female prairie vole induces a partner preference even in the absence of mating (Williams, Insel, Harbaugh, & Carter, 1994). Partner preference formation was blocked, even in the presence of mating, by the administration of oxytocin receptor antagonists (Williams et al., 1994), suggesting that oxytocin is both sufficient and necessary for a female to form a partner preference. Genital stimulation has been shown to cause oxytocin release in other mammals such as rats (Zhang, Filippi, Vignozzi, Morelli, Mancina, Luconi, et al., 2005), sheep (Kendrick, Keverne, Chapman, & Baldwin, 1988), cows (Williams, Gazal, Leshin, Stanko, & Anderson, 2001), pigs (Langendijk, Soede & Kemp, 2005), and horses (Nikolakopoulos, Kindahl, Gilbert, Goode, & Watson, 2000). Perhaps the mating-induced oxytocin increase partially accounts for the observed female preference for her mate following copulation.

Similarly, human oxytocin release has been implicated in the formation of female pair bonds. Not only is oxytocin released in females during orgasm (Young & Wang, 2004), but oxytocin is also found in male semen (Burch & Gallup, 2006). After unprotected sex, oxytocin is absorbed though the walls of the vagina and appears in the bloodstream; it then crosses the blood-brain barrier, and within an hour, binds to its receptors in the brain (Burch & Gallup, 2006). Oxytocin, therefore, could be important in the female bonding process because it facilitates attachment to and overall social affiliation with a particular partner.

In contrast, vasopressin seems to be both sufficient and necessary for male pair bond formation. Central

administration of vasopressin in a male prairie vole induced pair bond formation in the absence of mating, an effect blocked by the administration of a vasopressin antagonist (Winslow, Hastings, Carter, Harbaugh, & Insel, 1993). Vasopressin released during mating increases mate guarding and aggression toward same-sex voles as well as increases paternal behavior in both voles and humans (Williams, Catania, & Carter, 1992; Winslow et al., 1993; Bamshad, Novak, & DeVries, 1994; Storey, Walsh, Quinton, & Wynne-Edwards, 2000). Furthermore, the effects of vasopressin are androgen dependent (Bluthe, Schoenen, & Dantzer, 1990).

The gender-specific effects of vasopressin and oxytocin are complex, however, as both oxytocin and vasopressin are involved in the reproductive behaviors of both males and females. For example, central administration of a high dose of either oxytocin or vasopressin can induce pair bonding in both sexes (Cho, DeVries, Williams, & Carter, 1999). Moreover, pair bonding in both males and females can be blocked by antagonists specific to either oxytocin or vasopressin receptors (Cho et al., 1999), and surprisingly, oxytocin injected into the lateral septum of male prairie voles can induce pair bonding (Liu, Curtis, Wang, 2001). Although vasopressin pathways are sexually dimorphic, oxytocin pathways are not (Insel & Hulihan, 1995), and in the prairie vole there seems to be no gender differences in either distribution of oxytocin cell bodies (Wang et al., 1995) or receptors (Insel & Shapiro, 1992). Oxytocin may act to potentiate cellular responses to vasopressin, as repeated central administration of oxytocin enhances the behavioral effects of vasopressin (Poulin & Pittman, 1993). These observations suggest the action of both oxytocin and vasopressin receptors are essential to pair bonding in both sexes.

Although pair bonding is a simplification of human romantic attachment, pair bond formation does seem to provide insight into this complex phenomenon. Unlike most mammals, there do seem to be human couples that fall in love, form a strong attachment, and are faithful throughout their lives. What accounts for this monogamous attachment is the subject of much discussion in the scientific community. Both human and animal research implicates vasopressin and oxytocin in the formation of this complex social tie, and indicates they also contribute to romantic love; specifically, the activity of oxytocin and vasopressin influence areas associated with the dopamine reward pathway and social behavior. Additionally, these hormones, especially oxytocin, help solidify social memories of one's partner. The combined effect of oxytocin and vasopressin facilitate the formation of strong social attachment between two individuals and often leads to, if not sexual monogamy, then at least monogamous social affiliation.

Because of the obvious ethical limitations on the work allowed to be done with humans, most studies concerning romantic attachment have been done in animal models, especially the prairie vole (*Microtus ochrogaster*). The prairie vole model is important to the study of social attachment because, while monogamy is a rare social organization found only in about three percent of mammals, most of which are primates (Bales, Mason, Catana, Cherry, & Mendoza, 2007), the prairie vole exhibits monogamous behavior in the wild (Kleimen, 1977). Monogamous rodent behavior is characterized by an adult male and an adult female sharing a nest during breeding and nonbreeding seasons, preferential, if not exclusive, mating with the one partner, male participation in parental care, offspring that remain in the nest for several weeks after weaning, and selective aggression of both sexes against intruders (Dewsberry, 1981; Dewsberry, 1987; Young, Wang, & Insel, 1998). Prairie voles also exhibit monogamous behaviors in a laboratory setting as measured by partner preference and expressed aggression (Insel & Hulihan, 1995; Shapiro & Dewsbury, 1990).

The animal partner preference paradigm is a well established model for pair bonding because an expressed partner preference may indicate one of the earliest behavioral events in the development of a long-term, selective social attachment (Dewsbury, 1988; Insel & Hulihan, 1995). In a partner preference test a vole of one sex who has previously mated or cohabited with another vole of the opposite sex (the mate) is placed in a neutral, middle cage with access to both the mate and another opposite-sex vole with whom the first vole has no previous experience (the stranger). The amount of time the vole spends with either the mate or the stranger is recorded and the one with whom the vole spent the most time is determined to be the preferred partner (Insel & Hulihan, 1995). Monogamous vole species, such as the prairie vole or pine vole, tend to prefer the mate over the stranger; whereas, nonmonogamous vole species, such as the montane vole or meadow vole, show no preference for either opposite-sex vole and often prefer to remain in the neutral cage (Shapiro, Austin, Ward, & Dewsbury, 1986; Williams et al., 1992). Moreover, after copulation, monogamous vole species immediately prefer the mate and that preference is sustained over a period of time (Insel & Hulihan, 1995). The observed favoring of a mate when a partner preference is formed reduces the time an animal spends searching for another potential mate. Thus, developing a partner preference is considered the first step in developing a monogamous attachment.

Prairie voles are also useful in the study of social attachment and pair bonding because their close relatives in the *Microtus* genus exhibit a different social organization, namely promiscuity and no parental care (Young et al., 1998), which offering a good basis for comparison between the species. Unlike monogamous prairie voles, nonmonogamous montane voles do not form partner preferences when injected with oxytocin or vasopressin (Curtis & Wang, 2003). Injection of vasopressin into the lateral ventricles of prairie voles increases monogamy-related behaviors such as mate guarding and aggression toward same-sex voles; however, in nonmonogamous montane voles, vasopressin injection only increases autogrooming behavior (Young et al., 1998). Thus, different social structures seem to be dependent on the neural substrates available to process oxytocin and vasopressin.

Neurohormone receptor distribution could be part of the species-specific vasopressin and oxytocin processing systems. Although other behaviorally relevant receptors, such as benzodiazepine and μ opiate receptors, are similarly

distributed between all vole species (Zecevic, 1993), the distribution of oxytocin and vasopressin receptors is markedly different between monogamous and nonmonogamous voles (Insel & Shapiro, 1992; Young et al., 1998). Receptor distribution has important behavioral implications; for example, after giving birth, the distribution of oxytocin receptors in nonmonogamous female montane voles changes to resemble the receptor distribution of monogamous voles (Insel & Shapiro, 1992). Once this change has occurred, montane voles begin to exhibit affiliative and protective behaviors toward their offspring. This suggests that the distribution pattern of oxytocin and vasopressin receptors contributes to the kind of neurohormonal processing that results in social bonding.

Furthermore, unlike nonmonogamous voles, monogamous voles express oxytocin and vasopressin receptors in brain regions of the dopamine reward pathway such as the nucleus accumbens and prelimbic cortex (Insel & Shapiro, 1992; Young et al., 1998). This suggests that in monogamous voles neurohormone surges released after mating or during contact with a mate have some sort of rewarding property (Insel & Shapiro, 1992). Following the principles of operant conditioning, animals will increase these behaviors because they are rewarding. This may partially explain why in monogamous species there is an association between mates during nonbreeding times, biparental care of offspring, and male-female social affiliation.

A specific dopamine receptor that seems to be important in the rewarding properties of monogamous affiliations is the D_2 dopamine receptor. Gingrich, Liu, Cascio, Wang, and Insel, (2000) reported that blocking D_2, but not D_1, receptors in the nucleus accumbens inhibited the formation of mating-induced pair bonds, and site specific administration of a D_2 receptor agonist, but not a D_1 agonist, induced a pair bond in the absence of mating. Thus, the intense binding of oxytocin and vasopressin in the nucleus accumbens together with strong dopamine binding in these areas help form pair bonds. This is further supported by the fact that pair bond formation can be blocked with either a dopamine or oxytocin receptor antagonist (Liu & Wang, 2003). The binding of dopamine to its D_2 receptor along with the binding of vasopressin and oxytocin contribute to the rewarding nature of affiliative behaviors. Voles then increase these behaviors in the presence of one mate, to the exclusion of other possible mates, eventually leading to a monogamous social bond.

The combined influence of oxytocin and vasopressin in the dopamine reward pathway could also contribute to monogamous pair bonds in humans. For example, studies have shown that when people professing to be "truly, madly, and deeply in love" (Bartels & Zeki, 2004) view pictures of their significant other, the ventral tegmental area is highly active. As the ventral tegmental area is part of the dopamine reward pathway and is rich in vasopressin and oxytocin receptors (Loup, Tribollet, Dubois-Dauphin & Dreifuss, 1991), this data suggests that seeing one's significant other is rewarding and can lead to the selective affiliation seen in romantic attachment. Thus, as with pair bonds in animals, human pair bonds may be initiated by the combined action of oxytocin, vasopressin, and dopamine within the reward pathway.

The differential patterns of oxytocin and vasopressin receptor distribution between monogamous and nonmonogamous species is not due to differences in receptor structure, which is the same between species (Marín-Padilla, 1969; Somogyi & Cowey, 1984), but rather due to species differences in gene expression. The distribution pattern of receptor mRNA in all vole species is nearly identical to the pattern of receptor distribution, so localized gene expression, not differences in receptor transport to a final site, must be responsible for differences in receptor distribution (Somogyi & Cowey, 1984). Gene expression is influenced by the promoter region of the receptor coding sequence. Although the coding sequences for vasopressin and oxytocin receptors in both monogamous and nonmonogamous voles are 99% homologous, there is a difference on the 5' flanking region; monogamous prairie voles contain a 450 base pair sequence that is absent from the flanking region of nonmonogamous montane voles (Young et al., 1998). This 5' flanking region seems to be responsible for receptor distribution. For example, researchers inserted the extra prairie vole genetic sequence into the genetic code of mice, and mice subsequently showed a change from their species-typical receptor distribution pattern to a receptor pattern similar to that found in prairie voles. The mice also showed an increase in affiliative behavior after vasopressin administration (Young, Nilsen, Waymire, MacGregor, & Insel, 1999). This study suggests the extra sequence on the 5' flanking region of the prairie vole vasopressin receptor is enough to make notoriously promiscuous mice more inclined to pair bond.

Thus, researchers further examined the genetic code of the vasopressin receptor and its influence on receptor distribution and monogamous behavior. Vasopressin acts though a V1aR receptor subtype that has been implicated in the regulation of pair bonding in male rodents (Young & Wang, 2004). Moreover, studies suggest that partner preference and affiliative behavior is enhanced in nonmonogamous meadow voles when V1aR receptor density is increased in the ventral forebrain using viral vector transfer (Pitkow, Sharer, Ren, Insel, Terwilliger, & Young, 2001; Lim, Wang, Olazábal, Ren, Terwilliger, & Young, 2004). However, the implications for human research are not clear because, while humans do have three repetitive sequences in this region, they do not have a vasopressin gene with a 5' base pair sequence homologous to that of the prairie vole (Walum, Westberg, Henningsson, Neiderhiser, Reiss, Igl, et al., 2008). Perhaps these human genetic repeats have an effect similar to the prairie vole 5' sequence and are partially responsible for monogamous human tendencies.

Studies suggest that human vasopressin gene AVPR1A does influence social behavior. Mutations on this gene are associated with an increased likelihood of having autism (Kim, Young, Gonen, Veenstra-VanderWeele, Courchesne, Courchesne, et al., 2002; Wassink, Piven, Vieland, Pietila, Goedken, Folstein, et al., 2004; Yirmiya, 2006), a younger age of first sexual intercourse (Prichard,

Mackinnon, Jorm, & Easteal, 2007), and less altruistic behavior (Knafo, Israel, Darvasi, Bachner-Melman, Uzefovsky, & Cohen, 2007), although these findings have not been consistently replicated. In addition, 34% of men with two copies of a variant allele of the AVPR1A receptor gene reported marital crisis or threat of divorce as compared to only 15% of men carrying no copies of a variant allele, suggesting that being homozygous for the variant allele doubles men's risk of marital crises (Walum et al., 2008). Also, the frequency of nonmarried men was higher among men homozygous for the variant allele (32%) as compared to those with one for no copes of the allele (17%) (Walum et al., 2008). Thus, variations in the human vasopressin gene increase the likelihood of difficulty beginning and maintaining social attachments.

Walum et al. (2008) reported that variant alleles influenced perceived marital quality as judged by the women married to the men in the study. Women married to men with one or two copies of the variant allele reported significantly less affection, dyadic consensus, and dyadic cohesion in their marriages as compared to women married to men with no copies of the variant allele (Walum et al., 2008). Expression of the variant allele is also associated with increased amygdala activity, a brain region often deactivated during romantic love (Meyer-Lindenberg, Kolachana, Gold, Olsh, Nicodemus, Mattay, et al., 2008). Amygdala deactivation decreases social judgments of one's partner, so men with the variant allele could be overly critical or overly suspicious of their partners, negatively affecting their ability to bond with a potential mate (Baumgartner, Heinrichs, Vonlanthen, Fischbacher, & Fehr, 2008). Moreover, the high incidence of the variant allele in men with autism, suggest the variant allele is associated with impaired social relatedness and communication abilities, both behavioral deficits characteristic of autism (Walum et al., 2008). Taken together, this research suggests mutations on the human AVPR1A gene interfere with one's ability to successfully communicate and relate to others. Because these abilities are important in the maintenance of romantic relationships, those in whom these abilities are impaired may have unstable relationship histories.

Conversely, the presence of the nonvariant AVPR1A allele seems to be related to relationship success. While men with the variant vasopressin receptor gene tend to have impaired relational abilities, men with the nonvariant vasopressin receptor gene have a greater ability to empathize (Young & Wang, 2004). Moreover, a study examining people in a monogamous relationship for over two years noted that subjects with the nonvariant allele show increased activity in the ventral pallidum, a brain region involved in reward and motivation and rich in vasopressin receptors, as compared to controls (Fisher et al., 2006; Smith, Tindell, Aldridge, & Berridge, 2009). Thus, vasopressin's overall interaction with its AVPR1A receptor may influence the human ability to maintain and function within a social relationship.

Both vole and human studies suggest different gene sequences influence the expression and distribution of vasopressin and oxytocin receptors. Differential distribution and expression is also correlated with monogamous or nonmonogamous social behavior, suggesting that plasticity in gene expression could have played a role in the evolution of different social behaviors (Young et al., 1998). Differential expression could be due to differences in gene sequences or to differences in tissue-specific availability of transcription factors (Young & Wang, 2004). But overall, research does suggest that both human and animal monogamous behavior is influenced by receptor expression that creates regional sensitivity to endogenously produced oxytocin and vasopressin (Young et al., 1998).

Oxytocin and vasopressin receptors have been found in brain regions that modulate social memory (Marín-Padilla, 1969), suggesting that pair bonding requires memory processes. As simple as it sounds, if one does not remember one's mate, then how can he or she be expected to associate solely with that partner? Thus, some researchers suggest oxytocin and vasopressin, and their interaction with the dopamine reward pathway, influence monogamy by helping form a concept of one's partner that is then solidified and stored in long-term memory.

In rats both oxytocin and vasopressin play a role in consolidating memories of socially important individuals (Popik, Vetulani, & Van Ree,1992a; Popik, Vos, & Van Ree, 1992b; Dantzer, Koob, Bluthé, & LeMoal, 1988). In addition, oxytocin has been implicated in consolidation of a mother's memory of her offspring. Oxytocin released while a sheep is giving birth causes the mother to visually recognize her offspring and consequently behave in a nurturing way towards it (Keller, Meurisse, & Lévy, 2005). Oxytocin also seems to be important for consolidating socially relevant memories in humans. For example, oxytocin helps modulate recognition of semantic stimuli as reproduction-related or neutral (Heinrichs, Meinlschmidt, Wippich, Ehlert, & Hellhammer, 2004). Moreover, intranasal oxytocin spray improves facial recognition but not recognition of nonsocial stimuli, suggesting that oxytocin has a selective effect on strengthening the neuronal systems of social memory (Guastella, Carson, Dadds, Mitchell, & Cox, 2009; Rimmele, Hediger, Heinrichs, Klaver, P., 2009). Since neurohormones are important in memory formation of socially relevant stimuli, then perhaps they also help form memory concepts of an organism's mate.

Humans may also form social memories and partner concepts that have a stable, long-term memory component, which may be enhanced by vasopressin and oxytocin facilitation of the consolidation and maintenance of socially relevant memories, an effect dependent on hippocampal activity (Vianna, Alonso, Viola, Quevedo, de Paris, Furman, et al., 2000). Measurements of brain activity while viewing pictures of one's significant other reveal that the hippocampus is activated in romantic love (Bartels & Zeki, 2004). That activity is further enhanced when oxytocin and vasopressin bind to their receptors densely populating hippocampal regions (Loup et al., 1991), which increases the ability of the hippocampus to consolidate long-term memories.

Thus, the reinforcing properties of dopamine, oxytocin, and vasopressin are combined with neurohormone activation of brain regions associated with social memory and

long-term memory to influence monogamous pair bonding (Young et al., 1998). In voles, vasopressin and oxytocin play a crucial role in the formation of vole partner preference because their release during mating activates the dopamine reward pathway and conditions a vole to the odor of its mate (Young et al., 1998), facilitating the formation of a partner concept. A vole builds a strong profile of the partner based on smell, and once formed, that concept is easily recalled and remains stable over time (Ferkin, 1992).

The formation of human partner concepts are less dependent on odor than those of voles, but evidence does suggest the more in love a woman reports to be, the worse she is at identifying body odors of opposite-sex friends (Lundstrom & Jones-Gotman, 2009). Thus, the formation of a partner concept disrupts a woman's ability to distinguish odors of other potential mates, an effect that may be mediated by oxytocin. Since oxytocin and vasopressin are structurally similar enough to bind to each other's receptors, heavy binding of the more fast-acting oxytocin effectively decreases the availability of receptors with which vasopressin can bind. The resulting drop in levels of active vasopressin reduces the salience of opposite-sex odors, thus lowering a woman's capacity for olfactory social recognition and decreasing her selective attention to other potential mates (Lundstrom & Jones-Gotman, 2009). Decreased attention to potential mates combined with increased binding of oxytocin and the subsequent heightened feelings of attachment greatly facilitate the formation of a partner concept (Lundstrom & Jones-Gotman, 2009). This increases the likelihood of future affiliative behaviors that could possibly develop into an exclusive social attachment.

Vasopressin and oxytocin play such a crucial role in the formation of long-term social attachment, which makes one question the idea of human romantic love. Most people view love as an indescribable, almost magical, emotion beyond scientific explanation. Indeed, it is a complex emotion involving different levels of lust, physical and emotional attraction, and companionship. It is made even more complex by cultural institutions, often based on economic ties and rigid social norms. However, as much as people may scoff at the idea of such a complex human emotion being reduced to a molecular level, both animal and human research indicates that human romantic love and monogamous practices are influenced by neural substrates and the activity of oxytocin and vasopressin.

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Are Haemogregarines significant? An analysis on the effects of blood parasites on reptile behavior

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The effects of haemogregarine blood parasites on avians and their relation with erythrocytes has been studied greatly. Yet, the effects of parasite prevalence on reptiles and animal behavior is lacking in study. Several studies have found that haemogregarines negatively affect reptile physiology and also alter normal behaviors. A trade-off hypothesis can also be utilized to study the adverse affects of blood parasites on reptile behavior. A review of several original research studies illustrates the effect that haemoregarines may have on reptile behavior.

Introduction

Although their effects can be adverse, generalist, multi-host pathogens have been neglected by several fields of biology in favor of single-host pathogens (Woolhouse et al. 2001). Haemogregaines are a group of multihost pathogens that affect several types of animals and are very common in vertebrates while being the most common parasites found in snakes (Telford 1984; Appleby et al. 1999). The term "haemorgregarine" refers to a set of blood parasites that belong to the family Haemogregarinidae and contain several genera: *Heptozoon, Haemogregarina,* and *Karyolysys* (Telford 1984). *Heptozoon* appears to be the recurring haemogregaine in reptiles. Numerous studies have examined parasite prevalence, while fewer have shown the effects of haemogregarine infections on animal fitness levels, especially in reptiles. In addition, while original research (Oppliger & Clobert 1996 & 1997; Wileski 1999; Main & Bill 2000; Madsen et al. 2004) has illustrated a negative correlation between parasite prevalence and its effect on individual fitness, other studies (Brown et al. 2006; Sperry et al. 2009) have demonstrated no significant effect of blood parasites on reptiles. While the fact that haemogregaines are present in reptiles is undeniable (Brown 2006 et al.), their significance and effects on animal behavior are not as evident. Little information is available on the extent and mechanism of the effects of haemogregarines on host fitness (Sorci 1995; Brown 2006 et al). Investigators must understand that more than 10% of red blood cells must be infected for major physiological changes in blood chemistry (Brown 2006 et al.) and thus animal behavior. Hence, caution must be taken when creating an experiment examining blood parasites. Sometimes a benign relationship develops in which the host can tolerate the haemorgregarine, which is overall beneficial to the parasite (Cuadell et al. 2002). Consequently, a trade-off between cost and benefits has become clear (Oppliger & Clobert 1997; Wikelski 1999; Main & Bull 2000; Brown et al. 2006) in which parasite prevalence is tolerated by several species because of resource quality and availability.

Nevertheless, it is evident that blood parasites affect animal behavior to some extent. Haemogregarines have been studied in several avian species and affect body condition and reproductive success in Blue tits by distributing antibiotics to affected individuals and observing positive feedback in medicated individuals (2000). It has also been shown that bright bird feathers indicate parasite resistance and therefore cause sexual selection (Hamilton & Zuk 1989). Accordingly, haemogregarine prevalence also affects reptile behavior through sexual and natural selection, as males with high parasite loads are not as physiologically fit and therefore not suitable mates (Brown et al. 2006). Therefore, although there is a threshold of parasite prevalence that must be reached before haemogregarines affect animal behavior, several reptile behaviors are affected by these blood parasites. These behaviors include overall physiological fitness, predator avoidance, female reproduction, foraging patterns, home range and group behaviors (Sorci 1995& 1996;Oppliger & Colbert 1996 & 1997;Wikelski 1999;Main & Bull 2000; Cuadell et al. 2002; Madsen et al. 2004; Brown et al. 2006; Sperry et al. 2009). In addition, a trade-off hypothesis can be formed which predicts that a reptile will endure parasite prevalence if there is a net benefit, which may be resource abundance or quality.

Physiological Effects

Before discussing how haemogregaines specifically affect reptile behaviors, the pathology of this group of parasites must be examined. Blood parasites have a heteroxenous life cycle with sporogonic development, which later has asexual stages once in the vertebrate host (Telford 1984). In invertebrate vectors, which usually include ticks and mosquitoes, haemogregarines can have adverse effects on the

host (Dunlap & Mathies 1993), but because of its life cycle, a pathogen is more virulent in a second host than in the first (Woolhouse et al. 2001). Hence, haemogregarine are more damaging to reptiles than to the invertebrate vectors. Blood parasites are also usually intra-erythrocytic (Manwell 1997). Once in erythrocytes, blood parasites can cause severe damage. After entering red blood cells, parasites infect and multiply within host red blood cells (Madsen et al. 2004) and can destroy erythrocytes and consume hemoglobin (Atkinson & van Riper 1991). This destruction occurs when the blood parasites take up over half the volume of the erythrocyte cell volume (Wintrobe 1981). Also, haemogregarines can affect red blood cell production negatively as seen by a decrease in packed cell volume (PVC) (Sperry et al. 2009); lower PVC indicates anemia as well as decreased erythrocyte production (Strik et al. 2007). Hence, Haemorgregarines can have various adverse effects on erythrocytes (Brown et al. 2006; Fig. 1). Even though Brown et al. (2006) did not determine a statistically significant effect between haemogregarines and animal behavior, it was found that once two-thirds of the host's red blood cells were affected, the reptile could not function. Haemogregarines also affect lymphocytes and cause an increase of production (Sperry et al. 2009), which lowers individual immune function and makes affected reptiles more susceptible to other negative physiological effects, which will in turn affect reptile behavior.

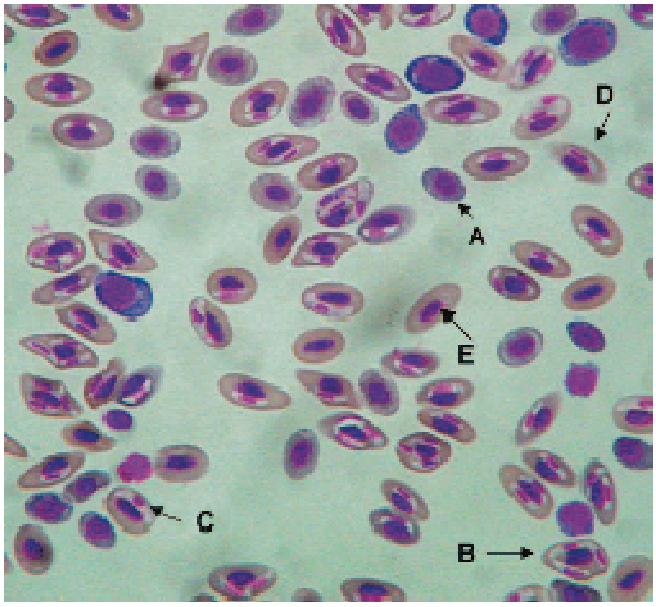

Fig. 1. Blood Smear from a heavily infected keelback snake, *Tropidonophis mairii,* showing (A) immature erythrocyte, (B) multiply infected erythrocyte, (C) large sausage-shaped gamont form, (D) small sausage gamont form, and (E) uninfected erythrocyte (Brown et al. 2006)

The most evident effect of haemogregarine prevalence in reptiles is the physiological cost, which results in lower growth rates and lower overall fitness. Once these aspects of hosts have been affected, several normal behaviors, such as predator avoidance, became negatively affected. In a study of water pythons, Madsen et al. (2004) determined a significant negative correlation between parasite prevalence and python growth rate. Haemogregarines were also shown to cause poor conditional status and sometimes death in these snakes (Fig. 2). This research experiment illustrates how haemogregarine levels deterred growth rate in a reptilian species that experiences indeterminate growth. Parasite prevalence also causes reduced tail regeneration, reduced running speed, and a decrease in resting oxygen consumption in common lizards (Oppliger & Clobert 1996, 1997). Reduced activity and emaciation are also direct effects of parasite prevalence (Main & Bull 2000; Cuadell et al. 2002). These haemogregarine-induced physiological effects, most frequent of which is reduced growth rate, all cause a variety of behavioral effects that are exhibited in several reptilian species.

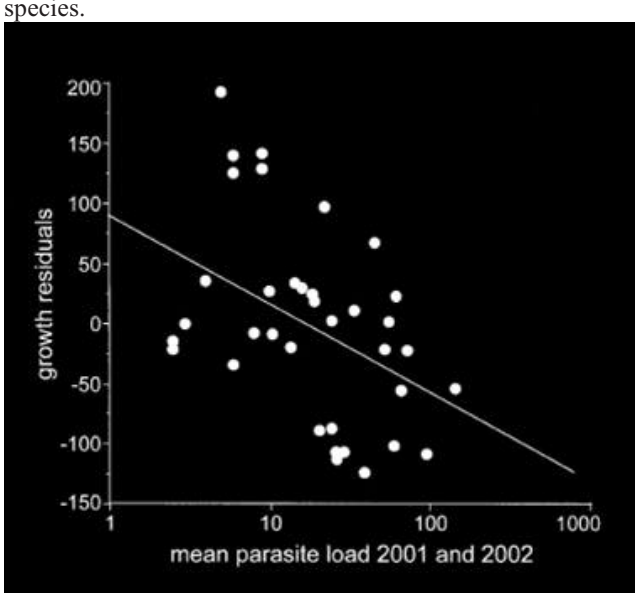

Fig. 2. Python growth residuals in relation to mean parasite load. Growth based on residual scores from a linear regression of daily growth increment and body length (Madsen et al. 2004).

Behavioral Effects

The immediate effect of physiological stress is reduced growth rate, which consequently affects predator avoidance behaviors. In common lizards, this imperative behavior is affected greatly when parasite prevalence causes reduced tail regeneration. Tail regeneration is a natural, critical process for common lizards (Oppliger & Clobert 1997), and predation may increase during this period (Dial & Fitzpatrick 1984). Haemogregarines also reduce oxygen consumption and ability to transport oxygen to muscle tissue, which reduces running speed (Oppliger & Clobert 1996). Reduced running speed caused by parasite prevalence affects predator avoidance behavior and will increase mortality rates in lizards. Another study found that tick-infested Australian tree lizards ran significantly slower and generally moved far less frequently than non-infested conspecifics and therefore had higher predation rates (Main & Bull 2000). The most immediate effects of reduced running speed and growth are

illustrated through predator avoidance in lizards, for these infected individuals will not be able to successfully escape from predators.

Yet, some reptiles do not have natural predators and parasite prevalence does not affect their predator avoidance behaviors. Water pythons experience indeterminate growth and have no natural predators; yet, Madsen et al. (2004) found that non-reproductive female pythons had significantly higher parasite loads than reproductive females. Reduced growth therefore does not affect predator avoidance in some species, but reproductive rates decrease because females are forced to devote more energy to parasite resistance. Tick-infested lizards also experienced less reproductive success than lizards whose ticks had been experimentally removed, with infested females also having less endurance (Main & Bull 2000). Several other studies have also shown that haemogregarines negatively affect female reproductive status (Sorci et al. 1996; Dawson & Bortolotti 2001). Reproductive rates are essential for several mating behaviors in reptiles and for species survival. Hence, haematozoan parasites affect reproductive behaviors in reptiles.

Foraging behaviors along with home ranges are also affected by haemogregarines (Oppliger & Clobert 1997; Cuadell et al. 2002). Although the direct result of parasite prevalence is reduced growth rates or movement, the indirect effect of these physiological stressors alters foraging patterns and hence home range. Reduced tail regeneration not only affects predator avoidance in lizards, but also hampers foraging behavior, which leads to further anemia and weak individuals (Oppliger & Clobert 1997). Parasite-infested snakes experience reduced ability to transport oxygen to muscle tissues and therefore cannot forage as efficiently as unaffected conspecifics (Cuadell et al. 2002). In addition, it is experimentally supported that tick-infested lizards have less daily activity and smaller home ranges than non-infested conspecifics (Main & Bull 2000). Main and Bull (2000) conclude that "Tick induced activity changes could reduce fitness, because lower movement may lead to fewer opportunities to sample or use resource patches or suitable refuges". Not only are foraging patterns indirectly affected by haemogregarines, but group dynamics and social status are also affected. Reduced social status and poor competitive ability in lizards is associated with high parasite loads (Schall 1992; Dunlap & Schall 1995). This reduced social status and competitive ability may be involved in sexual and natural selection and therefore thus affects mating systems as seen in avians (Merino et al. 2000). Furthermore, parasite infestation has been found to affect grouping in Galápagos marine iguanas; when the benefits of this distinctive behavior were examined, it was found that parasite resistance, not thermoregulation was the main cause of grouping behavior (Wikelski 1999). However, further investigation examining the extent of the correlation between haemogregarines and reptile behavior is needed. It is evident that a cause and effect relationship between haemogregarines and reptile behavior exists if parasite prevalence is over 10% (Brown 2006 et al.). It is apparent that an optimality or trade-off theory exists in haemogregarine blood parasitism as well.

Discussion

Research supports the idea that blood parasites do affect animal behavior in reptiles although the extent to which is not completely clear. However, at times different reptile species endure parasite prevalence if a parasite infested environment is abundant in resources. Just as predators affect the behavior and habitat selection of any animal, parasites impact the habitat use of reptile hosts (Main & Bull 2000). Living in a specific habitat that has high parasite prevalence may be more beneficial than choosing a home range that has low resource quality and low haemogregarine levels (Main & Bull 2000). For lizards and snakes, there are direct and indirect costs associated with haemogregarines that must be balanced with the benefits of a high quality habitat (Main & Bull 2000; Madsen et al. 2004). Thus, a trade-off theory exists in which reptiles utilize the optimality theory; haemogregarine presence and levels affect behavioral decisions. Yet, a threshold level that most likely exists warrants further study. In marine iguanas, grouping helps with parasite resistance, but once a group becomes large, parasites become more prevalent and thus decrease the fitness of the group (Wikelski 1999). The importance of a threshold level and trade-offs is not exclusive to habitat selection, but extends to physiological processes. In lizards with reduced tail regeneration, this slow growth may be induced by haemogregarines because more energy is allocated to parasite defense than to cell regeneration (Oppliger & Clobert 1997). This same principle is applicable to pythons, which are long-lived organisms with indeterminate growth; investing energy into self-maintenance and parasite resistance is highly beneficial (Madsen et al. 2004). Nevertheless, the difference between the costs of parasite resistance and parasitism is difficult to decipher and demonstrate experimentally (Oppliger & Clobert 1997). Therefore, more manipulative laboratory studies are needed to quantify a threshold level for the trade-offs involving heamogregarines, specific behaviors, and individual physiology.

Haemogregarines influence reptiles negatively through direct and indirect manners. The immediate effects of blood parasites are commonly reduced growth, condition, ability to transport, and overall fitness. These direct effects indirectly prompt several changes in common behaviors such as predator avoidance, foraging patterns, habitat selection and reproduction. These effects are indirect and subtle but can have severe effects on host fitness and behavior (Oppliger & Clobert 1997). Nevertheless, although several studies have found statistically significant results, the biological significance of haemogregarines has not always been fully determined (Cuadell et al 2002). In addition, a trade-off hypothesis and optimality theory can be utilized when examining the behavioral significance of blood parasites in reptiles. Further studies that focus on the behavioral effects, not solely the haemogregarine parasite load are necessary.

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Amplitude of P100 visual evoked cortical potential (VCEP) as a function of achromatic contrast stimulus in glaucomatous patients

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We recorded the visual evoked cortical potential (VECP) of seven glaucoma patients in different contrasts and spatial frequency ranges to study the VECP as function of the achromatic contrast stimulus and the contrast sensitivity function. Through this, we found that patients with glaucoma have impairment in their optic ganglion cells.

Introduction

The occipital lobe is the area of the brain that allows us to interpret visual stimulations caused by visible light, which ranges from 400-700 nm in wavelength. When light enters the pupil it travels all the way to the inner layer of the eyeball, the retina. Here, the photoreceptor cells called the rods and cones are found and are responsible for night vision and color vision, respectively. A nerve impulse travels through the retina in the following order: photoreceptors, bipolar and amacrine cells, and then the ganglion cells. The ganglion cells are neural cell bodies that when depolarized will send a nerve signal to the optic disc, which leads to the optic nerve (cranial nerve number 2). The optic nerve then goes through different pathways in order to reach the occipital lobe of the brain, also known as the visual cortex. The retinal ganglion cell axons extend to the thalamus, where the lateral geniculate nucleus (LGN) is located, via the optic tract.

It was Kaplan and Shapley in 1986 who first published their finding that the retina and the LGN had neurons with different contrast gain. They studied the retina and LGN of *Macaca fascicularis* and *Macaca mulatta*, species of macaque monkeys. Through their studies, it was determined that high contrast sensitivity ventures to the magnocellular ganglion cells in the retina and the cells of the magnocellular layers in LGN, while the low contrast sensitivity ventures to the parvocellular ganglion cells in the retina and the cells in the parvocellular layers in the LGN.

One disease involved in retinal damage is glaucoma. Generally, glaucoma is characterized by an increase in intraocular pressure. The fluid in the anterior chamber is not exchanged or drained and causes the pressure within the chamber to rise. Intraocular pressure impairs blood vessels of the optic nerve head and the ganglion cells, eventually causing ganglion cell death.

VECP is an electrophysiological method that measures an evoked potential using signal averaging, which are recorded by electrodes at the scalp. In Parisi et al. 2005, the clinical capability of pattern electroretinogram (PERG) and VECP was studied in patients with normal vision as well as in those with either ocular hypertension or open-angled glaucoma. They discovered that readings were delayed and/or decreased in the majority of abnormal patients. Combined readings showed retinal layer impairment and visual dysfunction.

Souza et al. 2007 used VECP to study the amplitude of the P100 component in a range of contrast and spatial frequencies. They suggested a differential contribution of the visual pathways to the VECP. Magnocellular pathway contributes mainly at low spatial frequencies in full contrast range and to low contrast at medium-to-high spatial frequencies. Parvocellular pathway contributes to the VECP in high contrast at medium-to-high spatial frequencies.

There is much controversy on which cell death occurs in the analysis of glaucoma disease. We used the same protocol of Souza et al. 2007 to analyze the cortical responses in glaucomatous patients. Since previous research suggests nonlinearity of VECP amplitude as a function of stimulus contrast, we were able to use the presence of straight-line, saturation, or double-slope amplitude functions to examine which pathway cells, magnocellular or parvocellular, are present in the patients.

Methods

Subjects

We tested seven adults with the mean age of 57±6.65 (four women and three men), all diagnosed with glaucoma, who were sent from their ophthalmologist's clinic to be tested in our laboratory. They were compared to a control group of

patients with normal vision from a previous study (Souza et al. 2007) with the same protocol, see Fig 1 and 2. We obtained the medical history and ophthalmologic records from all glaucoma patients. All patients had 2-3 sessions, within 7 days maximum from each other, in the laboratory where we first tested both eyes with the autorefractor (Humphrey 599; Carl Zeiss Meditec, Dublin, CA) to establish which was more acute. The more acute eye was the eye to be examined monocularly in the remaining tests. Secondly, each patient's visual field was psychophysically tested with the Humphrey Field Analyzer (Carl Zeiss, CA). Finally, we tested each patient electrophysiologically through VECP recordings with VERIS software (EletroDiagnostic Imaging, CA). All procedures were performed according to the tenets of the Declaration of Helsinki and were approved by the Committee for Ethics in Research, Tropical Medicine Institute, Federal University of Pará, according to Resolution 196/96 of the Health National Council of Brazil.

Visual Field Analysis

In order to ensure the diagnosis of glaucomatous disease for each patient, we used the Humphrey Field Analyzer, HFA (Humphrey 599; Carl Zeiss Meditec, Dublin, CA) to obtain analysis of the patient's visual field. We monocularly tested the patient with his/her more acute eye. HFA protocols 30-2 and 60-4 were used to test the central and peripheral vision fields, respectively, of the patient.

Visual Stimulation and Procedure for VECP recording

Three gold-plated electrodes were used to obtain the VECP recording. We placed the electrodes on the patient's head according to the International Society of Clinical Electrophysiology of Vision at F_Z as the reference electrode, F_{PZ} as the ground electrode, and O_Z as the active electrode. Then, each patient was positioned 1 m from the LG FlatronSlim $40cm_X35cm$ color monitor (LG, MA) that had the frame rate of 75 Hz and a spatial resolution of 800x1024 pixels. The visual stimuli were powered by VERIS software (ElectroDiagnostic Imaging, CA). The generated stimuli were achromatic, horizontal pattern reversal gratings. Each stimulus was in 5° visual angle, whereas the fixation point (a red X) was in the middle of the stimulus at 1° angle of central vision. The background of the stimulus had the mean luminance of the stimulus. The recordings took place in a dark room with nothing illuminated but the stimuli monitor and the computer monitor of the test administrator, which was turned away from the patient and lit at very low lighting with amber coloring.

Patients were monocularly tested with their more acute eye. We used segments of six different spatial frequencies; 0.4, 0.8, 2, 4, 8, and 10 cycles per degree (cpd); which were presented at 1 Hz square-wave contrast-reversal mode, and eight to ten Michelson contrasts were used at each spatial frequency, where contrast decreased from 100% down to 5 or 2.5%. Each contrast segment lasted 4 minutes.

Results

After successful recordings (based on ISCEV standard) were retrieved, we noted that three peaks were present, also in accordance with ISCEV—the N75, P100, and N135 peaks. The nomenclature of the peaks indicate whether it is a negative or positive peak (above or below axis) and the number represents its latency period. For example, a peak at P100 is a positive peak at 100ms. Because of the consistency of this peak, we used the P100 for our analysis.

When examining the VECP recordings, the P100 peak of all patients decreased as contrast decreased, and also the implicit time of the amplitude peaked increased as contrast decreased. The P100 peaked decreased but became sharper as spatial frequency increased—except at high spatial frequencies (See Figure 3). When comparing our data from the glaucoma group to the control group, glaucoma patients had significantly decreased amplitudes at P100 (ie, at 0.4 cpd and 100% contrast, patients with glaucoma: ~8 μV, control: 12 μV; Fig 2, Fig 4) and longer latencies for the P100 to occur (glaucoma: ~120ms, control: ~100ms). Many glaucomatous patients did not have signals at 2.5% and 5% contrasts.

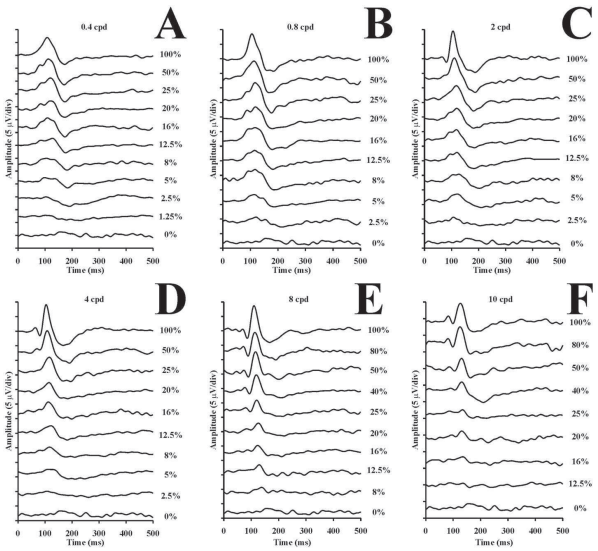

Fig. 1. shows VECP recordings from the control group. VECP recordings in different contrast levels obtained from subject SPG041127 at 0.4 (**A**), 0.8 (**B**), 2 (**C**), 4 (**D**), 8 (**E**), and 10 (**F**) cpd.. When spatial frequency was increased, the VEP waveform became sharper[6].

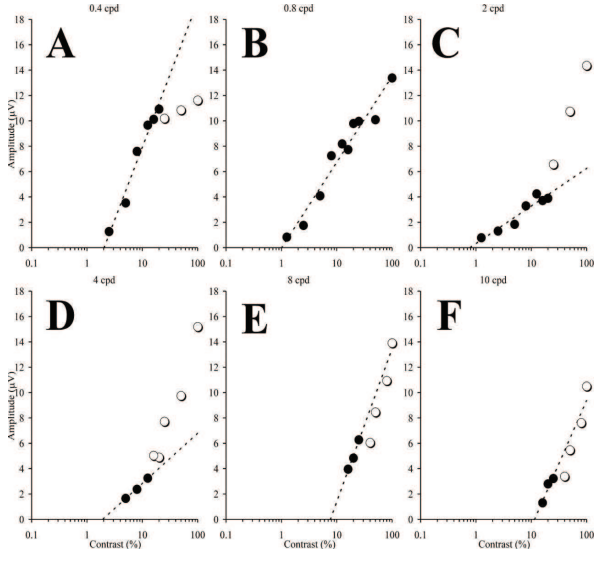

Fig. 2. This is the amplitude versus contrast function of a control subject. P100 amplitude as a function of log contrast, subject MGL041104. At 0.4 cpd (**A**), the amplitude saturated at high contrast (○). At 2 to 10 cpd (**C**–**F**), a double-slope function was used to describe the amplitude response along the contrast levels: one limb to high contrasts (○) and another limb to medium and low contrasts (•). *Dashed line*: the regression used to estimate the contrast threshold. In the presence of nonlinearity, regressions were fitted on the data obtained at medium to low contrasts[6].

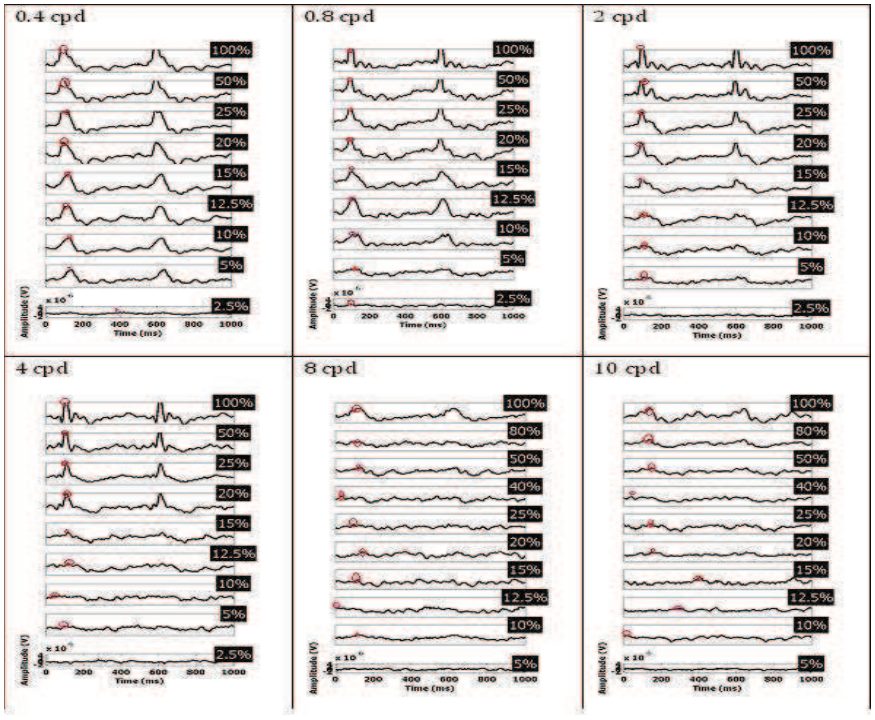

Fig. 3. VECP recordings of patient CMN090604 age 54, monocularly tested with right eye. Figure shows the different recordings from different spatial frequencies start from 0.4 cycles per degree (cpd) to 10 cpd. Each spatial frequency was recorded under nine or ten different contrasts starting from 100% contrast decreasing to either 5% or 2.5% contrast. The red circles show the highest peak from 0-500ms, and may not illustrate the P100 peak. Peak becomes sharper as spatial frequency increases from 0.4 to 4 cpd. However, peaks widen at 8 and 10 cpd. We used MATLAB for this analysis.

After recording the VECP of each patient at six different spatial frequencies with nine or ten subcategories of different contrasts, we graphed the amplitude as a function of contrast where amplitude (volts) was the dependent variable to the contrast (%). All showed saturation at 0.4 cpd and 0.8 cpd. All had a double-slope (when both saturation and linearity is apparent) function at 2 cpd. However, at 4 cpd, 8, cpd, and 10 cpd, patients varied with either double-slope function or linear function. Some patients had indistinguishable data at higher frequencies; 4cpd, 8 cpd, and/or 10 cpd.

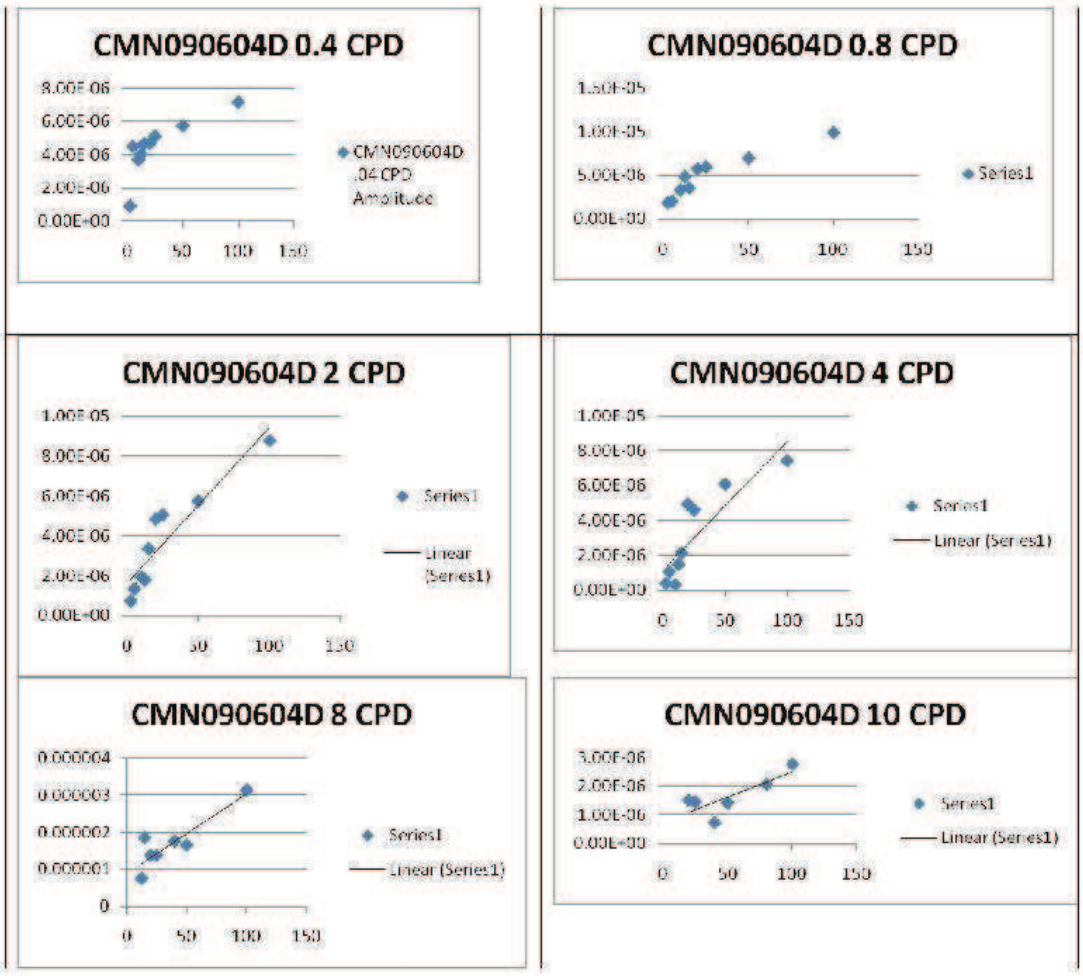

Fig. 4. Patient CMN090604, age 54, was monocularly tested with the right eye. VECP P100 amplitude recordings were measured in volts and were graphed as a function of the contrast in (%) where the amplitude was dependent to the contrast. Saturation is found in low frequencies, 0.4 cpd and 0.8 cpd. Characteristics of double-slope is found in 2 cpd and 4 cpd. Linearity is found in 8 cpd and 10 cpd. Patient was diagnosed with glaucoma and had 20/30 vision acuity in eye tested (OD).

The data in *Figure 4*, the shows a patient at early stages of glaucoma. Therefore, CMN090604 had similar readings to the control, where typical functions show saturation at low spatial frequencies, 0.4 and 0.8 cpd. Double-slope function is characterized in 2cpd and 4 cpd—middle spatial frequencies. Linearity is found at the high spatial frequencies of 8cpd and 10 cpd. Although graphical shape of the functions are similar, CMN090604 still demonstrated dramatically lower P100 amplitude peaks than those of the control.

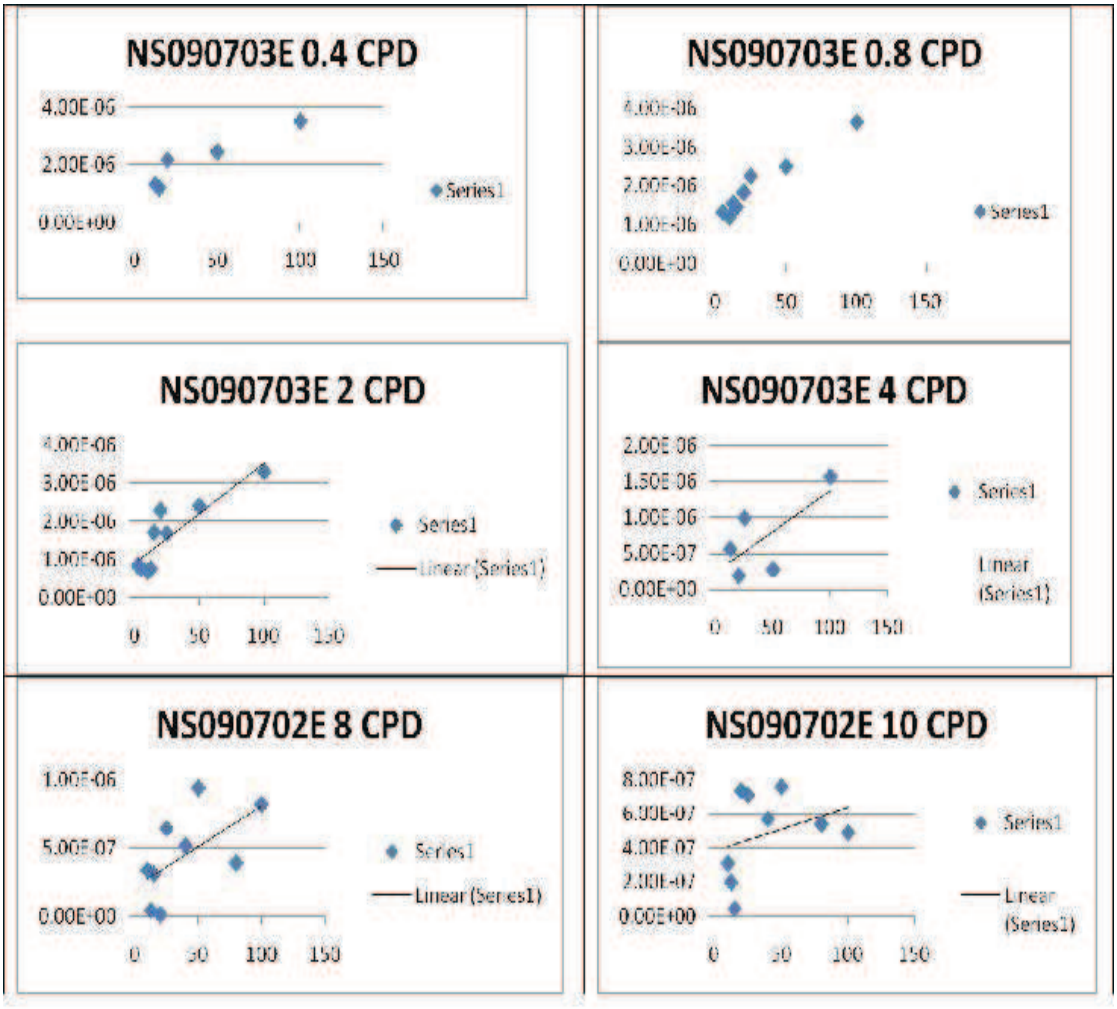

Fig. 5. Patient NS090702, age 66, was monocularly tested OS (with left eye). Amplitude (V) is dependent on contrast (%).

When recording the data of a patient in the later stages of glaucoma, data was more variable. In *Figure 5*, patient NS090702, age 66, still shows saturation at lower frequencies of 0.4 cpd and 0.8 cpd. Double-slope function is illustrated at 2cpd. However, in the higher frequencies of 4 cpd, 8 cpd, and 10 cpd, the data is very scattered and indeterminable. All patients at the later stages of glaucoma had similar data to *Figure 3*, where the higher frequencies could not be analyzed. Like CMN090604, this subject and all subjects had significantly lower P100 amplitudes of VECP than those subjects of the control group.

Discussion

There is much controversy whether there is magnocellular (M cell) death or parvocellular (P cell) death in the ganglion cells of glaucoma patients. In Ito et al., glaucoma was induced in Japanese monkeys, where M cells death was present at 2 weeks, and P cells death occurred at 12 weeks. According to Sun et al., contrast gain is reduced in M cells in glaucoma patients, and P cells are minutely affected.

According to our data, all saturation in lower spatial frequencies was decreased compared to the control groups, meaning M cells are less responsive and are possibly harmed or dead. Although double-slope function is present at medium spatial frequency, the low contrast P100 peaks were extremely reduced. Because the M cell pathway is functioning at low contrasts in middle-to-high frequencies (Souza et al. 2007), these functions (see *Figure 4* and *5*) could imply M cell damage or death. Also, when comparing subject NS090702 (*Figure 5*) to subject CMN090604 (*Figure 4*) in the medium spatial frequencies at double-slope functions, CMN090604's high contrast peaks had reached almost 9.0 μV while NS090702 high contrast peaks only reached around 3.0 μV. The P cell pathway operates at the high contrasts in the medium spatial frequencies. Thus, P cells are less operative in NS090702, whom has a longer history of glaucoma. Again, in the older patients with a longer history of glaucoma, the VECP at higher frequencies are undecipherable compared to the patients with more recent glaucoma diagnoses, and the control group, in which both groups had linear functions at higher frequencies. Comparing the amplitudes of the control group with the amplitudes of the glaucomatous group at higher frequencies, the glaucomatous group's peaks were extremely lower. Control subject's (*Figure 2*) high contrast in high

spatial frequency reaches amplitude of 16μV, while glaucomatous subjects (*Figure 4* and *5*) reach only up to ~3.0 μV. Though P cell pathway may be functioning in subject CMN090604 (*Figure 4*), P cells may be damaged due to the highly decreased P100 VECP amplitudes at high contrasts per high frequencies.

Our data correlates to Ito et al.'s findings of early M cell death followed by P cell death at later stages. In patients with earlier diagnoses of glaucoma (ie, CMN090604), P cells are functioning and M cells seemed to be degraded or destroyed. In patients with longer history of glaucoma (ie, NS090702), M cells are also degraded or destroyed; however, P cells also seem to be degraded or destroyed also. However, to further our findings, we should compare optic disc sizes to ensure the stages (early, late, etc.) of glaucoma the patients are experiencing.

Nonetheless, when comparing all glaucomatous patients to the control group, both pathways show impairment due to the reduced VECP recordings in P100 peak amplitude in all spatial frequencies and subsequent contrasts.

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D2 dopamine receptor expression in rat cortex

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Neurons communicate with each other across synapses via chemical messengers known as neurotransmitters. Each type of neurotransmitter binds to specific receptors on the surface of the postsynaptic receiving neuron. The neurotransmitter dopamine binds to five different classes of dopamine receptors. The localization and function of the D2 dopamine receptor has been extensively studied in subcortical sites but its distribution within the cortex has not been fully elucidated. The cortex receives dopaminergic innervation and is greatly affected by diseases and disorders leading to dopamine denervation. Dopaminergic regulation of cortex is complex and its effects are widespread. Currently, the localization of D2 dopamine receptor protein within the cortex is poorly understood. The purpose of this study was to determine the cortical expression pattern of D2 dopamine receptors using immunohistochemistry, a technique that uses antibodies to localize the proteins of interest. In order to identify the D2 dopamine receptor expression within the cortex, the retrograde neuronal tract tracer Fluorogold was injected into the striatum of the rat. Immunofluorescence was used to localize FluoroGold –filled neurons double-labeled for D2 dopamine receptors, thus revealing the expression of D2 dopamine receptors in the cortex.

Introduction

A neurotransmitter is a chemical messenger that allows neurons to communicate with each other over a synapse. Neurotransmitters are released from pre-synaptic neurons and bind to receptors that are located on the postsynaptic neuron. Dopamine is a type of neurotransmitter that binds to five different classes of dopamine receptors: the D1-like (D1 and D5) and D2 –like (D2, D3, D4). The localization of D2 dopamine receptor protein has been studied using indirect methods such as *in situ* hybridization and raclopride binding (Woodruff, et al. 1991), but a direct and conclusive analysis of D2 dopamine receptor localization using immunohistochemical methods is lacking.

An area that receives dopaminergic innervation that is affected greatly by diseases and disorders resulting in dopamine denervation, such as Parkinson's disease and schizophrenia, is the cortex. D2 receptor protein localization has been poorly characterized in this area of brain. The cortex promotes various functions such as sensory perception, motor function, and executive functions. It is divided into six layers. Dopaminergic regulation of cortex is complex and its effects are wide-ranging. One example, however, is dopaminergic regulation of prefrontal cortex (PFC) which appears to be essential for cognitive functions such as working memory. (Burgos, G. et al. 2005). Although dopamine appears be critical to many cortical functions, the laminar distribution of D2 receptor protein in PFC, as well as in other cortical areas, is poorly understood. Another example is the Substantia Nigra. Degeneration of dopaminergic neurons of the substantia nigra pars compacta is a cardinal feature of Parkinson's disease (PD). (Pellegrino, D.et. al 2007) The data from this study is essential in understanding the effects of dopamine depletion and the cortical activity during depletion in the cortex. Since the localization of D2 dopamine receptors in previous studies have not yielded reliable data, it is important to determine the expression of these receptors. Therefore, in this study, we sought to determine the cortical expression of the D2 dopamine receptors using immunohistochemical means.

Methods

All studies were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory animals. Adult male Sprague Dawley rats weighing about 280g-320g (Harlan, Birmingham, AL) were group housed on a 12h light/dark cycle with lights on at 6:00AM. Food and water were adequately available. The animals were iontophoretically injected with 4% Fluorogold. (a retrograde neuronal tract tracer) into the dorsal lateral striatum. A week after the rats were injected with the striatal injection of 4% Fluorogold, they were sacrificed. The rats were deeply anesthetized with isoflurane. Their brains were then removed and put in paraformaldehyde. 24 hours later, the brains were sectioned frozen on a sliding microtome at 50 µm. The sections were collected and stored in anti-freezing solution. Immunohistochemistry was performed in order to localize the D2 dopamine receptors. The sections were washed with 1x Tris- buffered saline (TBS) for 30 minutes and then blocked in normal horse serum for 60 minutes. The primary antibody was then added. The sections were incubated overnight at room temperature in primary antibody. The antibody used included rabbit anti-D2 dopamine receptor (1:3000; compliments of Dr. K Mackie). The sections were then washed with TBS for 30 minutes and incubated in a secondary antibody. The secondary antibody used was donkey anti-rabbit conjugated to biotin (1:1000; Jackson Immuno –

Research, West Grove, PA). After the sections were incubated in the secondary antibody for 2 hours, they were washed with TBS for 30 minutes and incubated in a 1:1600 solution of streptavidin conjugated to horseradish peroxidase (Jackson Immuno – Research, West Grove, PA). Sections were washed for 50 minutes, followed by a hydrogen peroxide reaction using diaminobenzidine as a substrate to reveal antibody location. Sections were washed thoroughly and mounted onto slides using a .15% gelatin solution. After being dried, they were cover slipped with DPX. Sections were visualized under visible light.

Results

D2 DA Receptor Expression in the Rat Cortex

An immunoperoxidase method for staining of D2 dopamine receptors revealed differential expression of D2 receptor protein within different areas of the cortex. The prelimbic PFC showed a moderate expression of D2 dopamine receptors. There was little, if any, receptor staining found in layer I, dense immunoreactivity in layer II/III, and moderate expression in layer IV, superficial layer V. Deep layer V and layer VI showed relatively weaker staining intensity. The cingulate cortex yielded denser staining across layers II-VI compared to prelimbic PFC, although it exhibited the overall same pattern of staining. The rostral aspect of motor cortex was completely devoid of D2 dopamine staining, an area which is known to be devoid of dopaminergic innervation (Gaspar P, et al. 1995). This finding lends support to the antibody being somewhat specific in its ability to localize the D2 receptor protein.

Cingulate Cortex **Motor Cortex**

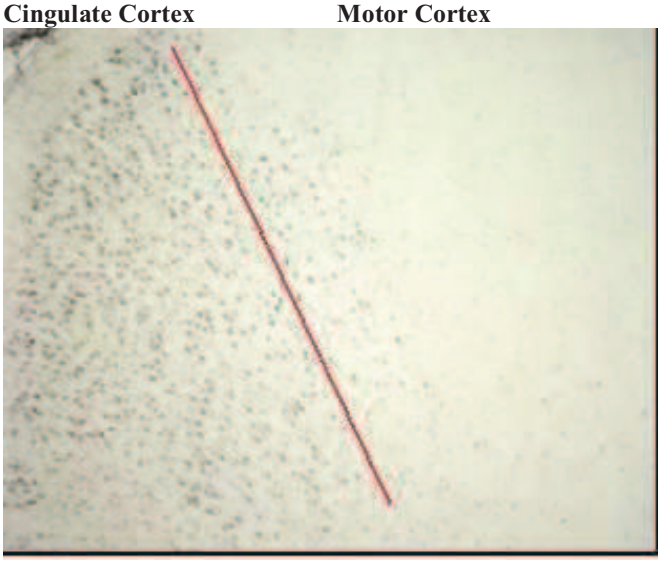

Fig. 1 D2 dopamine receptor expression within the cingulate cortex and the motor cortex.

D2 receptor immunoreactivity was absent in Layer I of somatosensory cortex. Layers II and III of this area exhibited a denser expression of D2 protein as compared with layers IV and V, which exhibited moderate expression levels. Layer VI showed very little immunoreactivity.

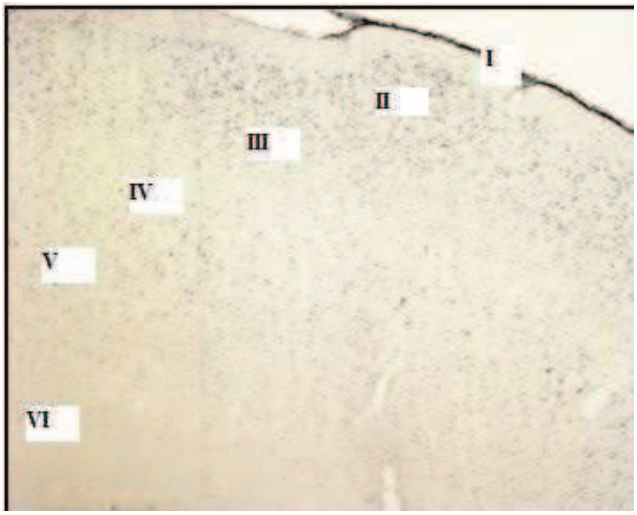

Fig. 2 D2 Dopamine receptor expression in the somatosensory cortex (in layer I-VI)

As a control, D2 dopamine receptor immunoreactivity was assessed in the midbrain dopamine neurons of the substantia nigra (SN), which are known to express this protein. 6-OHDA, a selective dopaminergic neuron toxin, was used to lesion midbrain dopamine neurons, unilaterally. Since midbrain dopamine neurons are known to express the D2 dopamine receptor, this experiment served as both a positive and negative control. Immunoperoxidase revealed D2 immunoreactivity in neurons located within the non-lesioned SN. On the lesioned side, where the dopaminergic neurons were destroyed by 6-OHDA injection, these cells were no longer visible following D2 dopamine receptor immunostaining. Furthermore, invading glial cells (gliosis resulting from lesion) on the lesioned side also expressed the D2 dopamine receptor, a finding consistent with previous reports.

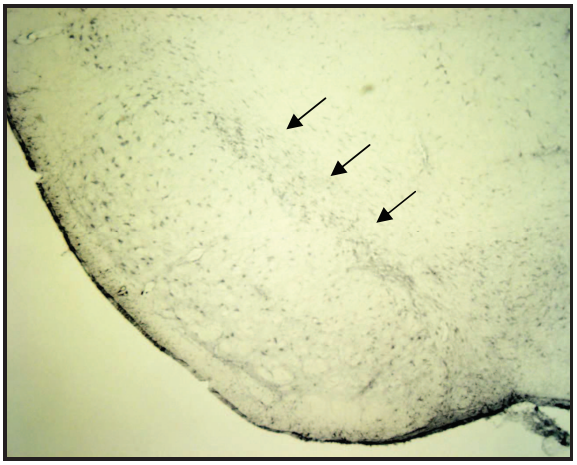

Fig. 3a Substantia Nigra (Control): D2 DA receptor expression. (arrows)

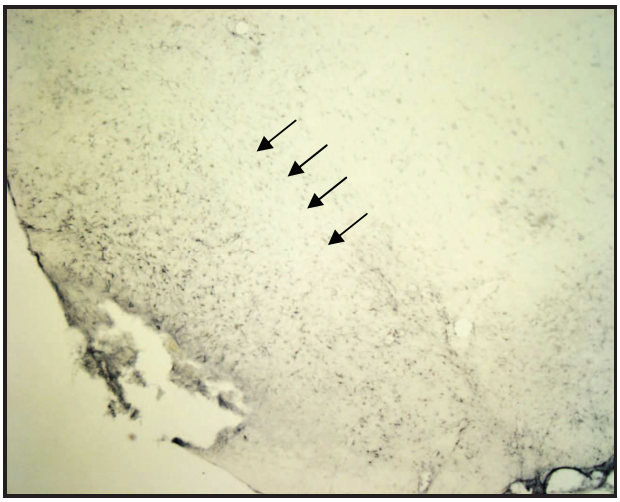

Fig. 3b Substantia Nigra: Lesioned with 6-OHDA. After the lesion, there is an absence of D2 DA receptors and presence of glial cells. (arrows)

Discussion

The results from these experiments indicate that D2 dopamine receptors are differentially localized within cortex. A moderate expression of D2 dopamine receptors was found in the prelimbic PFC. Our findings in layer V and VI are inconsistent with previous reports that employed *in situ* hybridization and found dense mRNA expression in layer V (Wang et al., 2002). This may be a result of differences between mRNA and final protein localization. The cingulate cortex and the somatosensory cortex also exhibited moderate staining. D2 receptor staining was absent in the rostral aspect of motor cortex. This finding is consistent with the *in situ* hybridization findings, which show no D2 receptor message in this area. Immunoperoxidase revealed D2 immunoreactivity on neurons located within the non-lesioned SN. On the lesioned side, these cells were no longer visible following D2 dopamine receptor immunostaining, indicating that our antibody was specific.

Our findings are generally consistent with the few studies that have examined D2 receptor mRNA and protein localization using raclopride binding studies within the cortex. Previous studies have shown in both the primate and rodent prefrontal cortices, D2 receptor mRNA expression was strongest in neurons located in layer V (Wang et al., 2002). Our findings showed a moderate expression of D2 dopamine receptors in the prelimbic PFC, with a greater expression in layer IV and superficial layer V. Studies using *in situ* hybridization to determine the cellular localization of D2 mRNAs in the rat cortices showed a greater expression of D2 mRNAs in the medial PFC, insular and cingulate cortexes and lower expression in the motor and parietal cortexes. Thus, these findings were largely consistent with our results. Furthermore, in a study done by Chen J et al., there was a high expression of D2 dopamine receptor mRNAs in the substantia nigra. Immunoperoxidase staining from our study revealed D2 immunoreactivity on neurons located within the non-lesioned SN.

While a previous study by Khan et al. (1998) demonstrated D2 receptor localization in rat cortex using a different antibody than the one used in this study, the results of this previous report have not been replicated using other antibodies to the D2 receptor protein. Khan et al. (1998) showed weak D2 receptor expression in cortex, with the heaviest labeling in layers IV and V. Unfortunately, they did not offer a complete overview of D2 receptor expression between cortical areas. In addition, the subcellular pattern of D2 receptor staining was largely perisomatic, indicating a membrane-bound localization of this protein. Our findings, however, differ. With regard to overall cortical expression, we found relatively strong expression of D2 dopamine receptors in the PFC, cingulate cortex, and the somatosensory cortex. Moreover, we often observed nuclear, as well as membrane staining of D2 receptor protein. The inconsistency among results seen when using different D2 antibodies continues to call the localization of true D2 receptor protein into question. However, our control experiments using unilaterally lesioned midbrain sections, as well as seeing a lack of expression in rostral motor cortex suggest that the antibody used in this study was reliably specific.

In summary, we have described the laminar localization of D2 receptor protein across various cortical sites with a degree of reliability not seen in previous reports. These findings will be critical in future studies of how dopamine regulates cortical function. Furthermore, in diseases and disorders such as Parkinson's disease and schizophrenia, where the function of the cortex is greatly affected by dopamine dysfunction, the information in this study will prove to be invaluable.

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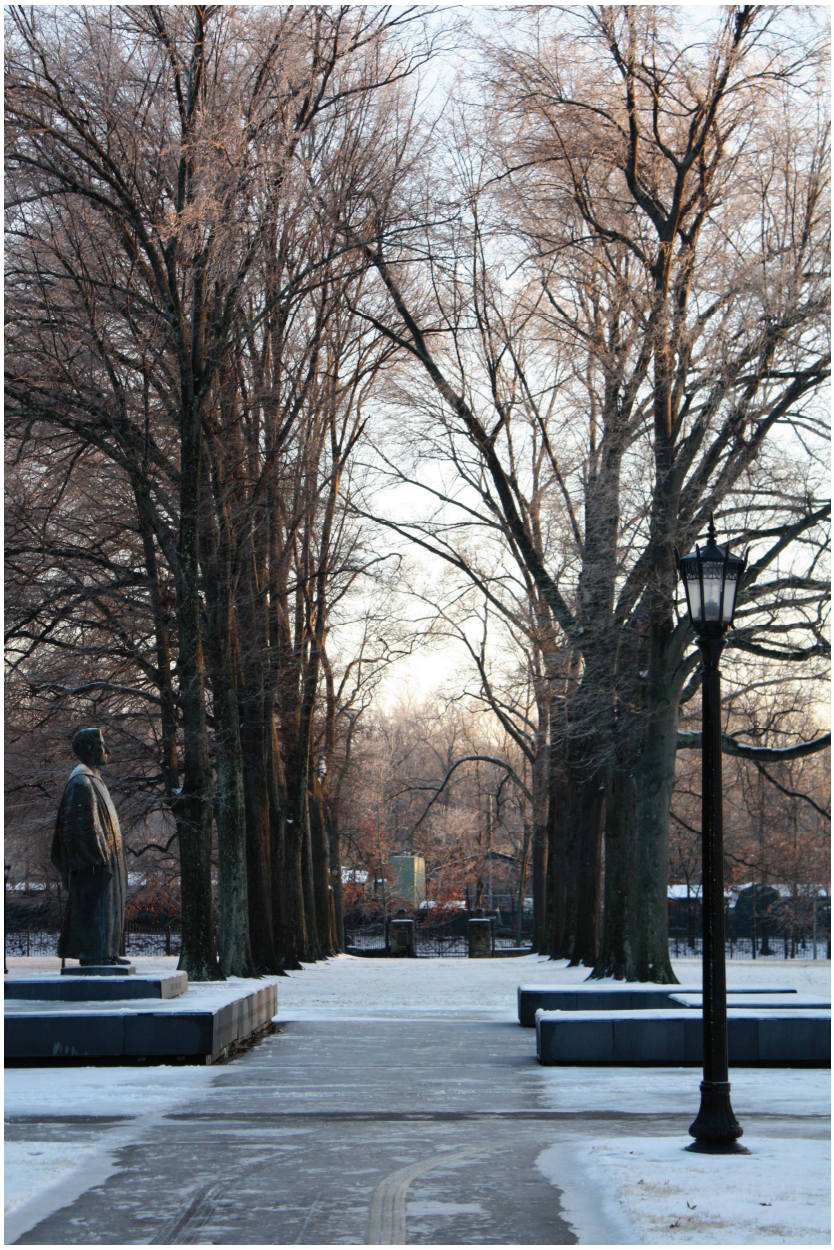

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