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Madeline Scott

Date 5/10/13

Neuroprotective effects of intranasally administered insulin against chronic-restraint stress induced oxidative stress in the cortex and hippocampus of mice

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2013

Submitted in partial fulfillment of the requirements for the Bachelor of Science degree with Honors in Neuroscience

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Acknowledgments

Thank you to Dr. Kim Gerecke and Megan Ververis for their extensive help in performing the techniques necessary for this research.

Also, thank you to the Neuroscience and Psychology departments for providing funding, as well as the Margaret Hyde Award in Psychology.

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ABSTRACT

Oxidative stress is a common toxic mechanism across neurodegenerative disorders, and can be caused by psychological stress. Chronic restraint stress (CRS) is one method of inducing oxidative stress in animal models. Insulin has been shown to be beneficial within both human and animal models of neurodegeneration, including protection against oxidative stress in *in vitro* models. Intranasal administration of insulin allows for insulin to enter the central nervous system without causing hypoglycemia, which can be dangerous. This study aimed to examine whether intranasal insulin administration is neuroprotective against CRS-induced oxidative stress. This question was examined by quantifying the number of reactive microglia, which play pivotal roles in the generation and perpetuation of oxidative stress, in the cortex and hippocampus of mice exposed to CRS. Reactive microglia were determined by the co-expression of the microglial marker Iba1 and the inflammatory marker Cox-2 using confocal microscopy. No significant differences were observed for any of the measurements. This suggests that insulin is not protective against oxidative stress. Comparisons with previous work indicate that intranasal treatment in general may be working as a moderate stressor, preparing the brain for severe stressors. This theory would explain why CRS did not significantly increase the number of reactive microglia in the brain. This research begins to flesh out how insulin works in the brain and within a model of oxidative stress, and calls for further work on this front.

INTRODUCTION

As advancements in science have extended the average lifespan, the elderly are becoming an increasing percentage of the global population (Zuehlke 2010). The disease mortality of neurodegenerative disorders, a major health issue for the elderly population, is predicted to increase by 119-231% by the year 2040 (Lilienfeld and Perl 1993), and this estimate is believed to be an underestimation (Lilienfeld and Perl 1993). While there are many different mechanisms that contribute to the pathology of each unique disorder, oxidative stress is a common mechanism across all neurodegenerative disorders (Gilgun-Sherki, Melamud & Offen 2001).

Oxidative stress is a process in which increased levels of reactive oxygen species (ROS), decreased endogenous antioxidants, and increased inflammatory factors contribute to cell death (Garcia-Bueno et al. 2008; Madrigal et al. 2006; Kubota et al. 2009; Serrano & Klann 2004). ROS are electrophilic molecules that cause the oxidation of nearby molecules (Garcia-Bueno et al. 2008; Madrigal et al. 2006). While both ROS and inflammation have roles to serve in the brain, excessive amounts of either can cause severe damage (Kubota et al. 2009; Serrano & Klann 2004).

Insulin may play an important role within neurodegenerative disorders. Both behavioral and physiological recovery from neurodegenerative damage is observed in human, rat and cortical tissue due to insulin administration (Moosavi et al. 2007; McNay et al. 2010; Martins et al. 2008; Duarte et al. 2005; Duarte et al. 2006; Duarte et al. 2008). However, diabetes is associated with a higher risk of developing neurodegenerative disorders (Arvanitakis et al. 2004; Hu et al. 2007), indicating that peripherally administered insulin is not sufficient to protect against neurodegeneration. In addition,

administering insulin peripherally can be dangerous, as it can cause hypoglycemia (Craft et al. 2011). In contrast, intranasal insulin may provide a method that bypasses the toxic effects of surplus insulin on the peripheral system while promoting a protective effect in the central nervous system. Therefore, this research tests the hypothesis that intranasal insulin administration in mice can protect against the neurodegenerative effects of CRS-induced oxidative stress.

Oxidative Stress and Neurodegeneration

Oxidative stress has been widely observed as a major problem in neurodegenerative disorders (Gilgun-Sherki, Melamud & Offen 2001). It has been shown to exacerbate the neurotoxic effects of traumatic brain injury, aging, and genetic conditions (Gilgun-Sherki, Melamud & Offen 2001). Signs of oxidative stress have consistently been found in the post-mortem tissue of Parkinson's patients (Zhou et al. 2008) and higher levels of reactive oxygen species (ROS) have been found in Alzheimer's and Parkinson's disease patients compared to controls (Vitte et al. 2004). Thus, understanding oxidative stress and its role in neurodegeneration has become imperative.

Physiological and psychological stressors induce a cascade of events that lead to oxidative stress. These stressors induce increased levels of catecholamines and glucocorticoids (Garcia-Bueno et al. 2008), both of which lead to elevated cytokine levels (Mattson et al. 2002). Cytokines promote increased reactive oxygen species (ROS), such as nitric oxide and superoxide. These molecules are highly electrophilic, resulting in the oxidation of nearby molecules (Garcia-Bueno et al. 2008, Madrigal et al. 2006). Normally, ROS are involved in important signaling cascades and there are factors within

the central nervous system that regulate ROS production (Kubota et al. 2009; Madrigal et al. 2006; Serrano & Klann 2004). However, a system is considered to have oxidative stress when ROS are released in an amount that the endogenous system cannot combat, and is thus toxic to neural cells (Kubota et al. 2009). Excessive ROS can cause protein misfolding, proteasomal malformation, mitochondrial dysfunction, glial cell activation, and programmed cell death (apoptosis) (Anderson 2004). These processes contribute to the weakening of cells as well as cell death.

In addition to increases in ROS, stressors increase production of and decrease reuptake of glutamate (Munhoz et al. 2008). An excessive amount of glutamate over-stimulates the *N*-methyl-D-aspartate receptor (NMDAr) (Nair & Bonneau 2006), which leads to excitotoxcity (Muhoz et al. 2008). Excitotoxicity is characterized by increased ROS, protein misfolding, and cytoskelton damage, all of which can potentially lead to cell death (Munhoz et al. 2008). NMDAr over-stimulation also activates microglia (Nair & Bonneau 2006). Excessive microglial activation increases ROS and neuroinflammatory factor production (Koutsilieri et al.2 002; Nair & Bonneau 2006). Microglia also signal for increased ROS production, creating a self-sustained feedback loop of amplification (Anderson 2004). Microglia are recruited to deliver ROS to damaged cells (Koutsilieri et al. 2002) and communicate through the release of inflammatory factors, resulting in increased inflammatory factors in locations of cell damage and death (Koutsilieri et al. 2002). Microglia that are producing excessive amounts of inflammatory factors are considered reactive (Koutsilieri et al. 2002). Reactive microglia are considered early signs of neural damage and death, and contribute to oxidative stress (Anderson 2004; Koutsilieri et al. 2002).

The hippocampus and cortex are both regions which have a high degree of glutamate receptors and are therefore especially sensitive to the damaging effects of oxidative stress (McEwan 2008). Consistent with this, patients with Alzheimer's have distinctive hypofrontality (Craft et al. 2011), suggesting that this region is particularly vulnerable to neurodegeneration. The hippocampus is involved with learning and memory, functions which require a high level of synaptic plasticity (Serrano & Klann 2004). Long-term potentiation (LTP), which is believed to be essential for learning and memory, is the mechanism that allows for more efficient and numerous synaptic transmissions (Serrano & Klann 2004). ROS act as cellular messengers during this process, and appear to regulate synaptic plasticity (Serrano & Klann 2004). Due to relatively high ROS activity within the hippocampus, it is particularly vulnerable to the effects of oxidative stress (Serrano & Klann 2004), making it a key region used to study oxidative stress.

The induction of oxidative stress using physiological challenges in animals is regularly used to generate animal models of neurodegenerative disorders. The neurotoxin MPTP induces ROS in toxic levels leading to the death of dopaminergic neurons in the substantia nigra, a brain region that is specifically affected by Parkinson's disease (Langston et al. 1983; Smeyne & Jackson-Lewis 2005). Intracerebroventricular (ICV) injection of streptozotocin (STZ) reduces cholinergic activity and energy metabolism in the brain by inhibiting adenosine triphosphate (ATP) production (Agrawal et al. 2009). Decreased ATP production disrupts the mitochondrial process, thus creating increased oxidative stress and neurodegeneration (Agrawal et al. 2009). This generates sporadic Alzheimer's disease-like conditions, such as decline in memory, decreased cerebral

glucose levels, and low energy metabolism (Agrawal et al. 2009). Cumulatively, these data indicate oxidative stress induced by toxins recreates many of the processes in neurodegenerative disorders.

Furthermore, psychological stressors also induce oxidative stress. Chronic-restraint stress (CRS) is the most common psychological stress model. In this model, rodents are immobilized in restraint tubes within a brightly lit room for a determined period of time on a chronic basis (Ejchel-Cohen et al. 2006; Jeong et al. 2006; Pham et al. 2003). Mice exposed to CRS have downregulated expression of several important genes in energy metabolism pathways and an increased amount of oxidative products (Ejchel-Cohen et al. 2006). Additionally, exposure to CRS increased Alzheimer's pathology and memory deficits in mice (Jeong et al. 2006).

Neuroprotective Factors

As the role of oxidative stress in neurodegenerative disorders has been discovered, methods to treat oxidative stress have been examined. Anti-oxidant administration counters the negative effects on cholinergic activity and energy metabolism caused by ICV injection of STZ (Tota et al. 2011). Exercise also protects against oxidative stress. A single bout of exercise raises levels of ROS but only to moderate levels and consequently prepares the system for coping with larger amounts of stress (Radak et al. 2001). Excessive exercise can create unhealthy ROS upregulation, but moderate exercise upregulates antioxidants (Radak et al. 2008). Additionally, exercise prevents dopaminergic neuronal loss after MPTP-induced oxidative stress (Gerecke et al. 2010) and improves behavioral task recovery after brain damage in mice (Carro et al. 2001).

Another recent route of study is whether insulin is protective against neurodegenerative disorders. Insulin deficiencies correlate with higher rates of neurodegenerative disorders (Ho et al. 2004; Kuuisto et al. 1997; Schubert et al. 2003; Xie et al. 2002). Insulin resistance is one type of insulin deficient system, and is associated with the risk of Alzheimer's disease (Kuuisto et al. 1997; Ho et al. 2004). This correlation appears to be linked to cardiovascular risk factors produced by insulin resistance (Kuuisto et al. 1997) as well as decreased insulin receptor signaling in the brain (Ho et al. 2004). Increased apoptosis in neuronal cells also occur in mice with a neuron-specific insulin gene receptor knockout (Schubert et al. 2003). Because problems with insulin are associated with increased neurodegenerative symptoms and pathology, insulin administration may therefore be protective.

Insulin administration is associated with recovery in cognitive behavior and memory in disease and brain-damaged models, as well as protection against oxidative stress. When insulin levels are elevated through infusion, Alzheimer's patients show increased performance on declarative memory and selective memory cognitive tests (Craft et al. 1999). Hyperglycemia enhances short-term memory in adults with Alzheimer's disease, though these effects are abolished when insulin production is blocked (Craft et al. 1999). In cortical tissue, insulin administration results in a decrease of necrotic and apoptotic cell death caused by ascorbate/Fe^{2+} treatment, which produces mild oxidative stress within cortical cell cultures (Duarte et al. 2005; Duarte et al. 2008). Oxidative stress is associated with decreased glycolysis, lower production of ATP, and diminished insulin receptor activity. These are prevented by the administration of insulin, most likely due to increases in ATP production (Duarte et al. 2005; Duarte et al. 2006;

Duarte et al. 2008). Therefore, insulin appears to be protective against the behavioral decline of neurodegeneration as well as oxidative stress.

Peripheral administration of insulin to diabetic patients does not appear to decrease the incidence of neurodegenerative disorders (Arvanitakis et al. 2004; Hu et al. 2007), and is unsafe for non-diabetic individuals (Craft et al. 2011). Intranasal administration allows insulin to be applied to the central nervous system while bypassing the hypoglycemic effects of peripherally administered insulin (Reger et al. 2006). Therefore, intranasal insulin is being tested as a possible treatment of Alzheimer's disease (Reger et al. 2006). Intranasal insulin has been shown to increase performance on memory tests in APOE-ε4, a genetic indicator of increased susceptibility to Alzheimer's disease, deficient Alzheimer's patients, while not changing plasma insulin or glucose levels (Reger et al. 2006). In addition, the distinctive hypofrontality exhibited by Alzheimer's patients is abolished with intranasal insulin treatment in humans (Craft et al. 2011). However, these studies do not examine how insulin is acting within these patients. There is very little evidence showing how insulin interacts with oxidative stress in brain tissue within animal models, suggesting a need for further research into intranasal insulin's role in within this important cellular process.

CRS reliably induces oxidative stress damage in mice. Insulin has been shown to be beneficial to both human and animal models of neurodegeneration as well as oxidative stress in cortical cell cultures. Exercise is neuroprotective against CRS induced oxidative stress, and increased insulin levels contribute to this protection. Therefore, this study seeks to examine whether intranasal insulin administration will protect against CRS induced oxidative stress as shown by decreased inflammatory microglia in the brain

tissue of mice. We hypothesize that stress will increase the number of microglia, the number of reactive microglia, and the proportion of reactive microglia. In addition to this, we hypothesize that intranasal insulin administration will decrease the number of microglia, the number of reactive microglia, and the proportion of reactive microglia.

METHODS

Animals

Sixteen female C57BL/6J mice were obtained from Jackson Laboratory, Bar Harbor, Maine, and housed 3 per cage in standard mouse cages in a temperature controlled room with a 12 hour light and dark schedule (6:30 a.m.- 6:30 p.m. light). Food and water were given *ad libitum*. All procedures were in compliance with the National Institutes of Health (NIH) standards, and approved by the Rhodes College Institutional Animal Care and Use Committee (IACUC Protocal #97). Mice were allowed to habituate for two weeks prior to experimental manipulations.

Treatment Conditions

Mice were randomly assigned to one of four treatment conditions: no stress saline, no stress insulin, stressed saline, stressed insulin. All mice were lightly anaesthetized through the inhalation of isoflurane every morning between 9 a.m. and 11 a.m. for sixteen days. Mice were then administered either 20 μL of insulin solution (2 μg Insulin/1 μL 0.1 M phosphate buffer saline [PBS]) or saline (PBS). The stressed condition mice underwent 2 hours of CRS every afternoon for fifteen days by being placed in restraint tubes (Broome Restraint Tubes, Harvard Apparatus, Holliston, MA) at randomized times within the daytime light cycle. These times all occurred at least 2 hours

after the daily morning drug administration. While in the tube, the mice were kept in their cages within a brightly lit room. To control for handling effects, the no stress mice were also picked up during this time. On the sixteenth day of treatment, the mice were administered their respective insulin and stress treatments as usual. One hour following the stress condition, the mice were deeply anaesthetized with tribromoethanol (250 mg/kg i.p.) and processed for immunohistochemistry analysis.

Immunocytochemistry

To quantify the level of microglia within these tissues, we are using the microglial marker ionized calcium binding adaptor molecule 1 (Iba1). Iba1 is a calcium binding protein that is exclusively expressed in microglia and macrophages (Ohsawa et al. 2000) and CRS increases the density of cells expressing Iba1 (Tynan et al. 2010). Thus we used Iba1 to stain for microglia, which are recruited during oxidative stress. We co-labeled these cells using Cox-2, which is a marker for inflammation and early stress (Boyd et al. 2007).

After being deeply anaesthetized with tribromoethanol, twenty four mice were transcardially perfused with 0.1 M PBS, followed by 4% paraformaldehyde in PBS. The whole brains were then removed and post-fixed for 24 hours in 4% paraformaledehyde in PBS. They were then allowed to soak in a 1 M sucrose solution at 4 °C for 24-48 hours to cryoprotect the tissues. Afterward they were rinsed in PBS for one hour, and then encased in Tissue-Tek Optimal Cutting Temperature Compound (Sakura Finetek USA, Inc., Torrance, CA) and frozen at -80 °C until being sectioned into 10 μm coronal sections and mounted on slides (SuperFrost Plus Slides, Fisher Scientific; Atlanta, GA).

For immunocytochemical labeling, frozen sections were warmed to room temperature and soaked in PBS with Tween 20 (PBST) to rehydrate for 30 minutes. After rehydration, the tissue was encircled with a PAP pen (Research Products International; Mt Prospect, IL), and incubated in 0.3% hydrogen peroxide for 15 minutes. Afterward the slides were soaked in dehydrogenized water for 5 minutes, followed by PBST for 15 minutes. Blocking buffer (PBST-BB [1% BSA, 0.2% nonfat milk, 0.3% Triton X-100, in 0.1 M PBS]) was then applied for 30 minutes at room temperature to block nonspecific binding.

Slides were then incubated in both rabbit anti-Iba1 polyclonal antibody (1:1000; Cat# 019-19741, Wako Chemicals, USA, Richmond, VA) and mouse anti-Cox-2 antibody (1:100; Cat# 610204, BD Transduction Laboratories, San Jose, CA) overnight at 4 °C. Slides were then washed 3 times for 5 minutes each in PBST and subsequently incubated in two fluorescently-labeled secondary antibodies for 2 hours at room temperature (Goat anti-mouse AlexaFluor 594, 1:250; donkey anti-rabbit AlexaFluor 488, 1:250; Molecular Probes). A PBS/glycerol solution (1:1) was used to apply a coverslip to the slides in order to ready them to be analyzed with a confocal microscope.

Confocal immunofluorescent images were obtained using a Zeiss Axio Imager.M2 confocal microscope coupled to a LSM 700 camera. Tissues were visualized using the 20x magnification plan-apochromat objective with a final magnification of 200x. Zen 2010 software (v6.0) captured digital images in Z stacks at 5 levels, 2 μm apart and a two-dimensional maximum intensity photomicrograph was created. Adobe Photoshop Elements software (v5.0) was then used while quantifying Iba1 and Cox-2 labeled cells.

Sections were analyzed similarly to that described previously (Lambert, Gerecke et al. 2000). The number of cells expressing Iba1 alone and those expressing both Iba1 and Cox-2 will be counted in the CA1 striatum pyramidale area in the hippocampus and in layers IV and V in the cortex directly dorsal in the sections beginning at Bregma -1.28 mm to Bregma -2.12 mm. For each brain, bilateral images acquired from five sections at regular intervals throughout the criterion area were quantified for expression. Two trained observers, who were blind to treatment groups, conducted the visual quantification. Multivariate ANOVAs with LSD post-hoc analyses (PASW Statistics 17.0, v 17.0.2, IBM Corp.) were used to quantitatively analyze the number of Iba1 positive cells, and the number of Iba1/Cox-2 coexpressing cells within each brain region. For all analyses, $p<0.05$ was considered significant.

RESULTS

No significant differences were found within the treatment groups. No significant differences in number of cells labeled with Iba1 were found within the cortex or hippocampus. No significant differences in number of cells co-labeled with Iba1 and Cox-2 were found within the cortex or hippocampus. In addition, we took percentage of cells labeled with Iba-1 that were also labeled with Cox-2 within each picture to account for variation within the 2 mm area we sampled from. Again, no significant differences were observed in the cortex and hippocampus.

DISCUSSION

No significant differences were observed in either the cortex or the hippocampus. These findings contradict our hypothesis that insulin would be neuroprotective against oxidative stress. In addition to reactive microglia, we also observed Cox-2 staining of blood vessels. Cox-2 is present in new angiogenic endothelial cells, though not in mature blood vessels (Masferrer et al. 2006)

Previous studies in our lab resulted in similar counts of microglia when comparing control conditions. However, the number of reactive microglia was greater in our control animals than in the control animals of the previous study. A possible explanation for this difference is that the intranasal treatment in general was a mild stressor. Mild increases in oxidative stress can actually be beneficial by increasing activity of the antioxidant system (Gomez-Cabrero et al. 2005). Perhaps the increased stress caused by intranasal treatment recruited more reactive microglia, regardless of treatment groups. The process of giving the mice intranasal treatment was a mild stressor against which the brain was able to compensate, leading to a decreased responsiveness to chronic-restraint stress, a more severe stressor. Exercise also appears to work this way, providing a manageable, mild stressor that prepares the system for a more severe stressor (Gerecke et al. 2013). If this is the case, it could explain the higher baseline of reactive microglia, and why the stressed mice did not contain significantly more reactive microglia than the no stress mice within the hippocampus.

While not significant, a surprising pattern was observed, particularly in the hippocampus. There was a consistent high amount of reactive microglia within the insulin stress treatment group. This is surprising, as previous literature has shown insulin

to be neuroprotective against Cox-2 and cell damage. Lipopolysaccharide-induced Cox-2 production is reduced by insulin treatment (Martins et al. 2008). In addition to this, recovery from chronic-restraint stress on spatial memory tasks can be induced by insulin administration to the CA1 (Moosavi et al. 2007), along with just general improvement on spatial memory tasks (McNay et al. 2010). While these findings suggest a neuroprotective role for insulin, there are a few results observed due to insulin administration which may shed light on what's occurring in this study. Insulin interacts with the NMDA receptor to produce long-term depression (LTD) (van der Heide et al. 2005). This suggests that insulin stimulates this receptor, which is also overexcited by the effects of chronic-restraint stress. Perhaps together insulin and stress combine to create an increased excitotoxic environment for NMDAr, resulting in increased oxidative stress and reactive microglia.

Insulin also appears to decrease endogenous norepinephrine reuptake protein expression (Figlewicz et al. 1993) resulting in an increase in norepinphrine within the hippocampus (Figlewicz and Szot 1991; Figlewicz et al. 1993). Without chronic-restraint stress, this may be just a mild stressor that the brain can handle with ease. However, with chronic stress, levels of norepinephrine may be inducing excessive stimulation of the endogenous stress system, resulting in increased oxidative stress and reactive microglia. Insulin's interaction with norepinephrine and its' ability to induce LTD within the hippocampus may explain why the hippocampus appears to be more susceptible to negative effects from insulin treatment than the cortex. If the increases in reactive microglia are due to excitotoxicity of the NMDAr, then a far greater effect would be seen in the hippocampus.

It is also possible that our divergence from the literature may be caused by strain/species differences. The behavioral recovery and reductions in Cox-2 production due to insulin treatment discussed above were found in Wistar rats (Martins et al. 2008; Moosavi et al. 2007; Figlewicz et al. 1993; Figlewicz and Szot 1991; van der Heide et al. 2005), while we used C57Bl/6J female mice. Perhaps there are differences between rats and C57Bl mice that would explain why we saw no protective effects of insulin. Differences in Cox-2 production in response to a physiological stressor have been observed between C57Bl mice compared to Swiss Webster mice (Boyd et al. 2007). The C57Bl mice had a strong, long-lasting stress response when treated with MPTP compared to the SW mice, which had a brief spike in the same response (Boyd et al. 2007). Due to this pattern, the C57Bl mice do not appear to attenuate quickly to stressors, so while other species may be able to adjust to stressors like chronic-restraint stress and intranasal treatment, the C57Bl's are particularly susceptible to neurotoxic damage (Boyd et al. 2007).

Further studies are necessary to elucidate the role insulin is playing when in combination with chronic-restraint stress. A high priority for us is a western immunoblotting analysis of apoptotic factors. Western blot quantification of Bcl-2 associated X protein (Bax) would be used to determine the extent of apoptosis in the frontal lobe and hippocampus. Apoptotic stimuli promote the formation of cytoplasmic Bax protein, which then integrates itself into the mitochondrial membrane (Haack et al. 2008) and forms a pore in the membrane, resulting in apoptosis (Haack et al. 2008). CRS has been shown to elevate Bax oligomer formation (Haack et al. 2008) and increased amounts of Bax protein (Gerecke et al. 2012). While reactive microglia appear to be an

early sign of neural damage and death, increased pro-apoptotic factors would be a more downstream effect (Hovatta et al. 2002) With the added information provided by the level of apoptotic factors within the brain, we would be able to better understand the role insulin in neuroprotection.

In conclusion, this study observed changes in microglia and reactive microglia expression in the hippocampus and cortex of mice due to chronic-restraint stress and insulin treatment. Insulin treatment did not appear to have a neuroprotective effect against oxidative stress. Most of the current literature on insulin treatment for neurodegenerative disorders relies on behavioral recovery in human patients and protection in cortical cell cultures (Reger et al. 2006; Craft et al. 2011; Duarte et al. 2005; Duarte et al. 2006; Duarte et al. 2008). This study seeks to begin filling a gap in the literature concerning animal studies focusing on intranasal insulin administration's interaction with oxidative stress, an important process present in all neurodegenerative disorders. Our finding that intranasal insulin is not neuroprotective against chronic-restraint induced oxidative stress suggests that insulin is not working through this method and is likely not an appropriate treatment for neurodegeneration. Further research is necessary to truly understand how intranasal insulin is acting in the brain.

Tables and Figures

Table 1. Hippocampus Iba1		
	Saline	Insulin
No Stress	M= 6.33	M= 6.78
	SE= 1.34	SE= 0.88
Stress	M= 6.00	M= 7.44
	SE= 0.99	SE= 1.18

Table 1. The Means and Standard Errors of cells labeled with Iba1 in the Hippocampus.

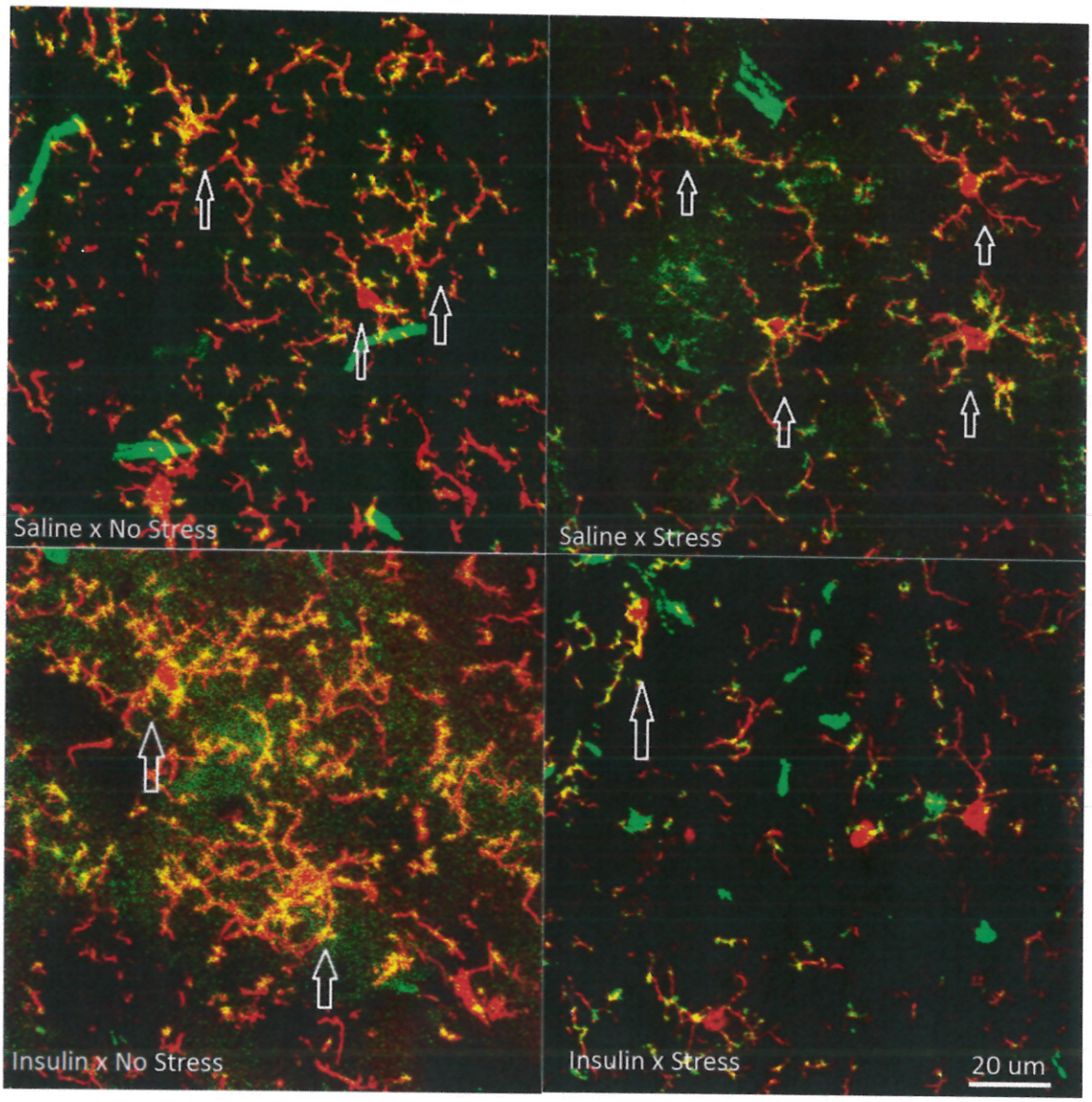
Table 2. Hippocampus Iba1+Cox-2		
	Saline	Insulin
No Stress	M= 2.44	M= 2.71
	SE= 1.35	SE= 1.50
Stress	M= 2.40	M= 3.95
	SE= 0.96	SE= 1.04

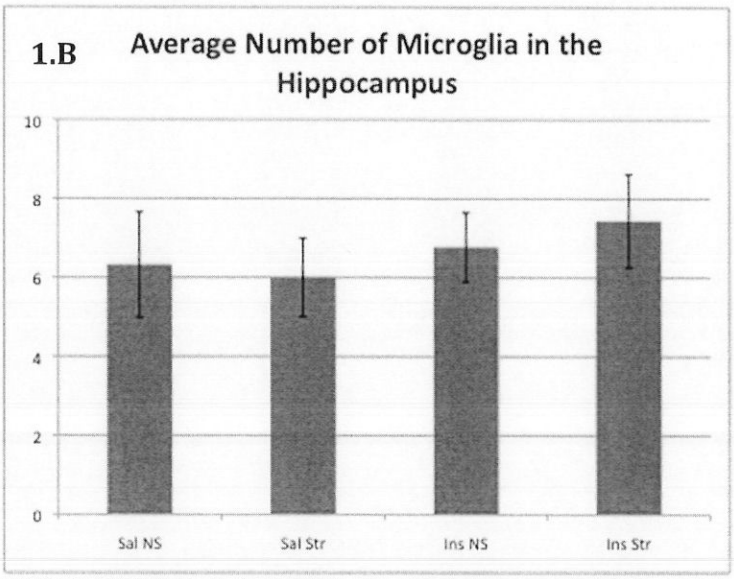
Table 2. The Means and Standard Errors of cells co-labeled with both Iba1 and Cox-2 in the Hippocampus.

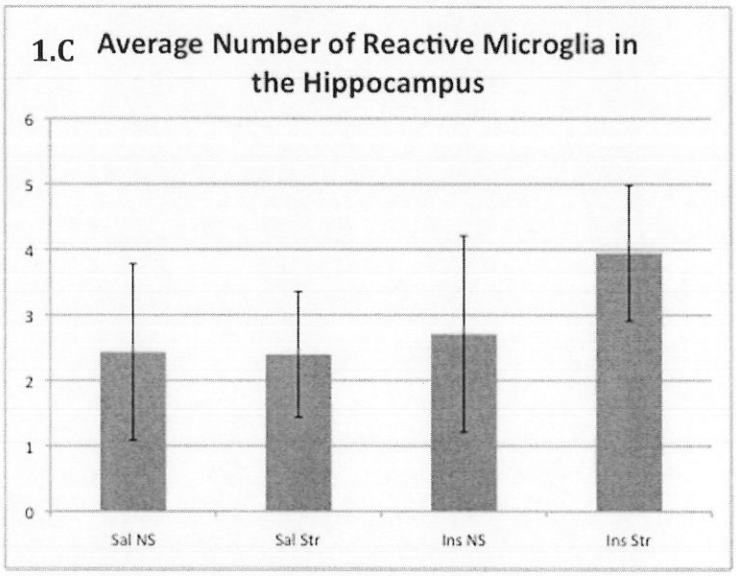
Table 3. Hippocampus Proportions		
	Saline	Insulin
No Stress	M= 39.06	M= 38.53
	SE= 14.60	SE= 16.38
Stress	M= 41.10	M= 53.22
	SE= 10.58	SE= 6.95

Table 3. The Means and Standard Errors of the proportion of Iba1 labeled cells co-labeled with Cox-2 in the Hippocampus.

1.A


Saline x No Stress
Saline x Stress
Insulin x No Stress
Insulin x Stress
20 um





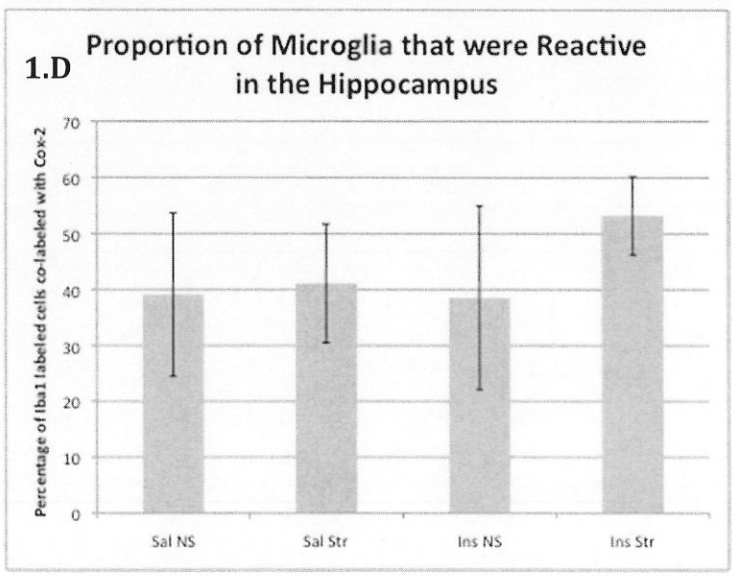


Figure 1. (A) These pictures display double-labeled immunofluorescence for Iba1 positive microglia (red) and Cox-2 (green) in the CA1 striatum pyramidale region. Arrows point to cells co-labeled with Iba1 and Cox-2 (yellow). Scale bar = 20 um, applies to all. **(B)** This graph displays the average number of microglia in the hippocampus **(C)** This graph displays the average number of reactive microglia in the cortex **(D)** This graph displays the average proportion of microglia that were reactive in the hippocampus. **(B,C,D)** Sal NS: Saline No Stress; Sal Str: Saline Stress; Ins NS: Insulin No Stress; Ins Str: Insulin Stress. All error bars represent 1 standard error away from the mean.

Table 4. Cortex Iba1		
	Saline	Insulin
No Stress	M= 6.39	M= 6.65
	SE= 0.88	SE= 0.48
Stress	M= 7.34	M= 8.29
	SE= 0.64	SE= 1.11

Table 4. The Means and Standard Errors of the number of Iba1 labeled cells in the Cortex.

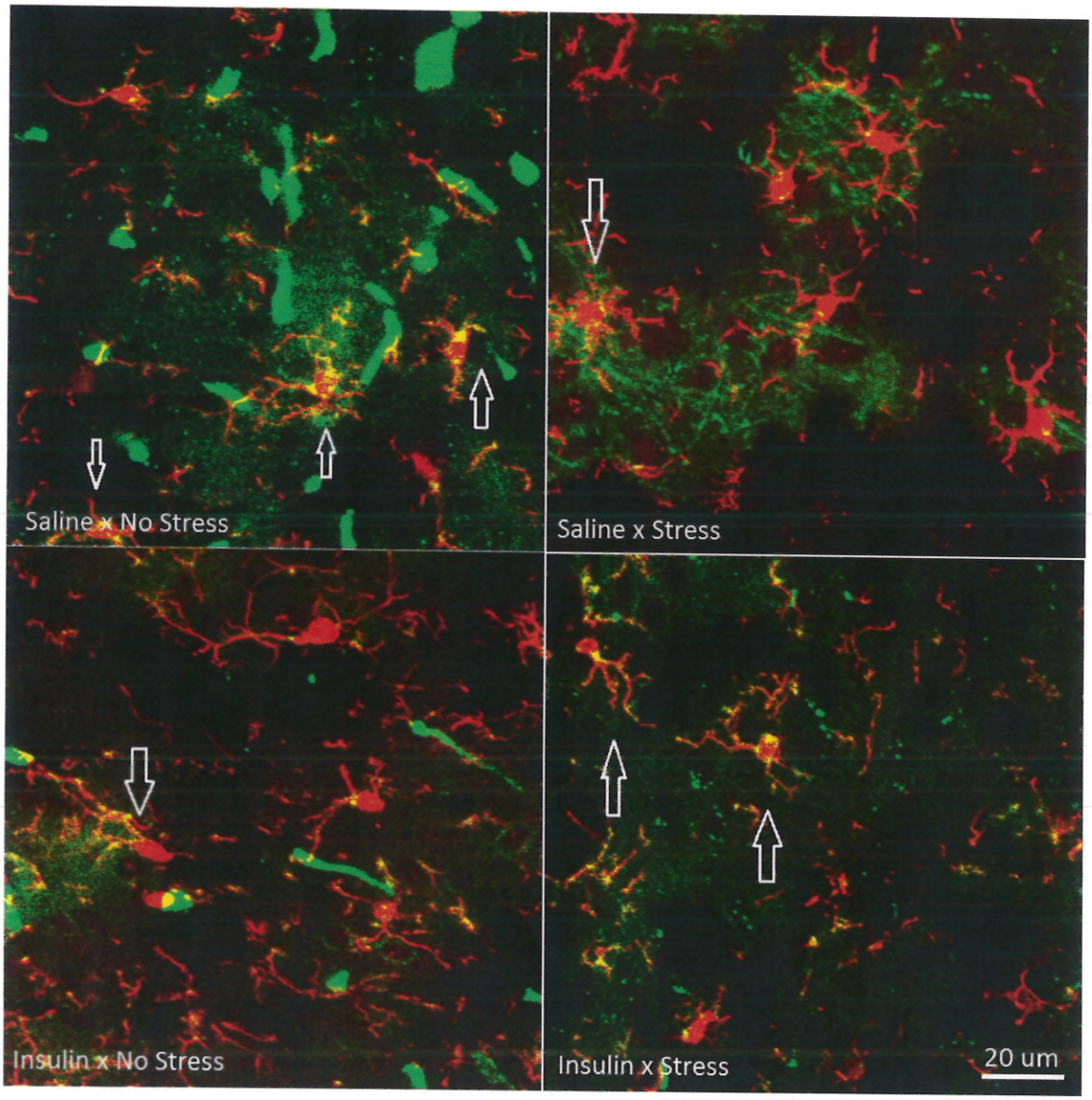
Table 5. Cortex Iba1+Cox-2		
	Saline	Insulin
No Stress	M= 2.38	M= 2.26
	SE= 1.88	SE= 0.93
Stress	M= 1.74	M= 3.11
	SE= 0.62	SE= 0.74

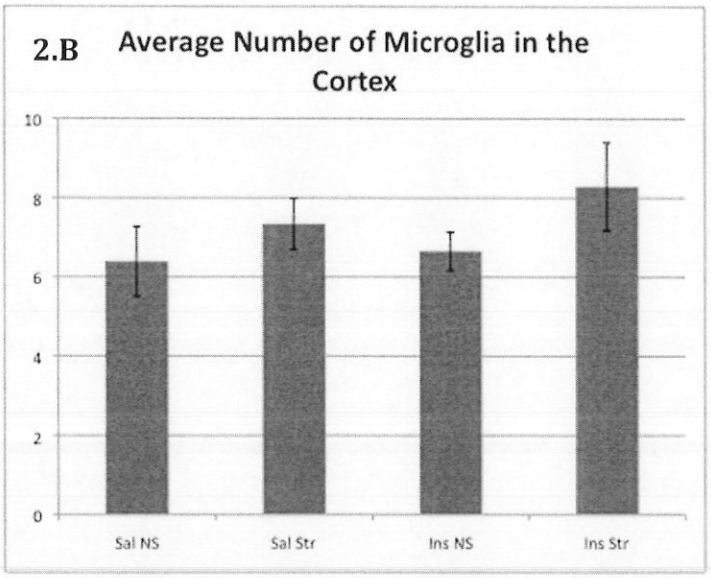
Table 5. The Means and Standard Errors of the number of Iba1and Cox-2 colabeled cells in the Cortex.

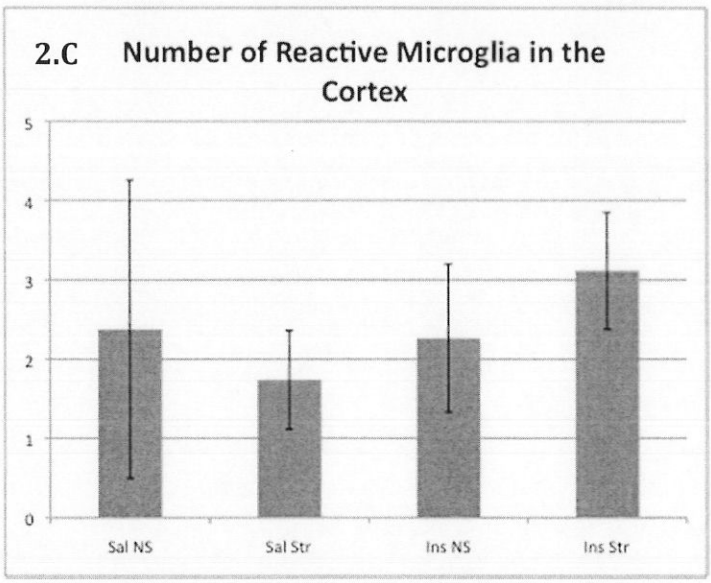
Table 6. Cortex Proportions		
	Saline	Insulin
No Stress	M= 36.29	M= 33.68
	SE= 19.50	SE= 11.29
Stress	M= 24.38	M= 37.39
	SE= 9.02	SE= 8.38

Table 6. The Means and Standard Errors of the proportion of Iba1 labeled cells co-labeled with Cox-2 in the Cortex.

2.A


Saline x No Stress
Saline x Stress
Insulin x No Stress
Insulin x Stress
20 um





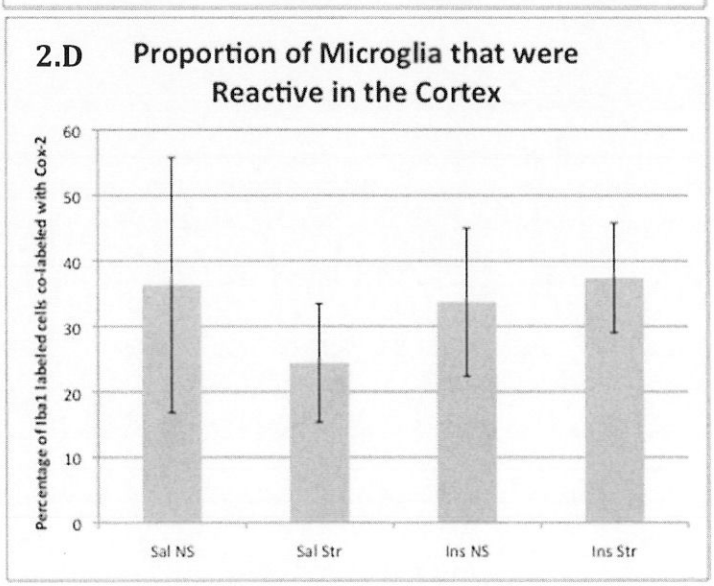


Figure 2. (A) These pictures display double-labeled immunofluorescence for Iba1 positive microglia (red) and Cox-2 (green) in the cortex. Arrows point to cells co-labeled with Iba1 and Cox-2 (yellow). Scale bar = 20 um, applies to all. **(B)** This graph displays the average number of microglia in the cortex **(C)** This graph displays the average number of reactive microglia in the cortex **(D)** This graph displays the average proportion of microglia that were reactive in the cortex. **(B,C,D)** Sal NS: Saline No Stress; Sal Str: Saline Stress; Ins NS: Insulin No Stress; Ins Str: Insulin Stress. All error bars represent 1 standard error away from the mean. Surprisingly large variance was observed in the saline no stress treatment.

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