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Effects of Zinc on Cognition

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Effects of zinc on cognition

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Abstract:

Insulin resistance is the defining symptom of type 2 diabetes and a key risk factor for Alzheimer's disease. Work from our lab and others has shown that insulin is a key component of brain pathways mediating cognition; specifically, mechanisms that transduce hippocampal memory processing. In the hippocampus, insulin regulates glucose metabolism through translocation of the glucose transporter GluT4 and modulates several other molecular cascades.

Studies where insulin is studied as a cognitive modulator have generally used a formulation that contains zinc to stabilize insulin. However, zinc itself modulates molecular signaling pathways including AKT and GSK3β, which are both downstream of insulin; as well as having a known effect in modulating neuronal glutamate release, suggesting a role in memory. Hence, delivery of zinc as an artifact of administering treatment to insulin-treated animals may confound the impact of insulin on cognition and metabolism. This is especially true since *in vivo*, the insulin hexamer, stabilized by zinc and calcium, breaks apart almost immediately after being released from beta cells in the pancreas; thus, insulin delivery would not produce an increase in zinc at the site of action.

This project therefore aimed to parse the distinct effects of zinc and insulin on hippocampal metabolism and cognitive processes. Sprague-Dawley rats received a micro-injection guide cannulae into their left hippocampus and were then treated with intrahippocampal delivery of Humulin (a zinc-containing insulin), Apidra (a zinc-free form of insulin), artificial extracellular fluid (aECF; a vehicle control condition), or zinc dissolved in aECF. Post injection the animals were placed in a plus maze for 20 mins for a spontaneous alternation test, followed immediately by a novel object recognition test spread over one day, both of which test cognition

and short-term memory. Brain samples from the left and right hippocampus along with the pre-frontal cortex were removed and were analyzed for the presence of key proteins in the activation cascades via western blots. Evidence from current literature indicates a strong possibility of finding a confound, to some degree, between the known actions of zinc and insulin in the hippocampus.

Acknowledgements:

I would like to thank Dr. Ewan McNay, not only for allowing me to join his lab and pursue my interests, but also for mentoring me over the past two and a half years. His guidance and constant encouragement pushed me to become better, not only as a scientist, but as a person. Thank you also to Dr. Ben Szaro, for agreeing to be the second reader on my thesis, but also for the great life advice given to me during my time at the university.

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Thank you to all of my friends who were able to keep me sane over the years and especially thank you to my girlfriend Hanna, for motivating me, and pushing me to go further than I believed I could.

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Introduction:

In 2015, over 30 million people in the United States had diabetes, with approximately 95% of those cases being type II diabetes according to the American Diabetes Association (ADA). This number is ever-growing and has a wide range of impact on society. Type II diabetes is marked by the body developing a resistance to insulin. Type II diabetes is not only itself a serious health issue, but it is also heavily linked with the prevalence of Alzheimer's disease (AD), the 6th leading causes of death in the country (Alzheimer's Association).

Alzheimer's is a neurological disease which as of now has no cure, and no actual diagnostic test. It causes changes in the personality of patients, increased anxiety, poor decision making and memory loss. The only way to get a positive diagnostic for AD is currently the post mortem examination of the brain, looking for the presence of Beta-Amyloid oligomers. Beta-amyloid, (A-Beta) occurs in the brain in three different forms and is currently used to determine physiologically the occurrence of Alzheimer's disease in patient. The presence of A-Beta in itself is not harmful, it is the A-Beta which has formed into oligomers which have been found to be problematic. A- beta is linked to insulin directly in that they are both removed and degraded by Insulin-Degrading Enzyme (IDE). It was long thought that deficit in IDE function in degrading A-beta caused the formation of oligomers leading to AD. However, further research seems to indicate that the regulation of IDE by the peroxisome proliferator-activated receptor γ (PPARγ) is down-regulated in mice with type II diabetes and AD (Li et al., 2017).

Insulin regulates blood glucose peripherally via translocation of GluT4 to the plasma membrane of fat and muscle cells. This process occurs via six specific steps, and the rate at

which insulin affects the translocation of Glut4 to the membrane depends on several variables like the type of cell and the amount of glucose present. We see that in ideal conditions and on the right type of cell, insulin can cause a 40 fold increase in the amount of Glut4 receptors on the cell surface (Brewer, Habtemichael, Romenskaia, Mastick, & Coster, 2014). Similar pathways appear to exist within the hippocampus and other insulin-sensitive brain regions and defects within this process are seen in insulin resistance and type II diabetes. Insulin regulates memory processing in the hippocampus via activation of PI3K through phosphorylation of Akt (McNay et al., 2010). Recruitment of PI3K by insulin in the hippocampus also showed production of long term depression (LTD) and alter the frequency of response of both LTD and LTP (McNay, 2007). Insulin has also been shown to modulate neurotransmission by altering the tyrosine phosphorylation of NR2A and NR2B subunits of the NMDA receptors via actions of its downstream pathways (Christie, Wenthold, & Monaghan, 1999). Growth factors src and fyn can cause phosphorylation of the NR2A subunit, but not the NR2B, and the brain-derived neurotrophic factor (BDNF) has the opposite effect of phosphorylating the NR2B subunit, and not the NR2A subunit. Hence, insulin is special because of its ability to act on a broader spectrum than most other growth factors, in this case both the NR2A and the NR2B receptors.

Insulin exists within the body as a hexamer composed of three sets of insulin dimers bound to zinc. Biologically proinsulin is packaged in vesicles filled with Zn^{2+} and Ca^{2+}, which perform vital roles in the stabilization and proper folding and assembly of proinsulin and insulin. (Dunn, 2005). This begins the formation of two insulin dimers which bind with two zinc ions producing the $(Zn^{2+})_2(In)_4$ tetramer; which then combines to the final dimer of insulin for the stabilized final insulin hexamer product (Coffman & Dunn, 1988). Zinc and calcium bonding become crucial after the tetramer formation, as there are incomplete binding site for both

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molecules with two His B10 side chains in the zinc location and four GlouB13 side chains at the calcium sites (Dunn, 2005). These incomplete sites destabilize the molecule which is stabilized by the completion of the binding sites when the third and final dimer of insulin attaches. Although it can be presumed that calcium is just as important in the functioning of insulin as zinc through their stabilizing actions, past work by our lab observing the effects of insulin on cognition has used Humulin, an artificial formulation of insulin with no calcium in its formation, leading us to investigate the effects of zinc on cognition due to its presence. Administering Humulin causes an increase in the baseline zinc concentration of the brain tissues surrounding the injection site, unlike what we could expect to see from the metabolic use of insulin. Although insulin *in vivo* is also stores as a hexamer, insulin breaks down into monomers almost immediately after being released from the beta cells of the pancreases and is most likely not part of metabolic insulin function. This increase in the zinc concentration could cause some confounds to interpretation of the effect of insulin administration.

Zinc is known to be an important modulator of metabolic and protein pathways, and a lack of zinc can have severe effects on the body. Mice suffering from a lack of zinc due to an inactive ZnT3 transporter have serious deficits in spatial working memory by reduction of signaling in the Erk1/2 MAPK signaling in hippocampal mossy fibers (Sindreu, Palmiter, & Storm, 2011). Zinc plays a role in the down regulation of neurons by activating Akt, which acts as an inactivator of GSK3β, a protein directly involved in apoptosis (Kyu Min, Eun Lee, & Chul Chung, 2007). The interaction of the zinc complexes was said to be insulin-mimetic, regarding the activation and regulation of the Akt/PI3K pathway (Barthel, Ostrakhovitch, Walter, Kampkötter, & Klotz, 2007), which points to possible confounds between its actions and zinc's.

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Zinc and insulin share common pathways in the mechanisms surrounding the development of β-Amyloid plagues. Aβ plaques are aggregated due to oxidation of iron by the ferroxidase activity of the amyloid precursor protein (APP), which is inhibited by Zn2+. Therefore, it was shown that an abnormal flow of Zn2+ in the brain could lead to development of Aβ plagues and Alzheimer's (Duce et al., 2010). Insulin degrading enzyme (IDE) is a zinc-metalloendopeptidase that degrades both insulin and Amyloid-β, preventing the latter from forming into plaques. Mice with IDE knocked out had impaired Aβ degradation, suggesting that hypofunction of IDE may lead to Alzheimer's disease (Farris et al., 2003).

Studies differentiating the mechanisms of insulin in afterhyperpolarization, were performed specifically differentiating the roles they played by zinc and calcium. Afterhyperpolarization (AHP) is a physiological marker of aging, which was used to investigate effects of intranasal insulin on cognition (Maimaiti et al., 2015). Using a zinc-less form of insulin (Apidra), Maimaiti et al. attempted to differentiate the actions of zinc and insulin on the Ca2+ dependent AHP. They found that insulin caused a decrease in AHP in the CA1 hippocampal field, increasing neuronal conductivity and translocation of AMPA and NMDA receptors amongst others. Their results showed that Humalog (zinc containing insulin) and Apidra had similar effects on AHP; however, the effects on AHP may not necessarily be representative of the whole cognitive process in the brain, and further action is required into the specificities of insulin acting on cognition.

Materials and Methods:

Adult Sprague-Dawley rats, aged 11 weeks old from Charles River Laboratory were used for the experiment and were kept on a 12-hour light/12-hour dark cycle (0700, 1900). Following a 48-hour habituation period to their new environment once they arrived at our facility, they were handled for five minutes daily for one week. They then underwent stereotaxic surgery to implant a microinjection guide cannula into their left hippocampus.

For the surgery, the animals were anesthetized with Isoflurane inhalation, a 5% concentration with oxygen, in a chamber and were maintained under anesthesia at a 2.5% Isoflurane inhalation through the nose cone for the duration of the procedure. Pre-operative weights were taken before the procedure. Aseptic procedures were used throughout the procedure, and the animals' heads were shaved to allow for proper sterilization of the incision site. The subjects were then mounted into the stereotactic device, where the nose cone was adjusted to assure proper delivery of Isoflurane and anesthetic depth was tested with toe pinches every five minutes. The rats were given a 5mg/kg dose of Rimadyl as a preemptive pain control measure, Enroflox to prevent infection, 3mL of saline to keep them hydrated and covered with a sterile surgery towel to keep them warm and form a sterile field for the procedure.

The shaved area was then prepped for the incision by a succession of washes with betadine and 70% ethanol, three times each. A midsagittal incision of the scalp was then performed to reveal the underlying skull. The subdermal membranes were broken with rigorous rubbing with sterile cotton swabs. Marcaine was used to control bleeding which allowed for

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visualization of the landmarks, Lambda and Bregma. The height of both of these locations (Z) were calculated to assure that the head was flat before moving on with the procedure.

The hippocampus was located from bregma, by moving the stereotaxis +4.6mm in the X direction, and -5.6mm in the Y direction. A hand-held drill was then used to make a hole through the skull at this location, as well as two other smaller holes above and to the right of the probe hole; into which two screws were inserted for the purpose of anchoring down the cap after the procedure. From the initial hole at skull level, the probe was lowered into the brain -3.3mm in the Z direction as per previous work done in this lab. A cap of dental cement was then applied to the skull, inside the animals' incision to close up the wound. Post-surgery the animals were put into a heated recovery chamber until the were mobile and had proper gait. They were then returned their new cage, individually housed to prevent removal of the cap by a cage mate.

Five mg tablets of Rimadyl, and Enroflox were given once a day for three days post-surgery as pain control and to continue the antibiotic treatment. Animals were also handled five minutes per day each for a full week before the beginning of the behavioral testing.

Behavioral testing began with microinjections, the animals were preemptively separated into four treatment groups (n=10). The first group received Humulin, a common pharmaceutical formulation of insulin, at a concentration of 1 unit/mL. The second group received Apidra, a fast-acting insulin formulation stored in monomers, also at a concentration of 1 unit/mL. The third group received $ZnCl_2$ dissolved in aECF, Zn at 0.015mg/mL and the final group was the control, which received aECF.

0.5 µL of the drug was administered over a 4-minute period at constant flow, and the probe remained in the animal for an additional two minutes to allow for the complete administration of the drug via diffusion. Immediately post completion of microinjection, animals

were placed into a plus maze for twenty minutes of spontaneous alternation testing. Spontaneous alternation counts the number of times and order in which the subject enters each arm of the maze. A successful alternation requires the animals to enter each of the four arms for every five total arm entries. An entry and exit from an arm was considered successful when the entire body of the animal, excluding the tail went through the threshold of the arm. A percentage of successful entries was compared to the total entries, and groups were then compared to each other, and to the baseline levels of successful alternations.

Upon completion the animals were immediately taken to the novel object recognition test; where they were placed into an open field with two identical objects in the center and allowed to roam freely for ten minutes. The test measured the amount of time the animal spent examining each object individually over the ten-minute period. They were then returned to their home cages for 24 hours after which the test was performed again, with one of the objects having been replaced. Their movements on both days were recorded using a camera perched directly above the field, and the amount of time they spent investigating each object was later analyzed from the video.

Directly following the completion of the second day novel object testing the animals were euthanized by overdose of Isoflurane inhalation followed by decapitation. Trunk blood was immediately collected and spun down for five minutes at 11,000 rpm to obtain plasma. The brain was removed from the skull and placed in ice-cold 1x PBS (phosphate buffered saline) for five minutes in an attempt to stop protein activity. After the five minutes the brain was dissected, and the left and right hippocampus were removed along with the pre-frontal cortex. These were homogenized on site in a cocktail of homo buffer with protease and phosphatase inhibitors.

Behavioral data from the spontaneous alternation test was input into excel and a percent of successful alternations for each animal was found. These percent scores were then input into SPSS and run through an Anova test. For the novel object recognition, videos of the animals' movements were coded using Solomon coder, the data was then exported to excel for reorganization before being input into SPSS and run as an Anova test.

Protein analysis of the samples will be done using Western blots to test the presence of Glut4, (p)AKT, and (p)IRS. Plasma membrane extractions were performed on the homogenized brain samples which will be followed by BCA analysis before Western Blots. Plasma membrane and total samples of the brains were needed to allow for the quantification of translocated Glut4.

Results:

While analyzing the behavioral data for both spontaneous alternation and novel object recognition, we failed to find any difference between the four treatment groups. The n for each group was 9,9,7 and 9 for the aECF, zinc, Humulin and Apidra respectively. The results for spontaneous alternation (figure 1) and novel object recognition (figure 2) show a fairly uniform performance along all groups. In figure 1 we can see that the zinc and Humulin groups performed slightly better than the control or Apidra group, and all groups performed better than chance level, as expected. For the novel object testing, we saw that the aECF, zinc and Humulin each spent about 50% of their total time exploring on the novel object. The Apidra group spent more time exploring the familiar object than the novel object, contrary to what would have been expected.
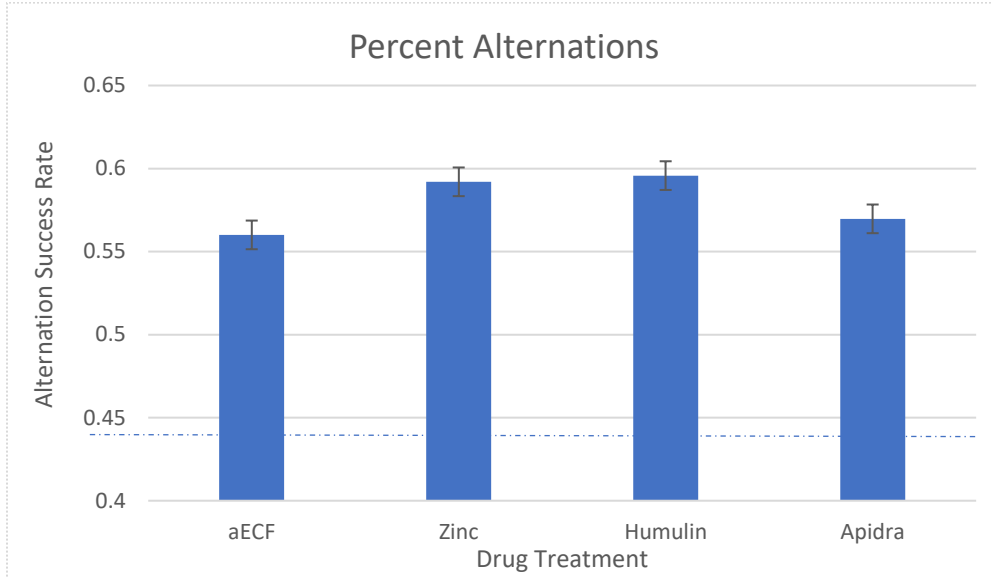
Figure 1: Percent of successful alternations for each group was displayed. We see that Humulin and zinc have a slightly elevated performance over the Apidra and aECF. Although there is a slight increase, it is not statistically significant. Also displayed on the graph is a dotted line at 0.44, which represent chance level of a successful alternation.
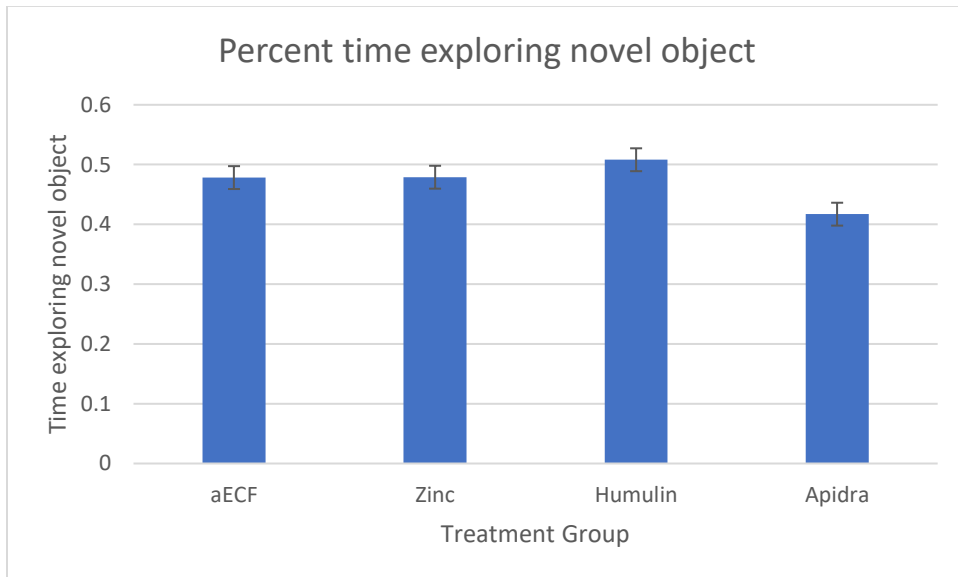

Figure 2: Representation of the time spent exploring the novel object as percent of the time spent exploring

Discussion:

Insulin has long been recognized for its roles past metabolism, as a key regulator of function in the brain, and more recently as a modulator in memory and cognition. Several past works by this lab and others have shown that animals treated with insulin have an increase in cognition, especially memory, in an inverted U curved manor. The U curve represents the effectiveness of the dosage, having little to no effect at high and low dosages while peaking at a middle concentration. This increase is not seen from the data collected from this experiment. No significant effects were noticed in any of the treatment groups. There are several potential reasons as to why this could have happened. It is possible that the microinjection procedure was not working properly, and therefore did not deliver the full amount of the drug. It could also be argued that if not enough, or too much of the drugs were delivered, the animals did not receive the optimal levels which would have increased cognitive power, laying instead on the far ends of the plot.

Looking specifically at the novel object recognition data, we find that the Apidra group, overall had the worse performance. Even though the data is not statistically significant, we can see that there was a general trend. A probable explanation is that Apidra is a fast-acting formulation of insulin. The stabilized monomers allow it to have a faster onset of action, but do not make it necessarily work longer. Therefore, it is realistic to think that due to the length of the spontaneous alternation test, the effects of Apidra wore off during novel object training due to a decrease in dosage. This could be further supported by the fact that data from spontaneous alternation is more level, indicating an action from the Apidra group not seen during novel

object. Future protein analysis will allow to quantify the presence of proteins and give us more insight as to the brain activation caused by the Apidra.

No matter where the error occurred, there is enough evidence in literature alluding to a relation between the actions of insulin and zinc to warrant further investigation. If the experiment were to be revamped and repeated, I believe that we will indeed notice that zinc contributes to the mechanisms associated with insulin, namely the proteins downstream of Akt. In redesigning the experiment, it will be important to keep in mind the rapidity of action Apidra shows, and plan accordingly. Ideally, a second microinjection would be given to the animals, possibly before the day two of novel object. This could allow us to attempt to measure recall of the memory.

Due to the formulation of the insulin hexamer with the zinc molecule, there is always a zinc molecule present where insulin is present. It was also shown in the Maimaiti paper on AHPs that there was some correlation between the two. Zinc has a significant role on the modulation of Akt without the presence of insulin, and it would seem to be there with insulin present as well. If this were to be true, the way we see insulin and what behaviors it is a part of could be completely changed. It would warrant more in-depth research into clearly identifying pathways were potential overlap could occur. Once the pathways are more clearly identified, this could lead to some medical advances in the treatment of diabetes, and maybe even in the treatment of Alzheimer's disease through a more thorough understanding of its functionality and mechanisms.

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