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Original Article

Intranasal Insulin Improves Age-Related Cognitive Deficits and Reverses Electrophysiological Correlates of Brain Aging

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Abstract

Peripheral insulin resistance is a key component of metabolic syndrome associated with obesity, dyslipidemia, hypertension, and type 2 diabetes. While the impact of insulin resistance is well recognized in the periphery, it is also becoming apparent in the brain. Recent studies suggest that insulin resistance may be a factor in brain aging and Alzheimer's disease (AD) whereby intranasal insulin therapy, which delivers insulin to the brain, improves cognition and memory in AD patients. Here, we tested a clinically relevant delivery method to determine the impact of two forms of insulin, short-acting insulin lispro (Humalog) or long-acting insulin detemir (Levemir), on cognitive functions in aged F344 rats. We also explored insulin effects on the Ca^{2+}-dependent hippocampal afterhyperpolarization (AHP), a well-characterized neurophysiological marker of aging which is increased in the aged, memory impaired animal. Low-dose intranasal insulin improved memory recall in aged animals such that their performance was similar to that seen in younger animals. Further, because ex vivo insulin also reduced the AHP, our results suggest that the AHP may be a novel cellular target of insulin in the brain, and improved cognitive performance following intranasal insulin therapy may be the result of insulin actions on the AHP.

Key Words: Calcium—Type 2 diabetes—Neuronal excitability—Memory—Afterhyperpolarization

Results from preclinical settings argue that peripheral insulin resistance, a component of type 2 diabetes and/or the metabolic syndrome, may have a negative impact on cognitive function. Emerging studies report that in diabetic models, insulin responses are attenuated in the brain, possibly indicating the presence of insulin resistance in this tissue. Clinical and preclinical studies show that insulin and insulin-like growth factor-1 (IGF-1) receptors, their message and their function, are often reduced in the hippocampus during normal and pathological aging ([1–6]). Similarly, in animal models of aging and Alzheimer's disease (AD), brain insulin receptor signaling is reduced, often in the presence of peripheral metabolic dysregulation ([7–14]). Importantly, several studies provide evidence that brain insulin therapy can greatly reduce cognitive decline with age and/or AD, and even improve memory in young adults [reviewed in ref. ([15])].

In recent studies examining the effects of insulin on cognition, insulin has been administered via the intranasal route to bypass the blood brain barrier. Notably, Born and colleagues ([16]) have shown that intranasal insulin is capable of entering the human brain rapidly. However, the underlying mechanism(s) leading to cognitive improvement are not yet clear. Potential molecular/cellular targets affected by insulin in the brain and capable of offsetting cognitive decline include changes in neuronal firing ([17]), Ca^{2+}-dependent K^+ channels ([18]), GABA currents ([19]) and tau and $A\beta$ metabolism ([20–22]). Insulin also alters synaptic structures ([23,24]), modifies synaptic plasticity ([25–27]) and increases delivery of NMDA receptors to the plasma membrane ([28]). Similar effects have been observed in response to IGF-1 replacement, showing increased AMPA-mediated excitatory postsynaptic potentials ([29,30]), and increased NMDA receptor trafficking ([31]), effects that appear capable of offsetting

age-dependent cognitive decline (32). Further, MRI data in men and women has provided evidence that intranasal insulin can have an impact on brain blood flow (33), and on whole-body insulin sensitivity via regulation of hypothalamic activity (34,35). Whether this later effect is purely mediated by central insulin effects or reflects permeation of small insulin amounts into the bloodstream is currently being investigated (36–38).

Another important candidate mechanism that may underlie the effects of intranasal insulin pertains to Ca^{2+} homeostasis. Ca^{2+} dysregulation is prominent in models of diabetes (39). In the dorsal root ganglion and the hippocampus of animals with STZ-induced diabetes (40,41), broader Ca^{2+} action potentials, larger Ca^{2+}-dependent afterhyperpolarizations (AHPs), and aberrant intracellular Ca^{2+} release are seen (40,42,43). Impaired memory and long-term potentiation maintenance is also present (44). Clinical data also supports a role for Ca^{2+} dysregulation in peripheral insulin resistance as evidenced by the presence of abnormal and sustained adipocyte Ca^{2+} elevations in hyperinsulinemic, obese subjects (45). Similar evidence for an increase in Ca^{2+} levels in skin fibroblasts from type 2 diabetics has also been reported (46). In recent work, we have shown that the Ca^{2+}-dependent AHP, which increases in amplitude and duration with aging (47–49), is sensitive to insulin (ex vivo), and that this sensitivity is reduced by a high fat diet (50).

While some studies in humans demonstrate the efficacy of intranasal insulin as a cognitive enhancer, studies in animal models are required to understand the potential mechanisms underlying the beneficial effects of intranasal insulin. However, only one prior study has measured the impact of intranasal insulin on brain function in the awake animal, focusing on olfactory discrimination in the young mouse (51). Because the greatest risk factor for AD is aging, research into how central nervous system insulin signaling affects brain aging may improve understanding of the biological conditions. Further, because of the sustained growth in the aging population, implementing strategies to reduce the impact of brain aging will likely benefit the aging population, and by extension, may reduce the incidence of age-related neurodegenerative diseases that share cellular mechanisms with brain aging (eg, AD).

We, therefore, assessed learning and memory performance in aged rats in response to two different insulin formulations (short-acting insulin Humalog and long-acting insulin Levemir), and quantified Ca^{2+} homeostasis. Insulin is available primarily in shorter- and longer-acting forms, and intranasal longer-acting insulin formulations (eg, Levemir) may potentially be superior to shorter-acting ones (eg, Humalog) especially in APOE-ε4 carriers (52). In addition, because insulin is often formulated with zinc, to more specifically address the impact of insulin, we studied a zinc-free insulin formulation (Apidra), as well as zinc alone on the AHP. Overall, our goals were to test whether intranasal insulin could reduce cognitive decline in aged animals, as well as redress the accompanying neuronal Ca^{2+} dysregulation seen in the hippocampus of aged animals.

Methods

Animals

Young male F344 (3 months old), aged male F344 (21 months old), and young male Sprague-Dawley rats (2–6 months old) were used for this study. Young F344 animals were not treated with insulin, and were used to help gauge the impact of intranasal insulin on memory function in aged F344. All animals were fed an 18% protein rodent diet (Harlan Teklad, diet #2018; Madison, WI).

In Vivo Insulin Delivery and Doses

Levemir doses were approximately 0.0715, 0.143, or 0.286 IU/day/rat delivered in two 5 µL applications applied to the tip of the right naris using a P10 Eppendorf pipette (Supplementary Figure 1) [adapted from (53)]. Humalog dose was chosen to deliver 0.0715 IU/day/rat for behavioral characterization. An intranasal 0.286 IU/rat dose was also administered once to aged animals (n = 3) to monitor the impact on peripheral glucose (Supplementary Table 1). Each application was separated by 1 minute, during which the animal was held supine and immobile in a DecapiCone (Braintree Scientific Inc., Braintree, MA). Each animal was acclimated to this procedure for 4 days prior to initiation of the Morris water maze protocol. All animal groups were exposed to the DecapiCone for the same duration, and no signs of stress from the bagging procedure were noted. The insulin doses were chosen to approximate several clinically relevant doses used in past studies ranging from 10 to 40 IU/day and were adjusted by brain weights. Assuming a 70-year old human brain weighs 1,360 g (54), 10, 20, and 40 IU/day are equivalent to 0.0074, 0.0147, and 0.0294 IU/day/g of brain, respectively. Assuming a 21-month old F344 brain weighs 2.06 g (55), our equivalent doses were 0.035, 0.069, and 0.138 IU/day/g of brain. It should be noted that these values may underestimate final insulin concentration given the greater olfactory epithelium surface in the nasal passages of the rat (50%), compared to man [8% (56)]. Insulin solutions were made fresh weekly from 100 U/mL vials (Humalog, insulin lispro, Lilly; Levemir, insulin detemir, Novo Nordisk) and diluted into sterile 0.9% saline.

Blood Glucose Levels

To ensure that the intranasal delivery did not result in reduced peripheral glucose levels, and to test whether stress from the acute restraint procedures (DecapiCone) could elevate blood glucose levels, we measured blood glucose (FreeStyle Lite glucometer; Abbott Laboratories, Abbott Park, IL) from dorsal tail veins in a subset of aged animals either exposed to intranasal Levemir (n = 5 from each treatment group) or Humalog (n = 3), both before and approximately 1 hour after dosing (Supplementary Table 1).

Animal Behavior, Treatment Groups and Morris Water Maze Procedures

Two cohorts of F344 animals were used for behavioral characterization (Supplementary Figure 1B). One cohort of animals (n = 50) was used to test the impact of long-acting insulin Levemir at three different doses (low, medium, and high; n = 10 per group) compared with an intranasal saline young group (3 months; n = 10) and an intranasal saline aged group (21 months old; n = 10). A second cohort of aged animals (n = 20) was used to test the impact of short-acting insulin Humalog against saline (n = 10 per group). For analysis, behavioral data from the two aged saline control groups in both cohorts were combined because memory performance on probe day was similar (path length to goal, p = .89 and cumulative search error, p = .913). One aged animal in the saline group died during acclimation prior to initiation of behavioral testing. The remaining 69 animals where trained on the Morris water maze (MWM). Animals were considered visually impaired or unable to learn the task if they could not find the platform on all three of the trials on the cue day (visual platform) and at least one of the remaining three training days. Based on these conditions, two aged Humalog animals and three aged low-dose Levemir animals were removed from the analysis. Behavioral results are therefore presented on 64 animals,

10 young saline, 19 aged saline, 8 aged low-dose humalog (10 IU/day), 7 aged low-dose Levemir (10 IU/day), 10 aged medium-dose Levemir (20 IU/day), and 10 aged high-dose Levemir (40 IU/day). On cue day, no significant differences were found across any of the groups on measures of path length (one-way ANOVA, $p > .05$) and swim speed across insulin dose (one-way ANOVA, post hoc $p > .05$), indicating motivation and motor activity were likely similar.

Insulin or saline treatment in both cohorts was initiated 4 days prior to the initiation of training (Supplementary Figure 1B) and was delivered 1–3 hours before testing on the MWM. The MWM protocol has been published elsewhere (50,57), but briefly, water temperature was maintained at 26°C –27°C, and black liquid tempera paint was used to make the water opaque and hide the submerged escape platform (~1.5 cm below water surface). A Videomex-V acquisition system and water maze analysis software (version 4.64, Columbus Instruments, Columbus, OH) were used to track and measure animal movements. Animals were allowed 60 seconds to find the platform, after which they were guided to it. Each animal stayed on the platform for 30 seconds before returning to a heated holding chamber for about 2 minutes. On cue day, a white cup hanging above the partially submerged platform helped orient the animals. Animals were subjected to three trials per day (semi-random drop location for each trial) for cue and training days. Twenty-four hours after the last training day, a probe trial was initiated with the platform removed (60 seconds max swim time). Swim speed was derived from the distance travelled over time on the last trial of the third training day and on the first trial of the cue day. On all cue and training days the intertrial interval was approximately 150 seconds. All behavioral experiments were conducted with the experimenters blind to the treatment groups using color coding of the insulin and saline vials.

Electrophysiology

Hippocampal slices were used across two electrophysiological setups, one monitoring extracellular field potentials and, the other measuring intracellular potentials (eg, AHP). All data were acquired using pClamp 8.0 (Molecular Devices MDS, Toronto, Canada) through a Digidata 1320A A/D converter (Molecular Devices). Potentials (eg, amplitude and duration of AHPs, field EPSPs) were quantified offline using Clampfit (Molecular Devices). Nineteen of the 20 behaviorally characterized aged animals (second cohort used to test the impact of short-acting insulin Humalog against saline) were used 2–5 weeks after the last day of behavioral characterization to determine the impact of single intranasal insulin dose in vivo. From those, a total of 30 slices used for extracellular field potential recordings (Figure 2D and E) and 13 cells used for intracellular recordings (Figure 2B and C) were used for statistical analyses and are presented using an *n* of 1 per cell/slice. In data presented in Figure 3, 16 Sprague-Dawley animals (2–6 months old) were used to monitor the impact of zinc and Humalog ex vivo both extracellularly and intracellularly (30 slices and 23 cells, respectively). Work presented in Figure 4 (intracellular physiology only) is compiled from a total of 17 F344 male rats split into young (*n* = 8; 3 months) and aged (*n* = 9; 22 months). This last group of animals yielded recordings from six cells for young artificial cerebro-spinal fluid (ACSF), eight cells from young Apidra, six cells for aged ACSF, and five cells for aged Apidra.

Field potentials

Rats were anesthetized with Isoflurane prior to decapitation. Brains were quickly removed and placed in oxygenated ACSF of the following composition (in mM): 114 NaCl, 3 KCl, 10 glucose, 1.25

KH_2PO_4, 26 $NaHCO_3$, 8 $MgCl_2$, and 0.1 $CaCl_2$. Hippocampi were sectioned (coronal) with a Vibratome 3000 (Bannockburn, IL) set at 400 µm. Slices were transferred to an interface-type recording chamber (static) containing oxygenated recording ACSF of the following composition (in mM): 114 NaCl, 3 KCl, 10 glucose, 1.25 KH_2PO_4, 26 $NaHCO_3$, 2.5 $CaCl_2$, and 1.3 $MgCl_2$. The recording chamber was kept at 32°C and warm moist 95% O_2 and 5% CO_2 was continually delivered to the chamber. Bipolar synaptic stimulation was delivered through a SD9K stimulator (Astro Med Inc., Grass Instr., Warwick, RI). Input/output curves (I/O) were recorded using a series of increasing voltage steps. The relationship between stimulus intensity and EPSP amplitudes (prior to dendritic spike contamination) was fit (linear, $r^2 > .9$) to yield a measure on the slope of the I/O. Data are also reported as the absolute amplitudes of the EPSPs measured as the maximum deflection from baseline. Insulin (1, 100, and 500 nM Humalog) and/or zinc treatment (130 nM—see AHP-Intracellular Recordings section) under these conditions was initiated 1 hour after placing the slices in the chamber (compounds remained in the chamber for the duration of the experiment.

AHP-Intracellular Recordings

Hippocampal slices (350 µm) were transferred from the static chamber and placed in a perfusion chamber (RC22C, Warner Instruments, Co., Hamden, CT) and maintained in a continuous flow of oxygenated ACSF preheated at 32°C using a TC2Bip/HPRE2 in-line heating system (Cell Micro Controls, Northfolk, VA). The ACSF contained 2 mM $CaCl_2$ and 2 mM $MgCl_2$ and in some experiments (Figure 4 only), the traditional 10 mM glucose concentration was replaced with 4.5 mM glucose. This was done to test whether higher glucose concentrations altered insulin sensitivity. We chose 4.5 mM to help maintain tissue health and physiology for extended hours (58), to approximate values in anesthetized preparations (59), and to consider fluctuations in brain glucose levels across a day (60). As previously described, cells were impaled with sharp microelectrodes filled with 2M $KMeSO_4$ and 10 mM HEPES, pH 7.4 [tip resistance 90–120 MΩ (47)]. Recordings of membrane input resistance (IR) were obtained in response to 800 ms, 200 pA hyperpolarizing current injections using an Axoclamp 2B amplifier (Molecular Devices) while holding the cell at −70 mV. To generate an AHP cells were held at −65 mV (baseline) and depolarized with a 100 ms current injection to generate four Na^+ action potentials. The medium AHP (mAHP) was measured as the peak hyperpolarization immediately after the offset of the depolarizing current injection, the slow AHP (sAHP) was measured 800 ms after the end of the current injection. The AHP duration was measured from the end of the depolarizing step until return to baseline.

Several concentrations and formulations of insulin were tested ex vivo by addition to the solution bathing the hippocampal slices. Humalog concentrations of 1, 100, and 500 nM were used. A zinc ($ZnCl_2$) concentration of 130 nM was used to mirror levels of free zinc attained in 250 nM Humalog. Apidra (insulin glulisine, Sanofi, a zinc-free insulin formulation) was used at 1 nM and results were compared to ACSF. Under these experiments, Apidra was used to treat slices both in the static chamber and during recording of the AHP. Apidra was continuously perfused into the flow of the oxygenated recording ACSF using a precision perfusion pump (Orion Research, Model 341B, Boston, MA) and resulting in a final concentration of 1 nM (diluted in sterile saline once a week from the 600 µM stock). Only neurons with IR > 25 MΩ, holding current < 500 pA and action potentials reaching above zero mV were included in the analysis.

Statistics

Statistical analyses testing for significance ($p < .05$) of main factors and interactions used a one-way or a two-way ANOVA as well as a *t*-test design (some electrophysiology). Fisher's PLSD was used for post hoc comparisons. All tests were conducted using StatView statistical package (version 5, Cary, NC) or GraphPad Prism V5 (San Diego, CA). Together the behavioral and electrophysiological data presented here are derived from a total of 106 animals.

Results

Blood Analyses

Several studies show that intranasal insulin either has no effect, or a moderate lowering effect (within the euglycemic range) on peripheral blood glucose (36–38,53,61–63). A two-way RM ANOVA shows intranasal insulin did not alter peripheral blood glucose levels in a subset of the aged animals ($n = 5$/group) 30–90 minutes after intranasal insulin Levemir delivery (Supplementary Table 1). In another group of aged animals, 40 IU Humalog also was unable to alter blood glucose levels. This result is consistent with transport of insulin to the perivascular space around blood vessels, indicating the hormone may not have crossed into the blood stream (56,64).

Behavioral Analyses

All groups learned to find the platform in the MWM as demonstrated by a significant decrease in path length to goal across the first 3 days of training [$F(2,116) = 27.2$; $p < .0001$]. During this learning phase, path length (Figure 1A) however, showed no significant differences across groups with age or intranasal insulin dose (two-way RM ANOVA). As shown previously (57,65), analysis of swim speed revealed a significant group effect [$F(5,58) = 12.5$; $p < .0001$] with post hoc tests indicating aged animals swam at slower speeds compared to young animals, but no difference between the insulin treatment groups was noted (Figure 1D). Latency to target, as predicted from slower speeds in aged animals, was significant across 3 days of training [$F(2,116) = 28.9$; $p < .0001$], with increased latency seen in all aged animals compared with young saline animals (data not shown). Because of this decrease in swim speed we chose to assess search errors and quantify memory performance on the probe day (no platform) using a proximity measure analysis. This approach is less dependent on swim speed than latency and is tabulated using a cumulative search error derived from the subject's distance to the platform summed over the time to reach the platform and is then subtracted from an ideal path (straight line); smaller values are indicative of better performance (66).

Compared to the learning component of the task, the 24-hour memory recall component measured during the probe trial (one-way ANOVA) revealed a significant group effect on path length to goal [$F(5,58) = 2.9$; $p < .05$) and on cumulative search error [$F(5,58) = 3.3$; $p < .05$). Post hoc analyses identified a significant increase on path length and cumulative search error in the aged saline ($p < .05$) and aged medium-dose Levemir ($p < .005$) groups compared with the young saline group (Figure 1B and C). A trend ($p = .059$) for improved performance was found for the high-dose Levemir animals on path length measures only. Notably, no significant differences were found between young saline treated and aged animals treated with either low-dose Humalog or low-dose Levemir on path length or cumulative proximal distance (Figure 1B and C), indicating that the lower dose intranasal insulin significantly reduced age-dependent memory loss. Additional post hoc analyses
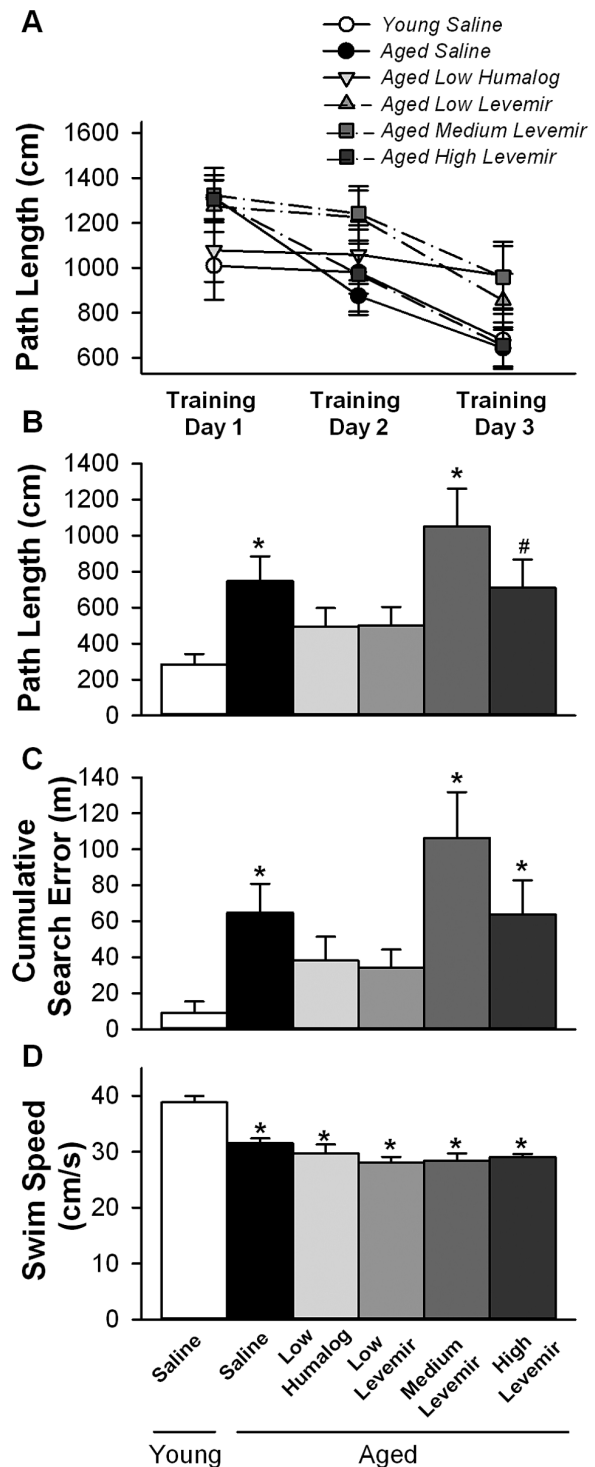
Figure 1. Morris water maze spatial learning and memory task. (**A**) Path length to submerged goal platform on the initial 3 days of training showed improved performance across all groups tested (RM ANOVA $p < .0001$), however, no group effect was seen. On probe day, with platform removed, animals receiving low doses of insulin (Humalog or Levemir) showed reduced path lengths to goal (**B**) and improved memory performance based on reductions in cumulative search error (**C**). Analysis of swim speed measured on the third training day shows aged F344 animals are slower [note no effect of insulin dose on speed (**D**)]. Mean ± SEM are shown. Asterisks indicate significant differences from the young saline group at $p < .05$; pound sign indicates a trend ($p = .059$) in comparison to the young saline group.

on memory performance in aged animals, comparing combined low dose insulins (Humalog and Levemir) with combined medium- and high-dose Levemir, revealed significant improvement in the low-dose insulin group on path length [$F(1,33) = 5.25$; $p < .05$] and cumulative search error [$F(1,33) = 5.91$; $p < .05$]. Thus, each of the different insulin formulations (Humalog and Levemir at low-doses) improved performance despite being administered to two separate cohorts of aged animals, indicating reproducibility of the insulin effect, and it appears lower, rather than higher doses of insulin are able to redress cognitive decline with aging.

Electrophysiology Analyses

Based on improved memory recall observed in aged animals treated with low-dose insulins, we next characterized the effects of in vivo intranasal insulin on hippocampal electrophysiology in a subset of the Humalog treated animals that were behaviorally characterized. Supplementary Table 2 shows that passive membrane properties were not different between groups and suggests cells with comparable baseline characteristics were analyzed. Intracellular recording of CA1 pyramidal neurons were obtained from animals treated with intranasal saline or Humalog (see Methods section). Approximately 3–7 hours following intranasal insulin delivery the Ca^{2+}-dependent AHP was not significantly different between groups (Figure 2B and C; *t*-test). This was true for measures of AHP amplitude (medium and slow AHP) and duration (not shown). Extracellular synaptic potentials also showed no significant difference across groups (Figure 2D and E; *t*-test). EPSP amplitudes measured at 33% of the I/O were slightly larger in the Humalog treated animals and I/O slopes based on EPSP amplitude measures were somewhat reduced (Figure 2D and E, respectively, $p < .1$). These results are surprising given our prior report that insulin application to hippocampal slices reduces the AHP (50). In the prior study, however, hippocampal slices were perfused for 15 minutes with insulin and then studied electrophysiologically. The results obtained in the present study raise the possibility that the delay between intranasal insulin exposure and electrophysiological recording (3–7 hours) was too long and that the effect of insulin on the AHP may have dissipated. Thus, to confirm that insulin must be present in order to alter hippocampal physiology, we tested a range of ex vivo insulin concentrations and formulations on hippocampal slices from a separate group of animals.

We used young animals in the next series of experiments, and examined three different doses of Humalog, comparing Humalog's actions to zinc and ACSF. In most insulin formulations, zinc is used to promote hexamer formation and prolong stability and duration of action in vivo. However, zinc is also known to modify ion flux across neuronal membranes, reducing activity at NMDA and $GABA_A$ receptors, ion channels associated with memory-related processes [reviewed in (67)]. Extracellular recordings in the presence of zinc or Humalog showed that neither EPSP amplitudes, nor I/O slopes were significantly altered when compared to ACSF controls (Figure 3E and F; one-way ANOVA). Similarly to data shown in Figure 3, these results indicate little if any, effects of insulin on extracellular postsynaptic potentials.

Intracellular recordings obtained from the same group of animals showed that the Ca^{2+}-dependent AHP was sensitive to treatment with zinc or insulin. As previously reported, measures of postsynaptic excitability demonstrated the sensitivity of the AHP to both zinc (68) and insulin (50). A main effect of treatment on the slow
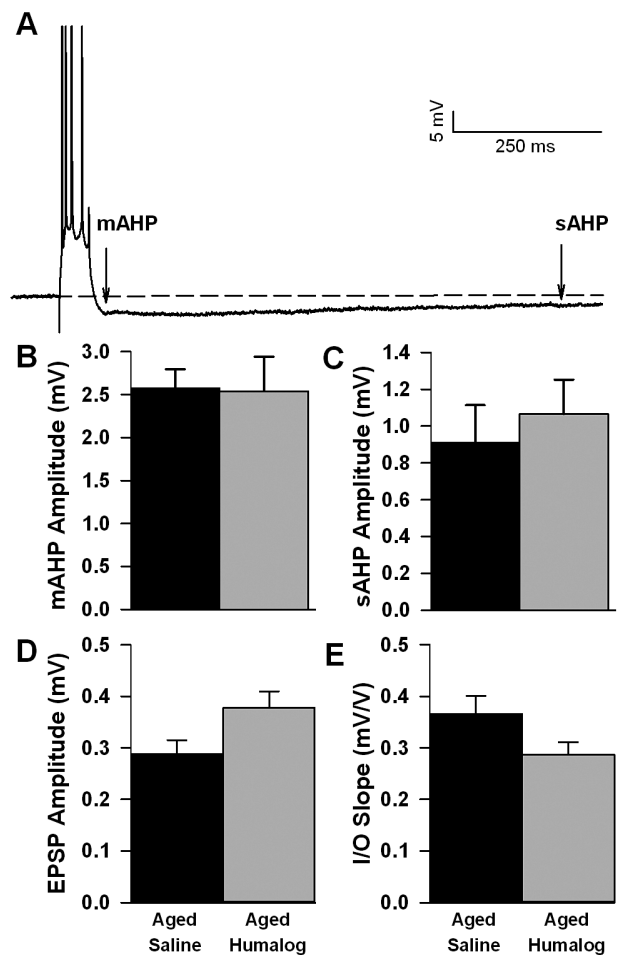
Figure 2. Impact of in vivo intranasal Humalog on intracellular and extracellular physiology. (A) Representative intracellular recording of the afterhyperpolarization (AHP) following a series of four action potentials (truncated to emphasize the AHP). The medium AHP (B) and slow AHP (C) amplitudes recorded 3–7 hours after intranasal Humalog in F344 rats were not significantly different. EPSP amplitudes (D) and I/O slopes (E) when recorded extracellularly 3–7 hours after intranasal Humalog also were not significantly altered. Mean ± SEM are shown.

AHP [$F(4,18) = 3.34$; $p < .05$] and its duration [$F(4,18) = 4.49$; $p < .02$] was seen across five groups of recorded cells (Figure 3C and D). Albeit being somewhat reduced, the mAHP was not significantly altered by insulin (Figure 3B). These results suggest a component of the insulin effect on the AHP could well be mediated by zinc found in this insulin formulation. To test the direct impact of ex vivo insulin on the AHP, we next investigated a zinc-free formulation of insulin (Apidra).

In this next series of experiments we obtained hippocampal slices from young ($n = 8$) and aged ($n = 9$) F344 rats and focused exclusively on intracellular potentials and the response to ex vivo Apidra exposure (1 nM). Further, a recent study identified that 0.1–100 nM exogenous insulin concentrations could reliably elicit dose-dependent activation of the insulin signaling pathway in human brain tissues (69), and approximated physiological levels near 1 nM. For these reasons, we used 1 nM Apidra. As previously reported (47–49,70) and shown here (Figure 4), mAHP and sAHP amplitudes recorded in CA1 pyramidal neurons were increased [$F(1,24) = 17.6$, $p < .0005$; $F(1,24) = 4.7$, $p < .05$, respectively] in aged, compared
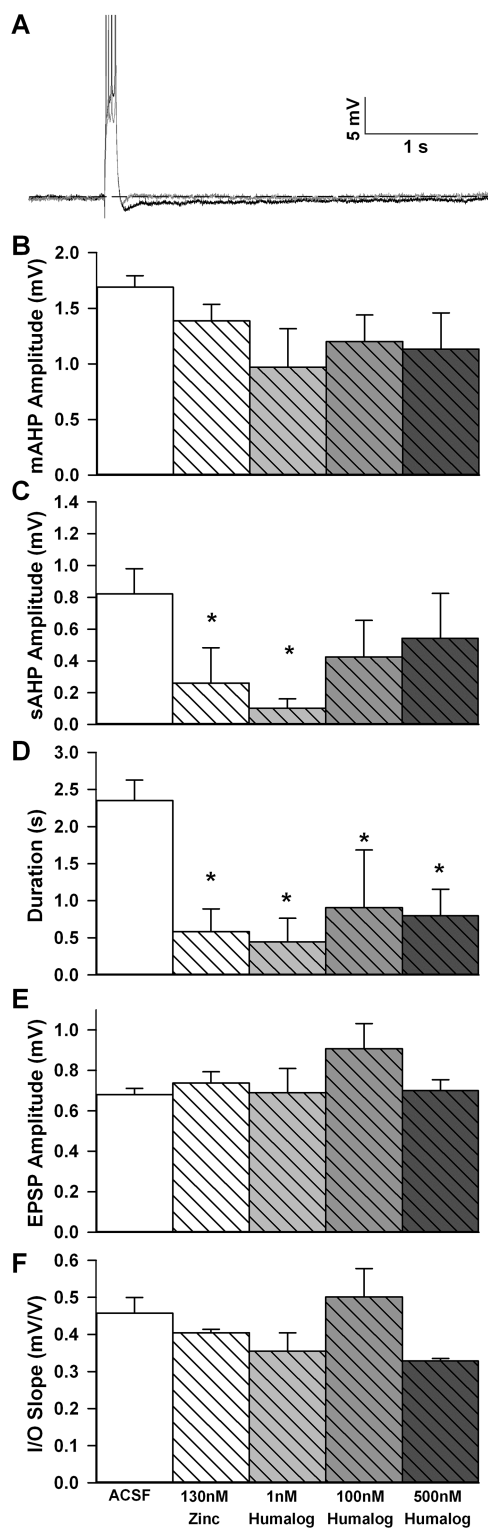
Figure 3. Impact of ex vivo Humalog and zinc on the afterhyperpolarization (AHP). (**A**) Representative voltage trace of the AHP recorded in a cell from an ACSF treated slice (black) and a Humalog treated slice (1 nM, gray). Data are derived from young Sprague-Dawley animals. Traces are truncated to emphasize the AHP. Significant reductions in the slow AHP (sAHP) amplitude (**C**) and duration (**D**) were seen in response to Humalog as well as zinc. Note that zinc and low concentration Humalog seem to work more robustly on the sAHP [only a trend was noted for the medium AHP (**B**)]. No significant effect was detected during extracellular recording of field EPSP amplitudes (**E**) or slopes of the I/O curve (**F**). Mean ± SEM are shown.

to young animals (two-way ANOVA). Treatment with the zinc-free Apidra insulin significantly reduced the AHP in slices from both young and aged rats as indicated by a main effect of treatment on the mAHP [$F(1,24) = 32, p < .0001$] and the sAHP [$F(1,24) = 29.1, p < .0001$]. However, the Apidra-mediated AHP reduction was larger in aged animals compared to young animals (Figure 4A and B) as evidenced by a significant interaction term in the two-way ANOVA [$F(1,24) = 10.9, p < .005$ for the mAHP]. These results are consistent with our prior work using Humalog showing that the hippocampal AHP is sensitive to insulin (50). In the experiments presented here however, it is clear that insulin alone has a direct effect on the AHP, and that this effect persists even when lower, more physiological glucose levels are used in the ACSF (experiments in Figure 4 were conducted with 4.5 rather than the typical 10 mM glucose concentration—see Methods section). Finally, it is interesting to note that Apidra did not alter holding current when compared to control cells (sup. Table 2), indicating insulin is neither activating nor inhibiting a tonic current near resting potential.

Discussion

Studies in humans have shown that intranasal insulin has promising cognition enhancing effects, alleviating, and perhaps even slowing the progression of age-related neurodegenerative disorders. However, the mechanisms underlying insulin's effects in the brain are unclear. In order to identify potential mechanisms, we treated aged rodents with intranasal insulin, attempting to model aspects of the clinical
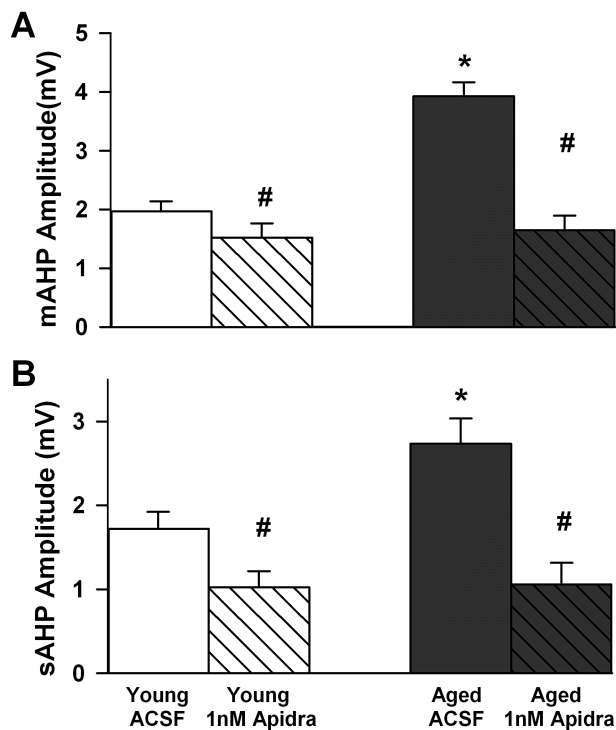
Figure 4. Zinc-free Apidra reduces the afterhyperpolarization (AHP) ex vivo. The medium AHP (mAHP) amplitude is shown in (**A**) and the slow AHP (sAHP) amplitude is shown in (**B**). Mean ± SEM are presented in response to 1 nM Apidra. These data are taken from young and aged F344 rats. Asterisks indicate significant age-dependent increases in the mAHP and sAHP, and pound signs illustrate significant insulin-mediated reductions in the AHP. Again, the impact of insulin seems more robust on the sAHP compared to the mAHP. These recordings were obtained with 4.5 mM glucose in the ACSF.

trials reporting on the beneficial impact of intranasal insulin. Here, we describe physiological actions of insulin in the brain, highlighting a potential new mechanism of action of insulin on hippocampal function in both young and aged rats. This first analysis of intranasal insulin in aged animals demonstrates that intranasal insulin improved memory recall in aged F344 rats. We show that the effect on memory was observed in two separate cohorts of animals, using two different insulin formulations (Humalog and Levemir) delivered at relatively low dose (0.0715 IU/day/rat). Thus, these studies also demonstrate reproducibility of the effect of insulin on behavior. We also show that the Ca^{2+}-dependent AHP, and in particular the slow AHP, a Ca^{2+}-related biomarker of brain aging which increases with age and cognitive decline, is reduced by acute insulin exposure in aged animals and could well represent a novel target of brain insulin underlying the improvement seen in memory recall.

Long-acting insulin (Levemir) was as effective as short-acting insulin (Humalog) on memory recall in aged animals, inducing levels of performance indistinguishable from those seen in young animals. Levemir's long duration of action is dependent on the presence of albumin in the periphery and a fatty acid modification of the insulin structure, increasing its' affinity for albumin. Humalog's shorter duration and faster onset are due to amino acid modifications in the β-chain of the insulin molecule. Because both versions of insulin were able to reverse cognitive decline to a similar degree in aged animals, our results suggest both insulins were able to gain access to the brain following intranasal administration despite structural differences. While results from clinical studies in memory-impaired older adults have reported improved word recall within 15-minute post-intranasal delivery (71), a prior clinical study on younger adults demonstrated the superiority of a faster acting insulin (Novolog) compared to regular insulin (Actrapid) on delayed word recall after 7 weeks of intranasal 160 IU/day Novolog (63). Using shorter delays (ie, 10 minutes) a recent study reveals 40 IU intranasal insulin delivery to young adult males improves odor-cued spatial memory (72). Clearly, the memory enhancing effects of insulin are depend on treatment regimen (acute vs subchronic), but also on the age and gender of the subject (73), as well as on the insulin formulation used (52,74). As mentioned above, a single, acute intranasal dose of insulin is capable of enhancing memory in humans and our electrophysiological studies showing an acute action of insulin on the AHP indicate that insulin can have rapid effects on neuronal processes critical to memory (see later). However, in the current study, it is unclear whether the effects on cognition observed were due to an acute action of insulin (ie, the dose received 1–3 hours prior to memory testing), or the result of the cumulative daily treatments received over the course of 8–11 days.

The decrease in the AHP by insulin was observed in field CA1 of the hippocampus, a synaptic zone which plays a key role in memory processing. Decreasing the AHP in this structure would be expected to increase neuronal excitability and facilitate throughput during physiological activation. Consistent with this, exogenous insulin increases excitatory neurotransmitter receptor trafficking including NMDA and AMPA receptors (25–27,75), thus promoting network activation. Furthermore, pure insulin has been shown to activate an inward current and depolarize hypothalamic neurons via activation of transient receptor potential channels, providing supporting evidence that insulin raises excitability (76). However, recent results provide evidence that insulin or other therapies that raise insulin (exendin-4 and glucagon-like peptide-1), increase functional $GABA_A$ conductances in CA1 (19) as well as in CA3 pyramidal neurons (77). This observation is in line with prior reports of enhanced $GABA_A$

function in response to insulin, a resulting decrease in synaptic activity (17,18) and a long-lasting enhancement of inhibitory postsynaptic currents [IPSCs (78)]. The net result, therefore, of enhancing both steady-state hyperpolarizing (GABA-mediated) and short-term depolarizing forces (eg, reduced AHP or direct depolarization) could well increase synaptic signal-to-noise, thereby improving throughput and neuronal communication. Clearly, as seen in hypothalamic arcuate nucleus neurons, it is important to note that insulin's modulatory influence on membrane potential/excitability, is insulin formulation dependent, as well as cell-type specific (76).

To extend slice health and allow for extended neuronal recordings, high glucose concentrations (10 mM) are commonly used in the ex vivo hippocampal slice. However, this superphysiological glucose concentration may alter insulin sensitivity in the brain. For this reason, we used lower glucose levels in some experiments while maintaining tissue health (Table 2), and more closely approximating physiological glucose levels. Importantly, significant AHP reduction was seen irrespective of recording conditions (10 or 4.5 mM glucose). Thus, the results presented here appear compatible with potential effects of insulin on hippocampal neurons in vivo.

The placebo group in almost all clinical trials studying the impact of intranasal insulin on memory function is generally exposed to either saline or HOE-31, a dilution buffer that does not contain zinc. Given that all insulin formulations except Apidra contain zinc, our results showing that Apidra is capable of reducing the AHP in hippocampal neurons ex vivo could have clinical significance. These data are consistent with insulin *per se* being able to alter hippocampal physiology, and suggest that insulin and not zinc may be responsible for the CNS effects of intranasal insulin on memory. Clearly further clinical trials with Apidra appear warranted.

Because the AHP is dependent on a Ca^{2+}-activated K^+ conductance, the insulin-mediated reductions in the AHP may be due to inhibition of Ca^{2+} influx (79) or inhibition of L-type Ca^{2+} channel currents as seen in pinealocytes (80). Taken together, these results suggest insulin acts through the insulin receptor to reduce calcium-mediated functions possibly through PI3 kinase- or tyrosine kinase-mediated phosphorylation of Ca^{2+} channel proteins. Indeed, insulin can enhance mitochondrial function and therefore, Ca^{2+} homeostasis through PI3 kinase mechanisms in the periphery (40). Insulin also increases sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) function in heart cells via an Akt (protein kinase B) pathway, and in neurons, insulin has been shown to reduce $KV1.3$ potassium channel function (81). Notably, all of these potential mechanisms are consistent with reductions in available Ca^{2+}, and hence, reductions in the amplitude of Ca^{2+}-dependent hyperpolarizing potentials.

Our data cannot address whether the beneficial actions of intranasal insulin on memory recall are mediated, in parts or in whole, by the effects of insulin on the AHP. While suggestive that this may be the case, we do not provide evidence to support this conclusion. Further, because young animals were not tested for the impact of intranasal insulin, future studies will be needed to test the hypothesis that the observed effect is age-selective, or generally nootropic. Finally, although a similar insulin dose yielded quantitatively similar enhancement on memory recall in two cohorts of aged animals, it is not clear that higher Humalog doses might also be ineffective, similarly to higher Levemir doses.

The present results shed new light on a previously unrecognized insulin mechanism in the brain. Reductions in the AHP could enhance neuronal communication and might represent a pathway through which low dose Humalog or Levemir improve memory recall in aged

animals. We show that insulin targeted the AHP, a Ca^{2+}-mediated conductance, and reduced AHP amplitude and duration, elevations of which are characteristic of brain aging. We propose that facilitating insulin signaling restores Ca^{2+} homeostasis in aged animals, resulting in optimal levels of membrane excitability and synaptic plasticity, in part by limiting the amplitude and duration of the AHP in CA1 neurons (Figures 4 and 5). During aging, reductions in brain insulin levels and/or insulin receptor function (see Introduction section) may help promote neuronal Ca^{2+} dysregulation, resulting in impaired membrane excitability and reduced synaptic plasticity (eg, larger AHP). Clearly, given the reductions in insulin receptor RNA and protein in the aged rodent (5,6), greater occupation of the leftover insulin receptors with insulin could well offset reductions in signaling through these receptors, or may even slow the age-related reduction in insulin receptor density. Collectively, these results indicate robust associations between brain insulin, Ca^{2+} homeostasis, and aging-related cognitive decline, and suggest the value of further developing "insulin-raising" strategies for treating cognitive decline in age and disease, perhaps curbing the development of AD.

Supplementary Material

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

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