Insulin bi-directionally alters NAc glutamatergic transmission; interactions between insulin receptor activation, endogenous opioids, and glutamate release

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Title: Insulin bi-directionally alters NAc glutamatergic transmission; interactions between insulin receptor activation, endogenous opioids, and glutamate release

Abbreviated Title: Effects of insulin on glutamate release

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Abstract:

Human fMRI studies show that insulin influences brain activity in regions that mediate reward and motivation, including the nucleus accumbens (NAc). Insulin receptors are expressed by NAc medium spiny neurons (MSNs), and studies of cultured cortical and hippocampal neurons suggest that insulin influences excitatory transmission via pre-synaptic and post-synaptic mechanisms. However, nothing is known about how insulin influences excitatory transmission in the NAc. Furthermore, insulin dysregulation accompanying obesity is linked to cognitive decline, depression, anxiety, and aberrant motivation that rely on NAc excitatory transmission. Using whole-cell patch clamp and biochemical approaches we determined how insulin affects NAc glutamatergic transmission in non-obese and obese male rats and the underlying mechanisms. We find that there are concentration-dependent, bi-directional effects of insulin on excitatory transmission, with insulin receptor activation increasing and IGF receptor activation decreasing NAc excitatory transmission. Increases in
excitatory transmission were mediated by activation of post-synaptic insulin receptors located on MSNs. However, this effect was due to an increase in presynaptic glutamate release. This suggested feedback from MSNs to presynaptic terminals. In additional experiments, we found that insulin-induced increases in presynaptic glutamate release are mediated by opioid receptor-dependent disinhibition. Furthermore, obesity resulted in a loss of insulin receptor-mediated increases in excitatory transmission and a reduction in NAc insulin receptor surface expression, while preserving reductions in transmission mediated by IGF receptors. These results provide the first insights into how insulin influences excitatory transmission in the adult brain, and evidence for a previously unidentified form of opioid receptor-dependent disinhibition of NAc glutamatergic transmission.

Significance Statement: Data here provide the first insights into how insulin influences excitatory transmission in the adult brain, and identify previously unknown interactions between insulin receptor activation, opioids, and glutamatergic transmission. These data contribute to our fundamental understanding of insulin’s influence on brain motivational systems and have implications for the use of insulin as a cognitive enhancer and for targeting of insulin receptors and IGF receptors to alter motivation.

Introduction:

Recent studies in humans suggest that insulin may enhance cognition and decision-making processes that influence reward-seeking (Reger et al., 2008; Freiherr et al., 2013). In addition, actions of insulin within mesocorticolimbic circuits influence hunger, motivation, and feeding behaviors (Liu and Borgland, 2015; Woods et al., 2016; Ferrario and Reagan, 2018). However, the mechanisms by which insulin affects neural function in the adult brain are poorly understood (Biessels and Reagan, 2015; Ferrario and Reagan, 2018). Studies in cortical and hippocampal neurons have shown that insulin influences excitatory transmission via pre-synaptic mechanisms that reduce glutamate release, as well as post-synaptic mechanisms that affect AMPAR trafficking (Beattie et al., 2000; Man et al., 2000; Passafaro et al., 2001; Huang et al., 2004; Labouebe et al., 2013; Liu et al., 2013). However, these studies were conducted in cultured neurons or juvenile rodents (18-30 days old). Thus, very little is known about effects of insulin on excitatory transmission in the adult brain.

Glutamatergic transmission within the nucleus accumbens (NAc) mediates many aspects of motivation and decision-making in response to food, sex, and drugs of abuse, as well as to environmental stimuli paired with these rewards. For example, food- and drug-seeking behaviors rely on activation of the NAc (Di Ciano et al., 2001; Kalivas, 2009; Wolf, 2016), and repeated exposure to drugs of abuse or palatable foods enhances NAc
Excitatory transmission that underlies food- and drug-seeking behaviors (Oginsky et al., 2016; Wolf, 2016; Dong et al., 2017; Derman and Ferrario, 2018; Alonso-Caraballo et al., 2020; Ferrario, 2020). Thus, identifying neural mechanisms that regulate NAc excitatory transmission is fundamental to understanding the neurobiology of normal and aberrant motivation.

Here, we used whole-cell patch clamp recordings in adult rat brain slices to determine how insulin affects excitatory transmission onto NAc medium spiny neurons (MSNs) and the mechanisms involved. Importantly, in addition to insulin receptors, insulin-like growth factor receptors (IGFRs) are also expressed in the NAc and can be activated by moderate to high concentrations of insulin (Unger et al., 1989; Schumacher et al., 1991). Thus, a wide range of insulin concentrations were examined, and the contribution of insulin receptor activation vs. IGFR activation to insulin’s effects were determined. In addition, given that obesity is associated with insulin dysregulation, altered NAc excitatory transmission, cognitive deficits and some psychiatric diseases (Biessels and Reagan, 2015; Kullmann et al., 2016; Stoeckel et al., 2016), we also determined how high-fat diet induced obesity alters insulin’s ability to influence NAc excitatory transmission.

Here, we found that insulin receptor and IGFR activation have opposing effects on excitatory transmission in the NAc, with insulin receptor activation increasing, and IGFR activation decreasing presynaptic glutamate release. Furthermore, insulin-induced increases in glutamate release occurred through a previously unidentified opioid receptor-dependent disinhibition that relied on GABAergic-receptor activation. Finally, diet-induced obesity resulted in a loss of insulin-induced increases in NAc excitatory transmission and a reduction in NAc insulin receptor surface expression. Together, these data reveal novel roles for insulin in the regulation of NAc excitatory transmission, provide new insights into opioid-dependent regulation of NAc glutamatergic transmission, and have implications for endogenous and exogenous insulin in modulating motivation and reward.

**Materials & Methods:**

**Animals:** Male Sprague-Dawley rats were purchased from Envigo (Indianapolis, IN), pair-housed (reverse light-dark; 12/12, lights off at 7am) with free access to food and water unless otherwise stated (70-80 days old).
All procedures were approved by the UM Institutional Animal Care & Use Committee (IACUC). See also https://sites.google.com/a/umich.edu/ferrario-lab-public-protocols/ for additional information.

**Electrophysiology:** Whole-cell patch-clamp recordings of medium spiny neurons (MSN) in the NAc core were conducted as previously described (Ferrario et al., 2011; Oginsky et al., 2016). Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.; slices prepared between 10-11 am), brains were rapidly removed and placed in ice-cold oxygenated (95% O2-5% CO2) aCSF containing (in mM): 125 NaCl, 25 NaHCO3, 12.5 glucose, 1.25 NaH2PO4, 3.5 KCl, 1 L-ascorbic acid, 0.5 CaCl2, 3 MgCl2, 295-305 mOsm, pH 7.4. Coronal slices (300 μm) containing the NAc were made using a vibratory microtome (Leica Biosystems, Buffalo Grove, IL, USA) and allowed to rest in oxygenated aCSF (40 min). For the recording aCSF (2 ml/min), CaCl2 was increased to 2.5 mM and MgCl2 was decreased to 1 mM. Patch pipettes were pulled from 1.5 mm borosilicate glass capillaries (WPI, Sarasota, FL; 3–7 MΩ resistance) and filled with a solution containing (in mM): 140 CsCl, 10 HEPES, 2 MgCl2, 5 Na+-ATP, 0.6 Na+-GTP, 2 QX314, pH 7.3, 285 mOsm. All recordings were conducted in the presence of picrotoxin (50 μM) to isolate excitatory transmission. Evoked EPSCs (eEPSCs) were elicited by local stimulation (0.05 to 0.30 mA square pulses, 0.3 ms, delivered every 20 s) using a bipolar electrode placed ~300 μm lateral to recorded neurons. The minimum amount of current needed to elicit a synaptic response with <15% variability in amplitude was used. If > 0.30 mA was required, the neuron was discarded. eEPSCs were recorded at -70 mV. Baseline responses were established (10 min) followed by a bath application of insulin in the presence or absence of antagonists (10 min). mEPSCs were recorded in the presence of tetrodotoxin (1 μM) at a holding potential of -65mV. To validate the paired-pulse facilitation procedure, eEPSCs were measured across a range of inter-pulse intervals (50, 75, 100, 200 and 400 ms; 6-8 pulses per interval) in a set of control cells. Facilitation was reliably produced at an interval of 50 ms, and thus this interval was used in our experiments. The probability of glutamate release was determined by dividing the averaged amplitude of the 2nd peak by the averaged amplitude of the 1st peak (i.e., paired pulse ratio). Recorded signals were amplified with a Multiclamp 700B (Molecular Devices, Union City, CA), digitized at 20 kHz and filtered at 2 kHz and collected with Clampex 10.4 data acquisition software (Molecular Devices). All drugs were bath applied for 10 min (Sigma Aldrich: insulin [91077C], phaclofen [114012-12-3], (-)-naloxone [51481-60-8], bestatin [65391-42-6], thiorphan [76721-89-6]), HNMPA and HNMPA-(AM)3 (Santa Cruz Biotechnology; sc-205714, sc-
picropodophyllotoxin (PPP, Tocris cat# 2956), (+)-naloxone was provided by Kenner C. Rice (Drug Design and Synthesis Section, NIDA IRP). In our initial studies, insulin concentrations ranging from 1 to 500 nM were used. This was done in part to facilitate comparison to effects of insulin in other brain regions including the VTA where concentrations of 100 and 500 nM have been used (Labouebe et al., 2013; Liu et al., 2013) and to avoid missing effects by examining just one concentration. Furthermore, while physiological concentrations of insulin are thought to be relatively low (~10-30 nM), how these levels may be affected by diet-induced obesity and/or the diabetic state is not understood and therefore levels could be much higher [see (Ferrario and Reagan, 2018) for additional discussion].

Single Cell RT-PCR and identification of D1- and D2-type MSNs: Single-cell RT-PCR was conducted on cell contents taken from MSNs after whole-cell recordings to identify D1- and D2-type MSNs. The first-strand cDNA synthesis was performed using the Superscript III First-Strand Synthesis System for RT-PCR (Life Technologies, Grand Island, NY) per the manufacturer's instructions. The reverse transcription product was kept at −20°C until PCR was performed. PCR primers used: prodynorphin forward: 5'-GCCTAGGAGTGGAGTGTTCG, reverse: 5'-GGGATAGAGCAGTTGGGCTG; proenkephalin forward: 5'-ATGCCATGCCATCGGGAAG, reverse: 5'-CAGGACCACGGGACAATC. PCR product lengths were >100 bp so as to not confuse them with primer dimers. Four μl (prodynorphin) or six μl (proenkephalin) of reverse transcription product were loaded into an Eppendorf tube with PCR solution containing 10 μl of 5× green GoTaq flexi buffer, 2 μl MgCl₂, 1 μl of 10 mM dNTP mix, 1 μl of 10 mM forward and reverse primers, 0.25 μl of GoTaq polymerase (Promega), and brought up to a final volume of 50 μl with nuclease-free water. The thermal cycling program was set to the initial denaturation for 5 min at 95°C for one cycle. The denaturation, annealing, extension cycles were done at 95 °C for 1 min, 58 °C (pENK) and 65 °C (pDYN) for 1 min, and 72 °C for 1 min, respectively, for 45 cycles. A final extension cycle was done at 72 °C for 5 min. Four microliters of the PCR reaction were placed into a second PCR tube with the same solution as before and the same cycling protocol was performed. Twenty microliters from the second PCR reaction was run on a 2% agarose gel containing ethidium bromide. Gels were imaged using UVP GelDoc-it² imager (UVP, Upland, CA). D1- or D2-type MSNs were defined by the presence of a PCR product band for either prodynorphin or proenkephalin, respectively.
High-fat Diet-induced Obesity: Rats were given free access to 60% high-fat diet (Open Source Diets D12492) in the home cage for a total of 8 weeks. Controls had free access to standard lab chow throughout (Lab Diet 5001, 13% fat). Weight was measured twice each week. In addition, after 7 weeks of high-fat or control diet, body composition was determined by NMR (Minispec LF90II, Bruker Optics), and fasted blood samples (16 hrs) were collected and used to determine plasma insulin levels. Blood samples were collected via tail nick into tubes containing EDTA (1.6 mg/mL, Sarstedt), and plasma was then isolated by centrifugation (1000 x G, 4 °C, 10 min) and stored (-20 °C) for subsequent analysis as previously described (Vollbrecht et al., 2015). Plasma insulin levels were determined by double-antibody radioimmunoassay using a 125I-Human insulin tracer (Linco Research St. Charles, MO), a rat insulin standard (Novo Nordisk, Plainsboro, NJ), a guinea pig anti-rat insulin first antibody (Linco Research), and a sheep anti-guinea pig gamma globulin-PEG second antibody (Michigan Diabetes Research Core). Blood collection and NMR were conducted at week 7 in order to avoid additional stress during the week of slice preparation or NAc tissue collection (week 8). Food was removed from the cage 1-2 hours before slice preparation or NAc tissue collection.

Biochemistry: Purification of Surface (bound) Proteins, and Western Blotting: NAc tissue was biotinylated and NeutrAvidin isolation of biotinylated (surface) proteins were conducted as previous described (Ferrario et al., 2011). For these experiments, verification studies were done to determine optimal pull down procedures and the amount of material to be loaded per lane. Briefly, bilateral NAc tissue (containing core and shell) from each rat was dissected and chopped (400 μM; McIlwain tissue chopper; the Vibratome Company, O’Fallon, MO). NAc tissue was added to ice-cold aCSF containing 1 mM sulfo-NHS-S-S-Biotin (Thermo Scientific, Rockford, IL) and incubated with gentle agitation (30 min, 4 °C). This reaction was quenched by the addition of glycine (100 mM, 10 min, 4 °C), tissue was pelleted, and re-suspended in ice-cold lysis buffer (in mM: 25 HEPES; 500 NaCl, 2 EDTA, 1 phenylmethyl sulfonl fluoride, 20 NaF, 1:100 protease inhibitor cocktail set I [Calbiochem, San Diego, CA], and 0.1% Nonidet P-40 [v/v]; pH 7.4), sonicated and stored at -80°C for subsequent use. Procedures to purify biotinylated (i.e., surface) proteins were adapted from Thermo Scientific product instructions and all steps were conducted on ice or at 4 °C unless otherwise noted. Protein concentrations were determined by Pierce BCA assay. 100 μg of NAc protein was added to high capacity NeutrAvidin agarose
beads (Thermo Scientific, Cat #29202) and incubated overnight with end-over-end rotation. Biotinylated proteins bound to NeutrAvidin beads (bound, surface fraction) were isolated from the non-biotinylated (unbound) fraction by centrifugation (3000 RPM, 1 min) and washed (3 times, 1 X PBS). The supernatant (unbound) was collected and fresh beads were added for a second overnight incubation and isolation of surface proteins as above. The bound fractions were combined in a total of 70 μL of Laemmli sample treatment buffer containing DTT (100 mM), and heated at 97 °C for 3 min to release the biotinylated proteins from the beads. The bound samples were then spun at 10,000 RPM for 5 min on a centrifugal filter unit (0.45 mm, #UFC30HV00, Millipore, Billerica, MA) to remove the NeutrAvidin beads from the solution. The samples were then stored at -20º C until used for Western Blotting.

For Western blotting, bound fractions (surface protein) or whole cell lysates (total protein) were heated (70°C, 10 min), loaded into gels (20 μg whole cell lysate, 20 μL bound fraction) and electrophoresed under reducing conditions. Proteins were transferred onto PVDF membranes (Millipore, Cat # IPVH 00010), membranes were rinsed, blocked (1hr, RT, 5% [w/v] nonfat dry milk in TBS-Tween 20 [TBS-T; 0.05% Tween 20, v/v]), and incubated overnight with primary antibody to the beta subunit of the insulin receptor (IRβ; 1:200 in TBS; Santa Cruz S711). To verify that intracellular proteins were not present in the bound fraction, the relative expression of tyrosine hydroxylase (TH, 1:30,000 in TBS; Life Technologies, P21962) was determined in the bound and unbound fractions. Membranes were then washed in TBS-T, incubated with HRP-conjugated secondary (Invitrogen, Carlsbad, CA; 1hr, RT), washed, and immersed in chemiluminescence detecting substrate (GE Health-care, Piscataway, NJ). Images were acquired on film and Ponceau S (Sigma-Aldrich) was used to determine total protein in each lane. Bands of interest were quantified using Image J (NIH).

**Experimental Design and Statistical Analysis:** eEPSCs were analyzed with Clampfit 10.4 (Molecular Devices), mEPSCs were analyzed using Mini Analysis program 6.0.4 (Synaptosoft) and verified by hand. No more than 3 cells were included/rat for any given measure to avoid over-representation of one subject. Two-tailed t-tests, one-way or two-way repeated measures ANOVAs, and Sidak’s post-hoc multiple comparisons tests were conducted using Prism 6-8 software (GraphPad). Statistical tests used for each data set are stated in the results section below and in brief in the figure captions. N’s are given in the results section, with the
number of cells followed by the number of rats used for electrophysiological recordings (e.g., 6,5 = 6 cells from 5 rats).

Results:

**Insulin bi-directionally influences NAc excitatory transmission:**

Using whole-cell patch clamping approaches from adult brain slices (Ferrario et al., 2011; Oginsky et al., 2016), we first determined how bath application of insulin (1-500 nM) affects the amplitude of evoked excitatory postsynaptic currents (eEPSC) in MSNs of the NAc core (Figure 1). We found that 30nM insulin significantly increased eEPSC amplitude (Figure 1A closed circles; two-way RM ANOVA main effect 30nM: $F_{(1,7)}=10.55$, $p=0.01$; $N=7,6$), whereas 100 or 500nM insulin produced a significant decrease in amplitude (Figure 1A triangles, diamonds; two-way RM ANOVA main effect 100nM: $F_{(1,4)}=19.56$, $p=0.01$; $N=5,4$; main effect 500nM: $F_{(1,4)}=43.50$, $p=0.003$; $N=5,4$). eEPSC amplitude returned to baseline following insulin washout. Furthermore, eEPSC amplitude was unchanged by 50nM (Figure 1A squares; two-way RM ANOVA main effect 50nM: $F_{(1,3)}>0.000006$, $p=0.99$; $N=4,3$), 1nM or 10nM insulin (Figure 1B heptagon, triangle; two-way RM ANOVA main effect 1nM: $F_{(1,6)}=0.093$, $p=0.77$; $N=6,4$; main effect 10nM: $F_{(1,3)}=1.617$, $p=0.29$; $N=4,2$). Thus, insulin produces bi-directional and concentration dependent effects on NAc excitatory transmission.

**Insulin receptor and IGFR activation have opposing effects on NAc excitatory transmission:**

In the adult brain, insulin activates insulin receptors and IGFRs (Vigneri et al., 2010). However, because of the different binding affinities of these receptors, low concentrations of insulin (~30nM) preferentially activate insulin receptors, whereas higher concentrations also activate IGFRs (Schumacher et al., 1991). We therefore hypothesized that increases in excitatory transmission elicited by 30nM insulin may be mediated by insulin receptors, whereas decreases following 100nM may be mediated by IGFRs. To test this, we applied selective antagonists of the IGFR (picropodophyllotoxin [PPP], 500 nM, Labouebe et al., 2013) or the insulin receptor blocker (HNMPA-(AM)3, 100 μM, Saperstein et al., 1989; Mebel et al., 2012) before or after insulin (Figure 1C-F). Additional controls were conducted to assess the effect of these drugs on baseline eEPSC amplitude. We found that PPP increased eEPSC amplitude on its own (data not shown; two-way RM ANOVA main effect PPP: $F_{(1,8)}=24.0$, $p=0.001$; $N=5,3$). Therefore, PPP was always applied prior to additional drug manipulations to
allow for a stable baseline to be established. Application of HNMPA-(AM)3 to the bath alone did not alter eEPSC amplitude (data not shown; two-way RM ANOVA main effect HNMPA-(AM)3: F(1,3)=0.004, p=0.95; N=4,3). Consistent with our hypothesis, application of 30nM insulin in the presence of the IGFR antagonist PPP resulted in a significant increase in eEPSC amplitude that was reversed by the subsequent addition of the membrane permeable insulin receptor blocker, HNMPA-(AM)3 to the bath (Figure 1C; two-way RM ANOVA condition x time interaction: F(8,48)=2.91, p=0.01; N=7,4). Furthermore, when the membrane-impermeable insulin receptor blocker HNMPA (300 μM, Baltensperger et al., 1992; Labouebe et al., 2013) was included in the recording pipette, 30nM insulin-induced increases in eEPSC amplitude were also completely blocked (Figure 1D, circles; two-way RM ANOVA: F(1,4)=2.7, p=0.17; N=5,4), while 100nM insulin-induced decreases in eEPSC amplitude were still observed (Figure 1D, triangles; two-way RM ANOVA main effect 100nM insulin: F(1,8)=19.2, p=0.002; N=9,5). As this manipulation would only prevent activation of insulin receptors within the recorded MSN, these data indicate that increases in excitatory transmission are due to activation of insulin receptors located on MSNs. This effect could be due to increases in post-synaptic glutamate transmission, or to increases in glutamate release due to feedback from MSNs to presynaptic terminals.

When the IGFR antagonist PPP was applied prior to 100nM insulin, previously observed decreases in eEPSC amplitude were absent (Figure 1E). Furthermore, under this condition, insulin produced a modest, but significant increase in eEPSC amplitude (Figure 1E; two-way RM ANOVA main effect 100nM + PPP: F(1,5)=7.55, p=0.04; N=6,4), likely due to activation of insulin receptors (which are not blocked by PPP). To verify this, PPP was included in the bath followed by 100nM insulin with and without HNMPA-(AM)3. Under these conditions, insulin-induced increases were completely reversed by the insulin receptor blocker (Figure 1F; two-way RM ANOVA main effect of condition: F(2,16)=12.5, p=0.0005; N=9,5). Together, these data demonstrate that insulin receptors and IGFRs work in opposition to enhance and reduce NAc excitatory transmission, respectively.

**Identification of D1-type and D2-type MSNs after whole-cell recording:** MSNs can be sub-divided by their expression of D1- and D2-like receptors that have dissociable roles in motivated behavior (Kravitz et al., 2012; Lenz and Lobo, 2013; Smith et al., 2013). Within the NAc, D1-type MSNs project to the substantia nigra and...
VTA (output nuclei), whereas D2- and D1-MSNs project to the ventral pallidum, which is a relay as well as an output nucleus. Compared to studies in the dorsal portion of the striatum, relatively little is known about potential differences in the regulation of excitatory transmission onto D1- vs D2-MSNs. Therefore, as a first step towards examining potential differences in insulin’s effects on these two populations, we established single cell RT-PCR approaches following whole-cell patch clamping to classify a subset of neurons as D1- or D2- type MSNs (cells from data in Figures 1 and 3-5). D2-MSNs were identified by the presence of proenkephalin (pENK) and absence of prodynorphin (pDYN), whereas D1-MSNs were identified by the opposite pattern (Figure 2A). We also determined that the sensitivity of pENK primers was lower than that of pDYN primers (compare Figure 2B, C), and that sensitivity of pENK primers can be enhanced by additional amplification (Figure 2D). Of the 72 cells collected, 13 cells could not be classified because bands were not visible (likely due to low starting RNA content). Of the cells identified, 41% were D2-MSNs and 47% were D1-MSNs, consistent with the literature (Sun et al., 2008). The remaining 12% were positive for both pENK and pDYN. This dual expression could be due to contamination from other cells as the pipette was removed from the slice. While this single cell RT-PCR method can distinguish D1- and D2-MSNs, statistical comparisons were not possible due to low N within a given measure in the current study (in part due to starting RNA content, and inability to collect cell contents from all cells). Thus, future studies utilizing transgenic rats specifically designed to identify MSN subpopulations (Pettibone et al., 2019) are needed to make strong conclusions about potential cell-type specific effects (see also discussion).

**Insulin-induced changes in excitatory transmission are mediated by alterations in presynaptic glutamate release:**

In Figure 1 we show that insulin bi-directionally alters excitatory transmission. In order to further understand this mechanism we determined if these changes in excitatory transmission were due to changes in presynaptic release or alterations in postsynaptic glutamate receptor transmission. Thus, we next examined the effect of 30 or 100nM insulin on miniature EPSC (mEPSC) amplitude and frequency, as well as on the paired pulse ratio (Figures 3, 4). 30nM Insulin increased mEPSC frequency (Figure 3A; two-tailed paired t-test: \( t_5=3.45, p=0.02 \); \( N=6,5 \)) without altering mEPSC amplitude (Figure 3B; two-tailed paired t-test: \( t_5=1.66, p=0.16 \)). In addition, the
frequency cumulative probability distribution was shifted to the left compared to baseline (Figure 3C), with no
change in the amplitude cumulative probability distribution (Figure 3D). These data suggest insulin-induced
increases in excitatory transmission are mediated by enhanced presynaptic glutamate release because we see
a change in frequency with no change in amplitude. Furthermore, including HNMPA in the recording pipette
blocked this insulin-induced increase in mEPSC frequency (Figure 3F; two-tailed paired t-test: \( t = 0.465, 
\)
\( p = 0.66, N = 6, 3 \) and prevented the leftward shift in the frequency cumulative probability distribution (Figure 3G),
one again confirming that effects are due to activation of post-synaptic insulin receptors on the recorded
MSN. Together, these data suggest that increases in presynaptic glutamate release are triggered by activation
of MSN-insulin receptors, resulting in feedback from the MSN to presynaptic terminals. To further confirm
effects on presynaptic release, we also determined the paired pulse ratio (PPR). We first verified the paired
pulse method in our hands by measuring eEPSCs across a range of inter-pulse intervals (50, 75, 100, 200 and
400 ms; 6-8 pulses per interval) in the same set of cells. As expected facilitation occurs at or below an inter-
pulse interval of 100 ms (Figure 3H), thus an interval of 50 ms was used to test the effect of insulin on PPR.
30nM insulin decreased the paired pulse ratio (Figure 3I; two-tailed paired t-test: \( t = 3.32, p = 0.03, N = 5, 4 \)),
indicative of an increase in the probability of glutamate release; this is consistent with the observed increase in
mEPSC frequency (Figure 3A). In addition, when HNMPA was included in the recording pipette and 30 nM
insulin was applied, a decrease in mEPSC amplitude was found (Figure 3J; two-tailed paired t-test: \( t = 7.75, 
\)
\( p = 0.006, N = 6, 3 \)) and the cumulative probability distribution of mEPSC amplitudes were shifted to the left
(Figure 3K). This suggests additional effects of insulin on post-synaptic transmission that are not mediated by
insulin receptor activation.

Effects of 100nM insulin on mEPSC amplitude and frequency (Figure 4A-E), and paired pulse facilitation
(Figure 4F, G) were also examined. We found that the frequency of mEPSCs was significantly reduced by
100nM insulin (Figure 4A; two-tailed paired t-test: \( t = 3.90, p = 0.008, N = 7, 6 \)), without altering mEPSC amplitude
(Figure 4B; two-tailed paired t-test: \( t = 0.94, p = 0.38 \)). In addition, the frequency cumulative probability
distribution was shifted to the right (Figure 4C), with no change in the amplitude cumulative probability
distribution (Figure 4D). Consistent with reductions in mEPSC frequency, 100nM insulin significantly increased
the paired pulse ratio (Figure 4F; two-way RM ANOVA main effect 100nM: \( F_{(1, 6)} = 21.41, p = 0.003 \); Figure 4G;
two-tailed paired t-test: \( t_6 = 4.67, p = 0.003; N = 7,5 \), indicating a reduction in the probability of glutamate release following 100nM insulin. Thus, activation of IGFRs by insulin reduces excitatory transmission in the NAc core by decreasing glutamate release onto MSNs.

Insulin-induced increases in excitatory transmission are due to opioid receptor-dependent disinhibition:

Endogenous concentrations of insulin in the brain are thought to range from 30-50 nM (Havrankova et al., 1978; Schulingkamp et al., 2000). Therefore, we focused studies of underlying mechanisms on insulin-receptor mediated increases in excitatory transmission following 30nM insulin. Because blockade of insulin receptors within the recorded MSN was sufficient to prevent increases in excitatory transmission due to increased presynaptic glutamate release (Figure 1D, 3F), we reasoned that presynaptic effects are likely mediated by a neuromodulator released by MSNs, such as GABA or endogenous opioids. Given that both of these transmitters are inhibitory, it is unlikely that effects of insulin are due to direct effects on presynaptic glutamatergic terminals, as activation of GABA or opioid receptors on glutamatergic terminals reduces presynaptic glutamate release, not enhances it (Nisenbaum et al., 1993; Hjelmstad and Fields, 2003). Therefore, we hypothesized that effects may be due to disinhibition of inhibitory inputs onto glutamatergic terminals (GABA\(_A\) but not GABA\(_B\) receptors were blocked during our recordings). Consistent with our hypothesis, addition of the GABA\(_B\) receptor antagonist phaclofen (20 \( \mu \)M) to the bath was sufficient to prevent insulin-induced increases in glutamate release measured using both PPR (Figure 5A; one-way RM ANOVA no effect of 30 nM insulin: \( F_{(2,8)} = 0.074, p = 0.93, N = 5,3 \)) and mEPSC frequency (Figure 5B; two-tailed paired t-test: \( t_5 = 0.082, p = 0.94, N = 5,3 \)). Thus, insulin-induced increases in excitatory transmission appear to rely on disinhibition, rather than direct enhancement of glutamate release. Importantly, the concentration of phaclofen used did not affect basal transmission, suggesting that we did not simply enhance excitatory transmission to a ceiling (data not shown; two-way RM ANOVA no effect of 20 \( \mu \)M phaclofen: \( F_{(1,10)} = 2.58, p = 0.14, N = 6,5 \)). GABA\(_B\) receptors are widely expressed, therefore phaclofen will act at both presynaptic and postsynaptic receptors on both glutamatergic and GABAergic neurons, perhaps explaining the lack of an effect of phaclofen alone.
However, changes that occur downstream of insulin receptors may be less ubiquitous and instead occur in a specified microcircuit, resulting in the observed increase in excitatory transmission.

Opioid receptors are found on GABAergic terminals in the NAc (Pickel et al., 2004), and activation of opioid receptors causes disinhibition in the VTA and hippocampus by reducing GABAergic transmission (Capogna et al., 1993; Hjelmstad et al., 2013). Thus, we speculated that insulin may trigger endogenous opioid release, which then activates opioid receptors on GABAergic terminals within the NAc to enhance presynaptic glutamate release (see schematic Figure 5C; note that recordings were done in coronal slices which contain cell bodies of cells intrinsic to the NAc, and terminals, but not cell bodies, from regions that project to the NAc).

Therefore, we next determined whether application of the opioid receptor antagonist (-)-naloxone (1 μM; Chieng and Christie, 1994) would prevent insulin-induced increases in excitatory transmission. Bath application of (-)-naloxone prior to 30nM insulin prevented insulin-induced increases in eEPSC amplitude (Figure 5D; two-way RM ANOVA no effect of 30nM insulin: F_{(1,5)}=0.82, p=0.41; N=6,3), and insulin-induced reductions in paired pulse ratio (Figure 5E; two-tailed paired t-test: t_6=0.43, p=0.68; N=7,4). Thus, effects of insulin rely upon opioid receptor activation. In addition, (-)-naloxone alone did not alter glutamate release in the absence of insulin (Figure 5F; two-tailed paired t-test: t_9=0.53, p=0.53; N=10,5). This suggests that opioid receptor activation is secondary to insulin receptor activation, consistent with the proposed microcircuit shown in Figure 5C. Given these results, it’s logical to suspect that enhancing basal opioid tone could partially occlude insulin’s effects. In an attempt to address this possibility, we utilized the peptidase inhibitors bestatin (10 μM) and thiorphan (1 μM), which can prevent the degradation of endogenous opioids (Birdsong et al., 2019). Bath application of these peptidase inhibitors decreased eEPSC amplitude from baseline, with no further changes observed when 30nM insulin was applied (data not shown; two-way RM ANOVA main effect of treatment: F_{(2,6)}=33.2, p<0.001; N=4,3; Sidak’s multiple comparisons post-test: BL vs. peptidase inhibitors, p=0.003; peptidase inhibitors vs. 30nM, p=0.31). The large reduction in eEPSC amplitude observed when the peptidase inhibitors were bath applied is consistent with a generalized inhibitory effect of increasing opioid tone, but complicates the interpretation of subsequent insulin application. Thus although the effect of insulin was occluded in these recording conditions, consistent with data above, this could merely be due to the overall inhibition caused by enhancing opioid tone. Interestingly, application of (-)-naloxone after 30nM insulin was not sufficient to reverse...
insulin-induced decreases in PPR (Figure 5G; two-way RM ANOVA main effect of treatment: $F_{(2,6)}=6.61$, $p=0.03$; $N=4,3$; Sidak’s multiple comparisons post-test BL vs. 30nM, $p=0.04$). This is consistent with the absence of effects of (-)-naloxone alone and suggests that once opioid receptor signaling is triggered, subsequent opioid receptor blockade cannot overcome ongoing signaling. Finally, to more conclusively test the role of opioid receptor activation, we bath applied (+)-naloxone (1 μM) prior to 30nM insulin. (+)-Naloxone is the structural enantiomer of (-)-naloxone, but does not have any action at opioid receptors (Iijima et al., 1978).

Consistent with the data above, (+)-naloxone did not prevent insulin-induced increases in glutamate release measured by paired pulse facilitation (Figure 5H; two-tailed paired t-test: $t_{7}=2.55$, $p=0.04$; $N=8.5$). Taken together, antagonist studies using (-) and (+)-naloxone show that insulin-induced increases in presynaptic glutamate release require opioid receptor activation.

**Diet-induced obesity blunts insulin receptor-mediated increases in excitatory transmission and reduces NAc insulin receptor surface expression:**

Circulating insulin reaches the striatum and NAc specifically, and diet-induced obesity is accompanied by chronic elevations in circulating insulin (Woods et al., 2016). In addition, obesity is associated with a reduction in the cognitive-enhancing effects of intra-nasal insulin in humans [see (Kullmann et al., 2016) for review] and impairments in hippocampal glutamatergic plasticity (Fadel and Reagan, 2016). Therefore, we predicted that high-fat diet-induced obesity may blunt insulin’s ability to enhance NAc excitatory transmission. For this set of studies, adult male rats were given free access to 60% high-fat diet in the home cage for a total of 8 weeks, while controls had free access to standard lab chow. As expected, high-fat diet resulted in significant increases in fasted plasma insulin levels (Figure 6A; two-tailed unpaired t-test: $t_{26}=3.65$, $p=0.001$; chow $N=13$, high-fat $N=15$) and fat mass compared to controls (Figure 6B; two-tailed unpaired t-test: $t_{26}=6.82$, $p<0.0001$). We next examined the effect of bath application of 30nM and 100nM insulin on eEPSC amplitude in slices from high-fat diet and control rats. Similar to results above, 30nM insulin increased eEPSC amplitude, whereas 100nM insulin decreased it in recordings from controls (Figure 6C circles; two-way RM ANOVA main effect insulin: $F_{(2,8)}=5.17$, $p=0.04$; $N=5.3$). In contrast, in MSNs from high-fat rats 30nM insulin did not significantly alter eEPSC amplitude, while significant decreases induced by 100nM insulin persisted (Figure 6C squares; two-
way RM ANOVA main effect insulin: $F_{(2,10)}=8.74$, $p=0.01$; BL vs. 30nM; main effect of insulin: $F_{(1,5)}=2.86$, $p=0.15$; BL vs. 100nM; main effect of insulin: $F_{(1,5)}=8.58$, $p=0.03$; N=6,5). This occlusion of insulin-induced increases in the high-fat group suggest physiological roles for insulin in the NAc, and are consistent with the idea that physiological shifts in circulating insulin secondary to diet-induced obesity impact neural insulin sensitivity (Ferrario and Reagan, 2018).

One potential explanation for the loss of insulin-induced increases in excitatory transmission is a reduction in NAc insulin receptor expression. Therefore, we next determined the effect of high-fat diet on surface expression of the obligatory $\beta$ subunit (IR$\beta$) of the insulin receptor using established biotinylation and pull down procedures (Ferrario et al., 2011). The intracellular protein TH was apparent in the unbound, but not the bound, fraction as expected (Figure 6D). In NAc tissue from chow and high-fat groups, we found a 21.7% (±7.6%) reduction in IR$\beta$ surface expression in high-fat vs. chow fed groups (Figure 6D; two-tailed unpaired t-test: $t_{18}=1.75$, $p=0.046$) without any changes in total IR$\beta$ (Figure 6E). This suggests that reductions in NAc insulin receptor expression may contribute to the loss of insulin-induced increases in eEPSC amplitude in the high-fat group.

**Discussion:**

**Bi-directional effects of insulin receptor and IGFR activation:** We found bi-directional effects of insulin on NAc excitatory transmission, with 30nM insulin increasing, and 100-500nM decreasing eEPSC amplitude (Figure 1A,B). Using antagonists, we show that increases in excitatory transmission are mediated by insulin receptors whereas decreases are mediated by IGFRs. Furthermore, insulin receptor-mediated effects were attributable to activation of insulin receptors on MSNs, as adding a membrane impermeable insulin receptor blocker in the recording pipette completely prevented insulin-induced increases in excitatory transmission. The magnitude of the insulin-induced increase in eEPSC amplitude was similar in the presence or absence of an IGFR antagonist (~25-30%). This suggests that 30nM insulin only activates insulin receptors whereas higher concentrations are required to recruit IGFR activation. Indeed, 50nM insulin did not alter excitatory transmission, presumably because the sum of enhancing (insulin receptor-mediated) and inhibitory (IGFR-
mediated) effects were offsetting (Figure 1A). These results suggest that the net effect of insulin on excitatory transmission in vivo may be influenced by local insulin concentration (see also, effects of high-fat below).

We next determined whether effects on excitatory transmission are due to alterations in pre- or postsynaptic function. Application of 30nM insulin increased mEPSC frequency without altering mEPSC amplitude, an effect that was blocked by preventing insulin receptor signaling (Figure 3 A,B,F). This same concentration of insulin also enhanced the probability of glutamate release (Figure 3I). Thus, reductions in the paired pulse ratio in combination with increases in mEPSC data strongly support insulin-induced enhancement in glutamate release. To our knowledge, this is the first time insulin has been found to enhance glutamate release. In contrast, in VTA insulin receptor activation produces rapid and persistent reductions in presynaptic glutamate release (Labouebe et al., 2013; Liu et al., 2013). Thus, effects of insulin are region specific, although studies in VTA were conducted in cultured neurons or juvenile mice, while studies here are in adult rats. IGFR-mediated reductions in excitatory transmission were also due to effects on presynaptic glutamate release (Figure 4). This is consistent with the ability of IGFR activation to inhibit L-type calcium channel activity which mediates presynaptic glutamate release (Subramanian et al., 2013; Sanchez et al., 2014), and with the suppression of spontaneous excitatory transmission in hippocampus by IGFR activation (Gazit et al., 2016).

**How does postsynaptic insulin receptor activation result in increased presynaptic glutamate release?** Blockade of insulin receptor signaling within the recorded MSN was sufficient to prevent insulin-induced increases in excitatory transmission (Figure 1D,3F), suggesting a mechanism involving feedback from MSNs to presynaptic glutamatergic terminals. Given that transmitters released by MSNs are inhibitory, we hypothesized that effects may be due to disinhibition of inhibitory inputs onto glutamatergic terminals. Indeed, our data support a previously unidentified mechanism whereby insulin produces disinhibition that is dependent upon opioid receptor activation (Figure 5).

Recordings were made in the presence of a GABA<sub>A</sub> antagonist, thus ionotropic inhibition cannot contribute. Addition of a GABA<sub>B</sub> antagonist prevented insulin-induced increases in release probability and mEPSC frequency (Figure 5A,B). While removing all GABA transmission is quite a “hammer”, this nonetheless provides additional support for disinhibition. Addition of the opioid receptor antagonist (-)-naloxone was sufficient to
prevent insulin-induced increases in excitatory transmission measured by mEPSC frequency, paired pulse ratio, and eEPSC amplitude (Figure 5A-E). The role of opioid receptors was further supported by the inability of (+)-naloxone (which does not have any action at opioid receptors (Iijima et al., 1978) to prevent insulin-induced increases in glutamate release (Figure 5H). Additionally, naloxone alone was not sufficient to alter glutamate release (Figure 5F), suggesting that there is not opioid-dependent tonic inhibition of presynaptic glutamate. This may be due to rapid degradation of endogenous opioids by peptidases (see also below). However, tonic inhibition is not necessarily required for the observed effects of insulin. Rather, we propose that activation of insulin receptors on MSNs leads to elevations in endogenous opioids thereby causing disinhibition of presynaptic glutamate release (Fig 5C).

Although few functional studies have examined the regulation of NAc glutamate release by endogenous opioids, this mechanism is consistent with anatomical and physiological data. Specifically, Mu opioid receptors are located on presynaptic GABAergic terminals within the NAc (Svingos et al., 1997; Pickel et al., 2004), and Mu opioid receptor activation reduces GABA release in the hippocampus and sub-thalamic nucleus (Lambert et al., 1991; Xie et al., 1992; Capogna et al., 1993; Lupica, 1995; Shen and Johnson, 2002). In addition, GABA\textsubscript{\textit{\text{b}}} receptors are located on glutamatergic terminals within the striatum where they inhibit excitatory transmission (Nisenbaum et al., 1993). Thus, it is feasible for endogenous opioids to produce the disinhibition observed here (Shen and Johnson, 2002; Banghart et al., 2015; Tejeda et al., 2017).

Naloxone is a non-selective opioid receptor antagonist. Kappa opioid receptors are located on terminals of excitatory and inhibitory synapses within the NAc (Svingos et al., 1999; Meshul and McGinty, 2000; Tejeda et al., 2017), and on dopamine afferents in the NAc (Spanagel et al., 1992), whereas delta opioid receptors are preferentially expressed on cholinergic interneurons within the NAc (Le Merrer et al., 2009; Bertran-Gonzalez et al., 2013; Castro and Bruchas, 2019). Thus, in addition to potential roles for Mu opioid receptors discussed above, effects could be mediated by one, or a combination of different opioid receptors. Future studies are needed to determine the receptor population(s) involved.

In the course of the studies above, we found that inclusion of peptide inhibitors reduced eEPSC amplitude by ~28% (±4.3). This suggests that under these conditions there may be an accumulation of endogenous peptides.
opioids within our slices. Although additional studies are needed to confirm this observation, these data are consistent with one previous report examining dorsal striatum (Atwood et al., 2014), although see (Birdsong and Williams, 2020) for additional discussion. Overall, reductions in eEPSC amplitude following peptidase inhibition provide indirect evidence for accumulation of endogenous opioids that warrants future study.

Finally, while studies using transgenics are needed to draw firm conclusions about potential heterogeneity of effects across D1- and D2-type MSNs, the consistency of insulin's effect across recorded cells suggest that potential differences may be subtle. That is, if the effects of insulin were isolated to one population, or were opposite in the two populations, one would expect that recording from both cell types indiscriminately would result in null effects. But this is not the case; instead we find consistent effects of insulin across different measures. This may not be entirely surprising given that the endogenous opioids dynorphin A17 (from D2-MSNs) and enkephalins (met-enkephalin and leu-enkephalin; from D1-MSNs) have activity at kappa, delta, and mu opioid receptors found within the striatum [e.g., (Gomes et al., 2020) and references therein].

Loss of insulin-receptor mediated effects following obesity: When effects of high-fat diet were examined, we found a loss of insulin receptor-mediated increases in excitatory transmission, but a maintenance of IGFR-mediated decreases (Figure 6C). Although slight trends were seen for reduced transmission following 30nM insulin in the high-fat group, the p value indicated a low probability of a true effect. The loss of insulin-induced increases may be due in part to modest reductions in NAc insulin receptor expression, as surface expression of IRβ was reduced following high-fat diet (Figure 6D). However, concomitant reductions in signaling downstream of the receptor could also contribute. This is an avenue for future investigation. A reduction in insulin receptor expression is consistent with the development of insulin resistance in the face of chronic elevations in circulating insulin resulting from obesity, and with impairments in hippocampal glutamatergic plasticity induced by insulin resistance (Fadel and Reagan, 2016). Although we cannot rule out the contribution of differences in basal insulin tone between chow and high-fat groups, these data nonetheless demonstrate that physiologically relevant increases in circulating insulin are accompanied by reductions in insulin receptor-induced effects on NAc excitatory transmission.
Summary and future directions: Studies above provide the first insights into how insulin influences NAc excitatory transmission. Based on these results, we propose that activation of insulin receptors on MSNs results in enhanced activity of endogenous opioids, ultimately producing disinhibition of pre-synaptic glutamate release (Fig 5C). In addition, data show that insulin receptors and IGFRs work in opposition to enhance and reduce glutamatergic transmission, respectively. This, in combination with recordings from obese rats, strongly suggests that shifts in the balance of activity at these receptors will influence the ability of insulin to regulate NAc activity. Thus, future studies will be needed to determine how insulin may affect motivation and feeding-related processes in the obese and non-obese state that are mediated by NAc excitatory transmission and endogenous opioids (Zhang and Kelley, 2000; Katsuura and Taha, 2013; Richard et al., 2013; Castro and Bruchas, 2019; Ferrario, 2020). Finally, the NAc receives inhibitory input from GABAergic neurons in the VTA (Van Bockstaele and Pickel, 1995), local collateralization of MSNs, and aspiny GABAergic interneurons (Smith and Bolam, 1990; Kawaguchi, 1993; Planert et al., 2010). Thus, in addition to identifying the opioid receptors involved, it will be important for future studies to determine whether disinhibition produced by insulin is selective to different sources of GABA within the NAc.
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Figure Captions:

Figure 1: Insulin receptor activation increases, whereas IGFR activation decreases, excitatory transmission onto MSNs in the NAc core. A) Average eEPSC amplitude during baseline, bath application of insulin (black bar) and following washout. B) Summary of average maximum change from baseline following insulin (1-500nM). Effects of insulin on excitatory transmission are concentration-dependent, and bi-directional. Recording location within the NAc core is shown at the right. C) Average eEPSC amplitude in the presence of the IGFR-antagonist PPP, before and after 30nM insulin, with and without the membrane permeable insulin receptor inhibitor HNMPA-(AM)3. D) Average eEPSC amplitude before and after insulin (30 and 100nM) with the membrane-impermeable insulin receptor inhibitor HNMPA included in the patch pipette. E) Average eEPSC amplitude before and after 100nM insulin administered in the presence of PPP. F) Average eEPSC amplitude in the presence of PPP, before and after 100nM insulin followed by the addition of HNMPA-(AM)3 to the bath. Data in all figures shown as average ± SEM. Statistical differences were determined by within subject, two-way RM ANOVA comparing baseline and treatment conditions. ** = main effect of treatment, p < 0.01; see results section for full statistical information.

Figure 2: Verification of single cell RT-PCR method. A) Example of single cell RT-PCR for a D2-MSN (left) and a D1-MSN (right) after whole-cell recordings in adult rat nucleus accumbens. β-actin was used as a positive control. B) Serial dilution of RNA from striatal tissue showing the sensitivity of prodynorphin primers (pDYN; 149 bp). C) Serial dilution of RNA from striatal tissue showing the sensitivity of proenkephalin primers (pENK; 220 bp). D) A second round of amplification is sufficient to allow for proenkephalin detection in samples containing 1 pg/μL of RNA.

Figure 3: 30nM insulin increases mEPSC frequency and the probability of glutamate release, but not mEPSC amplitude. A) Average mEPSC frequency before (baseline, BL) and after bath application of insulin (30nM). B) Average mEPSC amplitude before and after insulin (30nM). C) Cumulative probability distributions of mEPSC frequency before and after insulin (30nM). D) Cumulative probability distributions and histograms of mEPSC amplitude before and after insulin (30nM). E) Representative mEPSC traces before and after insulin (30nM), with and without the membrane-impermeable insulin receptor inhibitor HNMPA included in the patch pipette. Arrows in the upper traces indicate regions in which the time scale was expanded in the lower traces. F) Average mEPSC frequency before and after bath application of insulin (30nM) with membrane impermeable HNMPA included in the patch pipette. G) Cumulative probability distributions of mEPSC frequency before and after insulin (30nM) with HNMPA included in the patch pipette. H) Verification of paired-pulse facilitation in MSNs. Average PP ratio across increasing inter-pulse intervals (50-400 ms). Inset shows representative traces at a 50ms inter-pulse interval. As expected, the probability of glutamate release is relatively low in NAc medium spiny neurons, and facilitation occurs at inter-pulse intervals at or below 100 msec. I) Average PP ratio before and after insulin (30nM). Representative traces before (black) and after insulin (gray) are shown in the inset.
(50 ms inter-stimulus interval). J) Average mEPSC amplitude before and after insulin (30nM) with membrane impermeable HNMPA included in the patch pipette. K) Cumulative probability distributions and histograms of mEPSC amplitude before and after insulin (30nM) with HNMPA included in the patch pipette. Statistical differences were determined by two-tailed paired t-tests; * = p < 0.05.

Figure 4: 100nM insulin reduces mEPSC frequency and the probability of glutamate release without altering mEPSC amplitude. A) Average mEPSC frequency before (baseline, BL) and after insulin (100nM). B) Average mEPSC amplitude before and after insulin (100nM). C) Cumulative probability distributions of mEPSC frequency before and after insulin (100nM). D) Cumulative probability distributions and histograms of mEPSC amplitude before and after insulin (100nM). E) Representative mEPSC traces before and after insulin (100nM). Arrows in the left traces indicate regions in which the time scale was expanded in traces shown at the right. F) Average PP ratio before and after insulin (gray bar, 100nM) and following insulin wash out. Representative traces before (black) and after insulin (gray) are shown in the inset. G) Average change in the PP ratio following insulin (100nM). Statistical differences were determined by two-tailed paired t-tests (A, G; ** = p < 0.01) and two-way RM ANOVA comparing baseline and treatment conditions (F; * = main effect of treatment, p < 0.01).

Figure 5: Insulin-induced increases in glutamate release are GABA-B receptor- and opioid-receptor dependent. A) Average PP ratio at baseline (BL), and after insulin (30nM) in the absence and presence of the GABA_B receptor antagonist Phaclofen (20 μM). Phaclofen prevents insulin-induced decreases in PP ratio. B) Average mEPSC frequency before and after insulin (30nM) in the presence of Phaclofen (20 μM). Phaclofen prevents insulin-induced increases in mEPSC frequency. C) Proposed mechanism by which activation of insulin receptors on MSNs enhances glutamate release. We propose that activation of insulin receptors on MSNs results in the release of endogenous opioids (1) that reduces GABAergic transmission (2), thereby causing disinhibition of presynaptic glutamate release (3). D) Average eEPSC amplitude in the presence of the opioid receptor antagonist (-)-naloxone (1 μM) before and after bath application of insulin (30nM). (-)-Naloxone prevents insulin-induced increases in eEPSC amplitude. E) Average PP ratio in the presence of (-)-naloxone (1 μM) before and after bath application of insulin (30nM). (-)-Naloxone prevents insulin-induced decreases in PP ratio. F) Average PP ratio before and after bath application of (-)-naloxone (1 μM) alone confirms there is no effect of (-)-naloxone alone. G) Average PP ratio at baseline (BL), and after insulin (30nM) in the absence or presence of (-)-naloxone (1 μM). (-)-Naloxone does not reverse insulin-induced decreases in PP ratio. H) Average PP ratio in the presence of (+)-naloxone (1 μM) before and after bath application of insulin (30nM). (+)-Naloxone, which does not inhibit opioid receptors, does prevent insulin induced decreases in PP ratio. Example traces are shown within each panel. Statistical differences were determined by two-way RM ANOVA comparing baseline and treatment conditions (D), two-tailed paired t-tests (C, E, F, H), and one-way ANOVA followed by Sidak’s multiple comparisons post-test (B, G); * = p < 0.05.
Figure 6: High-fat diet-induced obesity results in a loss of insulin-induced increases in excitatory transmission and a reduction in NAc insulin receptor β (IRβ) surface expression. Average concentration of fasted plasma insulin (A) and fat mass (B) in chow and high-fat diet groups. C) Average eEPSC amplitude following bath application of increasing concentrations of insulin (gray bars) and following insulin wash out in MSNs from chow (circles) and high-fat groups (squares). Representative traces for each group before (black) and after (gray) each insulin concentration are shown at the right. D) Average NAc IRβ surface expression in high-fat and chow fed groups. Immunoblot for tyrosine hydroxylase (TH) in the bound (B) and unbound (UB) fractions is shown at the left. Consistent with its intracellular localization, TH protein levels were nearly absent in the bound (surface) fraction. E) Total NAc IRβ expression in high-fat and chow fed groups. Representative blot images are shown below each graph. Statistical differences were determined by two-tailed unpaired t-tests (A, B: **= p < 0.001), two-way RM ANOVA comparing baseline and treatment conditions (C: * = chow group, main effect of treatment, # = high-fat group, main effect of treatment, p < 0.05), and two-tailed unpaired t-test (D: p = 0.046).
A. Example of single cell RT-PCR for a D2- (left) and a D1-MSN (right) after NAc whole-cell recording.

B. Sensitivity of prodynorphin primers.

C. Sensitivity of proenkephalin primers.

D. Two rounds of amplification enhances sensitivity of the pEnk primers to 1 pg/μL of RNA.
A. mEPSC frequency

B. mEPSC amplitude

C. mEPSC frequency distribution

D. mEPSC amplitude distribution

E. Representative traces before (BL; HNMPA) and after insulin (30 nM)

F. mEPSC frequency + HNMPA

G. mEPSC frequency distribution + HNMPA

H. Verification of paired pulse facilitation

I. Paired pulse facilitation

J. mEPSC amplitude + HNMPA

K. mEPSC amplitude distribution + HNMPA
A. mEPSC frequency
B. mEPSC amplitude
distribution
C. mEPSC frequency
distribution
D. mEPSC amplitude
distribution
E. Representative mEPSC recordings before (BL) and after 100 nM insulin
F. Timecourse of paired pulse facilitation
G. Summary of paired pulse facilitation
**A.**

- Graph showing the average PP ratio (P2/P1) for different conditions.
- Conditions include: BL, Phac, Phac, 30nM Ins.
- Y-axis: Avg PP Ratio (P2/P1).
- X-axis: Conditions.

**B.**

- Graph showing the average normalized mEPSC frequency.
- Conditions: Phac, Phac, 30nM Ins.
- Y-axis: Avg Normalized mEPSC Frequency.
- X-axis: Conditions.

**C.**

- Diagram illustrating neural pathways and opioid receptor activation.
- IR activation increases endogenous opioid release from MSNs.
- Opioid receptor activation inhibits GABAergic transmission.
- Increased glutamate release via disinhibition.

**D.**

- Graph showing the average eEPSC amplitude (% baseline).
- Conditions: 1 μM (-)-Nal, 30 nM Insulin.
- Y-axis: Avg eEPSC Amplitude (% Baseline).
- X-axis: Time (min).

**E.**

- Graph showing the average PP ratio (P2/P1).
- Conditions: (-)-Nal, (-)-Nal.
- Y-axis: Avg PP Ratio (P2/P1).
- X-axis: Conditions.

**F.**

- Graph showing the average PP ratio (P2/P1).
- Conditions: BL, (-)-Nal.
- Y-axis: Avg PP Ratio (P2/P1).
- X-axis: Conditions.

**G.**

- Graph showing the average PP ratio (P2/P1).
- Conditions: BL, 30 nM Ins, 30 nM Ins, (-)-Nal.
- Y-axis: Avg PP Ratio (P2/P1).
- X-axis: Conditions.

**H.**

- Graph showing the average PP ratio (P2/P1).
- Conditions: (+)-Nal, (+)-Nal.
- Y-axis: Avg PP Ratio (P2/P1).
- X-axis: Conditions.
A. Fasted plasma insulin levels

B. Fat mass

C. eEPSC amplitude in chow and high-fat groups

D. IRβ surface expression

E. Total IRβ expression