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Insulin in the ventral tegmental area reduces hedonic feeding and suppresses dopamine concentration via increased reuptake

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Abstract

Mesolimbic dopamine (DA) signaling has been implicated in the incentive, reinforcing, and motivational aspects of food intake. Insulin receptors are expressed on dopaminergic neurons of the ventral tegmental area (VTA) and insulin may act in the VTA to suppress feeding. However, the neural mechanisms underlying insulin effects in the VTA are poorly understood. Here, we measured the effects of insulin on evoked DA concentration in the VTA using fast scan cyclic voltammetry. Insulin concentration-dependently reduced evoked somatodendritic DA in the VTA requiring activation of phosphoinositol 3-kinase and mTOR signalling. Insulin depression of somatodendritic DA was abolished in the presence of a selective DA transporter (DAT) inhibitor, GBR 12909, as well as in VTA slices of DAT knockout mice, suggesting that insulin upregulated the number or function of DAT to reduce DA concentration. Finally, insulin administered to the VTA depressed sated feeding of sweetened high fat food. Taken together, these results indicate that insulin depresses DA concentration in the VTA via increased reuptake of DA through DAT. Insulin-mediated decrease of DA in the VTA may suppress salience of food once satiety is reached.

Keywords

Dopamine; somatodendritic; insulin; VTA; DAT

Introduction

Consumption of food, especially when sated, is often driven by its rewarding properties, which likely involves activation of the mesolimbic dopamine (DA) pathway. DA neurons of the ventral tegmental area (VTA) have been implicated in the incentive, reinforcing, and motivational aspects of food intake (Ikemoto *et al.*, 1996; Brennan *et al.*, 2001; Ishiwari *et al.*, 2004; Ikemoto, 2007). In response to salient environmental stimuli, spikes of DA neurons are clustered into bursts resulting in increased extracellular DA in projection areas, including the nucleus accumbens and prefrontal cortex. However, DA can also be released within the VTA either somatodendritically (Rice *et al.*, 1994, 1997; Iravani *et al.*, 1996; Chen & Rice, 2002) or from synaptic DA input from its own axon collaterals and those from the substantia nigra pars compacta (Deutch *et al.*, 1988; Bayer & Pickel, 1990).

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Somatodendritically-released DA can act on somatodendritic D_2-like autoreceptors to regulate subsequent somatodendritic DA release (Cragg & Greenfield, 1997), DA neuron firing activity (Lacey *et al.*, 1988; Falkenburger *et al.*, 2001), as well as downstream axonal release in the striatum (Santiago & Westerink, 1991; Kalivas & Duffy, 1991). Furthermore, somatodendritically-released DA can act at presynaptic D_1-like receptors on GABAergic and/or glutamatergic inputs to the VTA to modulate release of those transmitters (Kalivas & Duffy, 1995; Miyazaki & Lacey, 1998; Koga & Momiyama, 2000). While these actions can have bidirectional effects on DA neuronal output to target regions, it is unknown how modulation of somatodendritic DA can alter feeding.

Circulating catabolic peptides, such as insulin and leptin, can regulate normal feeding by signaling satiety to the brain and promoting feeding cessation (Figlewicz & Benoit, 2009). Insulin released into the circulation in response to increased blood glucose levels, can permeate the blood-brain barrier by a receptor-mediated transport process (Woods *et al.*, 2003), concentrate and activate insulin receptors expressed throughout the brain (Woods & D'Allesio, 2008) including DA-containing neurons of the VTA (Figlewicz *et al.*, 2003).

How insulin interacts with DA neurons of the VTA is poorly understood. Several lines of evidence suggest that insulin may alter activity of VTA DA neurons and their output. Insulin administered directly into the VTA inhibits opioid-induced feeding (Sipols *et al.*, 2002) and palatable food ingestion (Figlewicz *et al.*, 2008). Moreover, insulin administered intracerebroventricularly (i.c.v.) alters reward-based feeding behaviours such as decreased motivation to obtain sucrose (Figlewicz *et al.*, 2006) and reduced conditioned place preference to high fat food (Figlewicz *et al.*, 2004). Furthermore, i.c.v. insulin had synergistic actions when paired with DA D2 receptor antagonism to reduce sucrose consumption (Sipols et al., 2000), suggesting that insulin may interact with DAergic systems to regulate sucrose consumption. Chronic hyperinsulinemia either by continuous i.c.v. insulin infusion (Figlewicz et al., 1994) or hyperinsulinemic obese Fa/Fa Zucker rats (Figlewicz et al., 1998) results in elevated DAT mRNA in the midbrain. Furthermore, insulin increased spike frequency in half of VTA/Substantia Nigra neurons (Konner *et al.*, 2011). Therefore, to elucidate the potential contributions of insulin signalling to DA transmission within the VTA, we explored the effects of insulin on evoked DA output ($[DA]_o$; a product of DA release minus reuptake) using fast-scan cyclic voltammetry. Furthermore, we tested the effects of insulin in the VTA on sated feeding of sweetened high fat food ('hedonic feeding').

Materials and Methods

Animals

All protocols were in accordance with the ethical guidelines established by the Canadian Council for Animal Care and were approved by the University of British Columbia Animal Care Committee. C57BL/6J mice were obtained from the University of British Columbia breeding facility. DAT (Giros *et al.*, 1996) or norepinephrine transporter (NET) (Xu *et al.*, 2000) knockout mice and wild-type (129SvJ/C57BL) mice were obtained from Dr. G. Uhl at National Institute of Drug Addiction.

Slice preparation

Male C57BL/2J mice (25–30g) were anesthetised with halothane, decapitated, and brains were extracted and prepared for slicing. Horizontal slices (250 μm) of midbrain containing the VTA were cut in ice-cold sucrose-containing artificial cerebrospinal fluid (aCSF) solution ([mM]: 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 7 $MgCl_2$, 0.95 $CaCl_2$, 75 sucrose) saturated with 95% O_2/5% CO_2 using a vibratome (Leica, Nussloch, Germany). Slices were incubated in warm (31.5°C) 95% O_2/5% CO_2 oxygenated aCSF ([mM]: 119 NaCl, 1.6 KCl, 1.0 NaH_2PO_4, 26.2 $NaHCO_3$, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 11 glucose; pH = 7.4) for at least one hour and transferred to a recording chamber that was constantly perfused (gravity flow: 1.5 ml/min) with oxygenated aCSF (30–32°C). The slices were allowed to equilibrate in the recording chamber with the superfusion medium for an additional 20–30 min before experimentation.

Fast-scan cyclic voltammetry

Evoked $[DA]_o$ was measured using fast-scan cyclic voltammetry with carbon-fiber microelectrodes. Carbon fibers (7 μm diameter; Goodfellow) were pulled in glass electrodes and cut to a final exposed length of ~150 μm. Triangular waveforms (holding at −0.4 V) at 10 Hz (−0.4 to 1.0 V vs Ag/AgCl at 400 V/s scan rate) were used. Catecholamine release was evoked using electrical stimulation (40 Hz, 5 pulses) applied with a bipolar stimulating electrode positioned flush with the tissue for local surface stimulation. The voltammetric electrode was positioned between the tips with the aid of a binocular microscope, then lowered 50–100 μm into the tissue. DA was identified by characteristic oxidation and reduction peak potentials (+600 and −200 mV vs Ag/AgCl). To determine the time course of DA, the current at the peak oxidation (+600 mV) was plotted against time. Relative electrode sensitivities for DA and norepinephrine (NE) were determined by obtaining voltammograms from exogenous application of DA (0.1 – 1 μM) and NE (1 – 10 μM) to a flow cell specifically designed for microelectrode calibration. Calibration solutions were made from stock solutions in 0.1 M $HClO_4$ immediately before use.

Surgical Procedures

Animals (C57BL/6J males; 25 to 27 g at start of experiment) were housed individually on a 12-hour light/dark cycle (lights on at 6 am) at a constant temperature (21°C). Mice were given access to standard laboratory rodent chow and water *ad libitum* prior to and for 14 days following surgery. Before bilateral intracranial cannulae implantation, animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg i.p.): xylazine (10 mg/kg i.p.) and placed in a stereotaxic frame (Kopf; Tujunga, CA). 26 gauge bilateral guide cannulae (Plastics One, Roanoke, VA) were lowered into the VTA (AP, −3.2 mm; ML, ±0.5 mm; DV, −4.6 mm). Cannulae were anchored to the skull surface with dental cement and occluded with metal obturators of the same length. Mice were treated post-surgically with ketoprofen (5 mg/kg, s.c.) and children's Tylenol (orally). Weights were monitored daily to ensure appropriate weight gain. Mice recovered for 14 days.

Feeding Experiment

After recovery from surgery mice were given standard mouse chow (6 % fat, 44 % carbohydrate, Harlan Laboratories diet 2018) and entrained to consume their daily caloric needs within 4 hours per day (12 – 4pm, PST) in a novel entrainment cage with kitty litter bedding. The amount of chow consumed was weighed hourly and the animals were weighed daily after the 4 hour entrainment period. Mice were entrained for 19 days prior to VTA microinjections. Over the course of the experiment, mice maintained their weight between 27–30g. Microinfusions were conducted using a 33 gauge cannula that protruded 0.2 mm below the base of the cannulae to a final DV coordinate of 4.8 mm. Insulin (0.3 μg in 10 % DMSO) or vehicle (10 % DMSO in saline) was infused bilaterally into the VTA (0.2 μl at 0.1 μl/min). Microinjectors were left in place for 2 min and then mice were placed in home cages for 10 min prior to access to feeding in the entrainment cages. On test days, mice received either vehicle or insulin. The order of drug delivery was reversed on subsequent test days in a crossover experimental design.

Sated High Fat Feeding Experiment

Mice were maintained on a restricted feeding schedule as above. 4 days prior to testing, mice were habituated to a small quantity of sweetened high fat food (60 % fat, 20 % carbohydrate; Bioserv, NJ; Diet D12492). On test days, mice were given 4 hour access to regular chow. Mice were then microinfused with insulin or vehicle. 10 min after the injection, mice were replaced in the entrainment cage and given access to sweetened high fat food for 1 hour. The quantity of food consumed and the animals' weight were measured following high fat feeding. To prevent high fat food binging, animals were returned to the 4 hour entrainment schedule with access to regular chow for 4 days before a subsequent test day with sweetened high fat food. At the end of the behavioral experiments, mice were deeply anesthetized with halothane and then decapitated. Brains were removed, fixed in 4% paraformaldehyde, sectioned (60 μm, coronal) and stained with cresyl violet to verify placement of cannulae and injector tips. Injection sites were located under a light microscope and recorded on atlas figures adapted from Paxinos and Watson (2001).

Drugs

Rapamycin, PKI and GBR 12909 were obtained from Tocris Bioscience. HNMPA$[AM]_3$ was purchased from EDM Biosciences. All other chemicals were obtained from Sigma-Aldrich. Insulin or HNMPA$[AM]_3$ were dissolved in DMSO and used at 1/1000 of the stock solution.

Statistics

Values listed are means ± SEM. Statistical significance was assessed using Student's t tests and one-way ANOVA for multiple comparisons using a Bonferroni post hoc test unless otherwise indicated. A difference of $p < 0.05$ was considered significant. Unless otherwise indicated, n refers to the number of slices recorded from. Data were graphed and statistical tests were performed using GraphPad Prism v.5.

Results

Insulin inhibits evoked somatodendritic DA concentration

To determine the effect of insulin on somatodendritic DA concentration, we bath applied insulin to VTA slices while evoking DA release. Cyclic voltammograms characteristic of DA (peak potentials: oxidation +600 mV; reduction, −200 mV vs Ag/AgCl) demonstrated that bath-applied insulin (500 nM, 10 min) depressed somatodendritic $[DA]_o$ (Fig. 1A). The insulin-mediated decrease in $[DA]_o$ reached a maximum of 27 ± 3 % 40 min after insulin application (n=6, $P < 0.05$), whereas application of vehicle (aCSF, 0.001% DMSO) in control slices did not produce significant changes in evoked DA (Fig. 1B; n=6; $P > 0.05$). The decay of the current, represented by Tau (τ), has been demonstrated to be positively correlated with Km, suggesting that tau, is an appropriate measurement of DA uptake (Yorgason *et al.*, 2011). Averaged current-time plots of evoked somatodendritic $[DA]_o$ indicated that insulin significantly increased the decay of the current ($\tau_{baseline}$ = 0.6 ± 0.05 s, $\tau_{insulin}$ = 0.4 ± 0.06 s; n=6; $P < 0.05$) (Fig. 1C). Furthermore, Vmax was significantly increased in the presence of insulin (baseline: 20 ± 2 μM/min, insulin: 26± 2 μM/min; n=6; P <0.05).

DA neurons of the VTA can receive noradrenergic inputs originating from the locus coeruleus (Grenhoff *et al.*, 1993). Because voltammograms for DA and NE appear indistinguishable, we tested the relative electrode sensitivities for exogenously applied DA (0.1 −1 μM) and NE (1 – 10 μM) using a flow cell. Consistent to that reported by others (Heien et al., 2003), we found our electrodes were 10 fold more sensitive to DA than NE with our waveform parameters (n=3 electrodes; Fig. 1D), suggesting that the evoked concentrations measured were that of DA. Furthermore, bath application of yohimbine (1 μM), an α2 adrenergic receptor antagonist, did not modulate $[DA]_o$ (5 min: 97 ± 3 %; 45 min: 93 ± 4%; n=4; $P > 0.05$). Taken together, these results suggest that insulin suppresses somatodendritic DA in the VTA.

The suppression of somatodendritic $[DA]_o$ produced by insulin was concentration-dependent with an IC_{50} = 62.0 ± 0.1 nM and a maximum of 78 ± 2 % of baseline (n = 5 or 6; Fig. 2A). To verify that the effect is mediated through activation of insulin receptor tyrosine kinase, we measured the effect of insulin on $[DA]_o$ in the presence of $HNMPA[AM]_3$ (300 μM), a cell permeable inhibitor of insulin receptor tyrosine kinase (Saperstein *et al.*, 1989). $HNMPA[AM]_3$ applied to slices alone had no significant effect on $[DA]_o$ (data not shown). When slices were preincubated with $HNMPA[AM]_3$ 60 min prior to recording and throughout the experiment, insulin did not depress somatodendritic $[DA]_o$ (n = 7; Fig. 2B).

Insulin decreases evoked somatodendritic DA via PI3K and mTOR activation

Insulin receptor activation triggers a variety of signal transduction cascades upon dimerization, autophosphorylation, and recruitment of insulin receptor substrates. One pathway involves activation of phosphatidylinositol 3 kinase (PI3K) and subsequent stimulationof Akt kinase. This pathway also triggers activation of mammalian target of rapamycin (mTOR) (Taha & Klip, 1999). PI3K has been implicated in DAT trafficking (Carvelli *et al.*, 2002; Garcia *et al.*, 2005; Simon *et al.*, 1997) therefore, we tested the effects

of insulin on $[DA]_o$ in the presence of wortmannin, a PI3K inhibitor. Wortmannin (100 nM, Qiu *et al.*, 2006) alone modestly suppressed $[DA]_o$ (5 min: 98 ± 3 %; 45 min: 93 ± 3%; n=4; $P < 0.05$; Fig. 3A), suggesting the possibility of constitutive DAT trafficking. However, when slices were preincubated with wortmannin for 30 min prior to and during insulin application, insulin did not significantlyinhibit $[DA]_o$ (5 min: 102 ± 5%; 45 min 99 ± 6%; n=6; $P > 0.05$; Fig. 3B). Averaged current-time plots of evoked somatodendritic $[DA]_o$ indicated that insulin did not significantly alter the decay of the current in the presence of wortmannin ($\tau_{baseline}$ = 0.7 ± 0.1 s, $\tau_{insulin}$ = 0.6 ± 0.1 s; n=6; $P > 0.05$). To determine if mTOR signaling was required for insulin-induced suppression of $[DA]_o$ in the VTA, we prevented activation of mTOR by bath application of rapamycin. Rapamycin (50 nM) alone did not significantly alter $[DA]_o$ (5 min: 105 ± 2 %; 45 min: 96 ± 10 %; n=5; $P > 0.05$; Fig. 3C). When slices were preincubated with rapamycin (50 nM, 20 min), insulin did not inhibit somatodendritic $[DA]_o$ (5 min: 100 ± 3 %; 45 min: 105 ± 9 %; n=7; $P > 0.05$; Fig. 1D), nor did insulin alter the decay of the current ($\tau_{baseline}$ = 0.8 ± 0.1 s, $\tau_{insulin}$ = 0.8 ± 0.1 s; n=7; $P < 0.05$). Taken together, these results suggest that PI3K and mTOR signaling are necessary for insulin suppression of somatodendritic $[DA]_o$.

Insulin-mediated suppression of somatodendritic $[DA]_o$ is independent of altering DA release

To determine if insulin-mediated suppression of somatodendritic $[DA]_o$ was due to an insulin-induced inhibition of DA release, we employed two strategies. Because release of somatodendritic DA is significantly less, but not absent, in low Ca^{2+} (Chen & Rice, 2001), we first tested if insulin could modulate evoked $[DA]_o$ in 0.5 mM Ca^{2+}. We consistently evoked DA in 0.5 mM Ca^{2+} for up to 1 hour, albeit the signal was 4-fold smaller than in 2.4 mM Ca^{2+} (n=4; Fig. 4A). In low Ca^{2+}, insulin significantly depressed evoked $[DA]_o$ (5 min: 99 ± 5 %; 45 min: 79 ± 6 %; n=6; $P < 0.05$; Fig. 1B). This was not significantly different from insulin-mediated inhibition of $[DA]_o$ in 2.4 mM Ca^{2+} (*c.f.* Fig. 1A, B, $P > 0.05$). Secondly, we tested if insulin modulated the frequency-dependence of DA release. Evoked somatodendritic $[DA]_o$ increased with increased frequency reaching 140 ± 8 nM at 40 Hz (n = 4; Fig. 4C). Significant insulin-mediated depression of $[DA]_o$ was observed only at frequencies greater than 20 Hz (n = 4; $P < 0.05$; Fig. 4C). The amount of insulin-mediated depression of $[DA]_o$ was significantly greater at 30 and 40 Hz compared to that at 1 and 5 Hz (n = 4, $P < 0.05$; Fig. 4D). Taken together, these results demonstrate that insulin suppresses $[DA]_o$ even in the presence of low Ca^{2+} and that insulin-mediated suppression of $[DA]_o$ was greater in the presence of higher DA concentration.

Insulin-mediated suppression of somatodendritic $[DA]_o$ is due to increased DA reuptake

Because insulin-mediated suppression of $[DA]_o$ was not consistent with the typical hallmarks of inhibition of DA release, we next tested if insulin altered DA reuptake. We measured the effects of insulin on $[DA]_o$ in the presence of a selective DAT blocker, GBR 12909, which is known to increase the time course of DA reuptake (Cragg *et al.,* 1997). GBR 12909 (500 nM) did not alter peak $[DA]_o$ when evoked every 5 min (n=6; $P > 0.05$; Fig. 5A), suggesting that there was enough time for DA diffusion away from the electrode, monoamine oxidases to break down extracellular DA, *de novo* synthesis of DA between pulses, and/or DA to be taken up by NET (Cragg *et al.*, 1997; Cragg *et al.*, 2001). Consistent

with reported effects of GBR 12909 (Cragg *et al.*, 1997; Chen & Rice, 2001), we found that GBR 12909 (500 nM) maximally prolonged DA reuptake 50 min after application ($\tau_{baseline}$ = 0.6 ± 0.1 s, $\tau_{GBR\ 12909}$ = 1.8 ± 0.5 s; n=5; $P < 0.05$; Fig. 5B). Therefore, we preincubated VTA slices with GBR 12909 for 1 hour prior to and during the experiment. In the presence of GBR 12909, insulin-mediated reduction in $[DA]_o$ was abolished (n = 5; $P > 0.05$; Fig. 5C). Further, insulin did not alter the GBR 12909-potentiated DA reuptake (Fig. 5D).

To confirm that insulin-mediated suppression of $[DA]_o$ was due to an increase DA reuptake through DAT, we tested the effects of insulin in the VTA of homozygous DAT or NET knockout mice or their littermate wildtype controls. Insulin did not alter somatodendritic $[DA]_o$ of DAT −/− mice (5 min: 99 ± 2 %; 45 min: 101 ± 3 %; n=5; $P > 0.05$; Fig. 6A). Further, somatodendritic DA reuptake was not altered by insulin in DAT −/− mice ($\tau_{baseline}$ = 1.7 ± 0.3 s, $\tau_{insulin}$ = 1.7 ± 0.3 s; n=5; $P > 0.05$; Fig. 6B). In contrast, insulin suppressed somatodendritic $[DA]_o$ in VTA of NET −/− mice (5 min: 97 ± 2 %; 45 min: 80 ± 7%; n=5; $P < 0.05$; Fig. 6C, D) to a similar extent as in wild-type mice (5 min: 99 ± 1%; 45: 81 ± 2%; n= 6; $P < 0.05$; Fig. 6E, F). Furthermore, insulin significantly increased the decay of the current in both NET −/− ($\tau_{baseline}$ = 1.3 ± 0.2 s, $\tau_{insulin}$ = 0.8 ± 0.1 s; n=5; $P < 0.05$; Fig. 6D) and wild-type mice ($\tau_{baseline}$ = 0.9 ± 0.1 s, $\tau_{insulin}$ = 0.5 ± 0.1 s; n=6; $P < 0.05$; Fig. 6F). There was no significant run down of evoked $[DA]_o$ over time in DAT (n=4), NET (n=4) or wild-type (n=3) mice in the absence of insulin (Fig. A, C, E). Taken together, these results suggest that insulin suppresses $[DA]_o$ by increasing DA reuptake via DAT.

Insulin can potentially increase DA reuptake by increasing the DAT function, trafficking or *de novo* expression. Because insulin-mediated suppression of $[DA]_o$ required mTOR signalling, a mediator known to promote protein synthesis (Asnaghi *et al.*, 2004), we tested if insulin could alter somatodendritic $[DA]_o$ in the presence of the protein synthesis inhibitor, cycloheximide. Cycloheximide (100 nM; Argilli *et al.,* 2008) applied to VTA slices for one hour did not produce significant changes in evoked $[DA]_o$ (5 min: 100 ± 2 %; 55 min: 98 ± 4 %; n=8; $P > 0.05$; Fig. 7A). When VTA slices were preincubated with cycloheximide 20 min prior to and for the duration the experiments, insulin suppressed somatodendritic $[DA]_o$ (5 min: 106 ± 4 %; 45 min: 77 ± 6 %; n=8; $P < 0.05$; Fig. 7B). These results suggest that insulin does not alter DA reuptake by *de novo* increases in DAT expression.

Insulin intra-VTA suppresses hedonic feeding

Activity of VTA DA neurons as well as increased DA in target regions of the VTA have been implicated in promoting ingestive behavior (Palmiter, 2007). To test the behavioral significance of insulin action in the VTA, we explored the effects of insulin in the VTA of hungry and sated mice. We bilaterally cannulated the VTA (Fig. 8A). After recovery, mice were entrained to consume their daily caloric needs within a 4h period per day in a novel feeding cage. To determine if intra-VTA insulin altered feeding in hungry mice, we microinjected insulin or vehicle to the VTA 10 min prior to chow exposure. Intra-VTA administered insulin did not alter food consumption within the first hour or total 4 h period compared to vehicle-administered or naïve controls (Fig. 8B; $P > 0.05$). To test if intra-VTA insulin modulated sated feeding, we used a feeding paradigm whereby sated mice

voluntarily overconsume a palatable test diet (Choi *et al.*, 2010). Insulin or vehicle was administered intra-VTA after mice consumed their caloric needs in the 4 h period and prior to a 1 h exposure to sweetened high fat food. Mice consumed over half their total intake within the first hour of chow access and gradually tapered their consumption over the remaining 3 h (Fig. 8C, left). After exposure to sweetened high fat food, vehicle-treated mice consumed a similar quantity of sweetened high fat food as during the first hour of regular chow access (1.9 ± 0.1 g versus 2.1 ± 0.1 g, respectively; Fig. 8C; $P > 0.05$), indicating that mice can consume sweetened high fat food even when sated. In contrast, insulin administered intra-VTA significantly reduced the amount of sweetened high fat food consumed during the 1 hr exposure compared to vehicle-treated mice (0.8 ± 0.1 g; Fig. 8C; $P < 0.001$). Taken together, these data suggest that insulin in the VTA can inhibit sated consumption of sweetened high fat food.

Discussion

Circulating peptides such as insulin, leptin, and ghrelin can alter feeding behavior by modulation of reward circuitry (Figlewicz & Benoit, 2009). Here, we demonstrate that insulin suppressed $[DA]_o$ in the VTA in a concentration-dependent manner by activating PI3K and mTOR signalling. Insulin-mediated suppression of $[DA]_o$ resulted from increased DA reuptake through DAT, but not NET. Furthermore, insulin in the VTA attenuated sated feeding of sweetened high fat food.

Insulin suppresses DA in the VTA via an increase in DAT number or function

We tested if insulin-mediated suppression of $[DA]_o$ was due to a decrease in release of DA from somatodendrites or a result of increased DA uptake through either DAT, NET or both transporters. Several lines of evidences suggest that insulin-mediated suppression of $[DA]_o$ is due to an effect on DAT. Insulin-mediated suppression of $[DA]_o$ was calcium-independent, suggesting that insulin does not alter a release mechanism of DA. Further, insulin did not reduce $[DA]_o$ at all frequencies indicating that insulin-mediated suppression of $[DA]_o$ does not occur in a frequency-dependent manner. Insulin suppressed $[DA]_o$ at higher stimulation frequencies with no significant effect at lower frequencies, indicating that insulin-mediated inhibition of $[DA]_o$ is more effective in the presence of increased extracellular DA. Thus, one can speculate that insulin may suppress $[DA]_o$ during times of burst firing, a firing pattern which facilitates DA release in somatodendritic regions of the midbrain (Bjorkland & Lindvall, 1975; Kalivas & Duffy, 1991; Rice *et al.,* 1997; Jaffe *et al.*, 1998). In the striatum, peak $[DA]_o$ at high stimulation frequencies is primarily due to increased DA release as there is limited time for DA uptake between each stimulation pulse (Cragg & Greenfield, 1997). Therefore, the greater insulin effect at high stimulation frequencies could suggest that insulin may also alter DA release. However, in contrast to this idea, insulin-mediated suppression of $[DA]_o$ was completely abolished in the presence of the selective DAT inhibitor GBR 12909 or in mice lacking DAT. Notably, while GBR 12909 significantly increased the decay of the evoked current, there was not a significant increase in amplitude over the time course of the experiment. This may be because dopamine a) was able to sufficiently diffuse away from the electrode within the 5 min simulation intervals, b) was degraded to its metabolites that are less sensitive to the electrode, or c) was taken up by other

monoamine transporters. Regardless, in the presence of the selective DAT inhibitor or in mice lacking DAT, insulin did not suppress evoked $[DA]_o$. Taken together, these data support the hypothesis that insulin-mediated suppression of $[DA]_o$ is due to an increased reuptake of DA. These data are consistent with the effects of insulin in the striatum. Rats made hypoinsulinemic display decreased DA clearance (Owens *et al.*, 2005). Depletion of insulin reduced the ability of amphetamine to release DA via DAT (Williams *et al.,* 2007). Furthermore, insulin increased DA clearance via human DAT expressed in HEK 293 cells (Garcia *et al.*, 2005).

Interestingly, insulin inhibited somatodendritic $[DA]_o$ to a similar extent in VTA of mice lacking NET as in that of wild-type mice, suggesting that, in VTA, insulin does not modulate NET. In contrast, insulin decreases [^{3}H]norepinephrine uptake in hippocampal slices (Figlewicz & Szot, 1991), PC12 cells (Figlewicz *et al.*, 1993) and whole-brain neuronal cultures (Boyd *et al.*, 1985, 1986). Conversely, insulin has been demonstrated to stimulate uptake of NE via NET in SK-N-SH cells (Apparsundaram *et al.,* 2001) and decrease NET surface expression in mouse hippocampal slices and superior cervical ganglion neuron boutons (Robertson *et al.,* 2010). Insulin in the nucleus accumbens potentiated the NE release-enhancing effects of desmethylimipramine (Schoffelmeer *et al.,* 2011). In summary, while our experiments found no effect of insulin on NET in the VTA, insulin likely modulates NET in other brain regions. This discrepancy may be due to decreased NET in the VTA compared to other brain regions (Lorang *et al.,* 1994) and sparce en passant rostral norepinephrinergic processes in the VTA (Cragg *et al.*, 1997.

Insulin activates insulin receptor tyrosine kinase, PI3K and mTOR to reduce $[DA]_o$

Insulin-mediated suppression of $[DA]_o$ was blocked by the cell permeable insulin receptor inhibitor, $HNMPA[AM]_3$. This compound acts at the tyrosine kinase phosphorylation site and does not distinguish between insulin-like growth factor 1 (IGF-1) and insulin receptors that are both present on VTA neurons and can both be activated by insulin (Figlewicz *et al.,* 2003; Quesada *et al.,* 2007). Thus, it is possible that IGF-1 receptors may contribute to the insulin-mediated decrease in somatodendritic $[DA]_o$.

Insulin administration in the VTA can lead to activation of PI3K and increases formation of phosphatidylinositol 3,4,5 phosphate (PIP3), the lipid product of activated PI3K (Figlewicz *et al.*, 2007). A recent study demonstrated that insulin-activation of PI3K is reduced in VTA of mice lacking insulin receptors in tyrosine hydroxylase-containing neurons (Konner *et al.,* 2011). We found that insulin-mediated suppression of $[DA]_o$ was blocked in the presence of wortmannin, a PI3K inhibitor. Consistent with these findings, insulin and subsequent signalling through the PI3K-dependent pathway has previously been implicated in increased expression of membrane-bound DAT and increased DAT clearance in the striatum (Carvelli *et al.*, 2002; Garcia *et al.,* 2005; Simon *et al.*, 1997). Taken together, our results support the notion that insulin signalling in the VTA via PI3K can regulate DAT function resulting in decreased somatodendritic DA.

Formation of PIP3 activates Akt which then activates the downstream mTOR pathway (Taha & Klip, 1999). We found that pretreatment with rapamycin, an mTOR blocker, inhibited insulin-mediated suppression of $[DA]_o$. Insulin signalling through rapamycin-sensitive

mTOR complex 1 has been implicated in a variety of activities in the brain including increasing hippocampal spine formation (Lee *et al.,* 2011), as well as integrating nutrient and hormonal signals in the hypothalamus with food intake (Cota, 2009). While the functionally distinct rapamycin-insensitive mTOR complex 2 has been implicated in trafficking of NET due to its ability to be phosphorylated by Akt (Siuta *et al.*, 2010), our results demonstrate a novel role for rapamycin-sensitive mTOR in regulating DA clearance.

Because mTOR signalling is implicated in protein translation and previous work has shown that insulin administered intracerebroventricularly increases DAT mRNA in the VTA (Figlewicz *et al.,* 1994), we tested if protein synthesis was required for insulin-mediated suppression of $[DA]_o$. We demonstrated that *de novo* synthesis of DAT was not required, because insulin-mediated suppression of $[DA]_o$ was unaffected in the presence of cycloheximide, a non-selective protein synthesis inhibitor used at a concentration and in a manner which blocks protein synthesis in VTA slices (Argilli *et al.*, 2008). Notably, cycloheximide did not interfere with evoked DA release or clearance when applied alone. Thus, it is likely that insulin-mediated suppression of $[DA]_o$ is due to an increase in DAT function or trafficking to the plasma membrane. DAT is known to be dynamically regulated at the cell membrane by various intracellular signals (Carvelli *et al.,* 2002; Foster *et al.,* 2006; Quick, 2006; Blakely & Bauman, 2000). Furthermore, membrane-bound DAT can be functionally altered by phosphorylation of various protein kinases including PKA, protein kinase C, PI3K, Akt, tyrosine kinases and protein phosphatase PP1/PP2Ac (Carvelli *et al.*, 2002; Foster *et al.*, 2003; Moron *et al.*, 2003; Melikian, 2004; Hoover *et al.*, 2007). Taken together, our results are consistent with an insulin-mediated increase in DAT trafficking or membrane function resulting in enhanced DA clearance.

Insulin in the VTA attenuates sated hedonic feeding

We investigated the behavioral significance of insulin-induced suppression of $[DA]_o$ in the VTA to examine how insulin alters hungry or sated feeding. Insulin administered into the VTA did not alter hungry feeding. Consistent with this finding, Figlewicz *et al.* (2008) determined that the arcuate nucleus of the hypothalamus, but not the VTA, was responsible for insulin-mediated reduction of food self-administration. Furthermore, insulin in the VTA suppressed feeding only modestly at the highest dose within a 24 hour feeding period (Bruijnzeel *et al.*, 2011).

Interestingly, in contrast to the lack of effect of insulin on hungry feeding, insulin administered to the VTA reduced sated consumption of sweetened high fat food. This finding is supported by several other studies demonstrating that insulin in the VTA can modulate hedonic feeding. Firstly, intra-VTA insulin reduced opioid-stimulated feeding, a model known to stimulate consumption of palatable foods (Figlewicz *et al.,* 2008). Secondly, insulin in the VTA decreased sucrose self-administration in rats that were not food deprived (Figlewicz *et al.,* 2008). Third, rats given intracerebroventricular insulin spent less time in a context associated with high-fat food consumption (Figlewicz *et al.*, 2004). Fourth, mice lacking insulin receptors on tyrosine hydroxylase-containing neurons had increased sensitivity to sucrose solutions (1% and 2%) compared to controls (Konner *et al.,* 2011).

Palatable food consumption increases DA levels to a greater extent than hungry feeding (Wilson *et al.,* 1995). Because insulin was more effective at reducing $[DA]_o$ in the VTA under higher stimulation frequencies resulting in increased DA release, one can speculate that potentiated DA levels associated with sweetened high fat food consumption may be suppressed by insulin's effect on DAT. In contrast, the effects of insulin on feeding in hungry mice may be minimal due to less DA availability.

Conclusion

In summary, the present findings demonstrate for the first time that insulin reduced somatodendritic DA in the VTA via PI3K and mTOR signaling which led to an increase in trafficking or function of DAT. Furthermore, insulin in the VTA reduced hedonic feeding. A recent study demonstrated that application of exogenous insulin increased tonic firing in approximately half of VTA/substantia nigra neurons (Konner *et al.*, 2011). This effect may be due to increased insulin-induced DAT activity leading to higher DA clearance resulting in reduced DA action at D2 autoreceptors and disinhibition of DA neurons. However, this effect would seem inconsistent with insulin suppression of hedonic feeding.

Somatodendritically-released DA can act at presynaptic D_1-like receptors on glutamatergic inputs to the VTA to increase glutamate release (Kalivas & Duffy, 1991, Kalivas & Duffy, 1995). Increased glutamate is required for burst firing of DA neurons (Suaud-Chagny et al., 1992). Burst-like firing and associated transient increases of DA in VTA and its projection areas is triggered by salient environmental stimuli and can facilitate motivated behaviours, such as food intake (Overton & Clark, 1997). Thus, an insulin-mediated reduction of somatodendritic DA in the VTA may reduce stimulation of presynaptic D_1-like receptors controlling glutamate release (Kalivas & Duffy, 1991). This may reduce the effectiveness of excitatory inputs into the VTA in mediating burst firing (Johnson *et al.,* 1992; Murase *et al.*, 1993). As a result, the reduced probability of burst firing in the presence of insulin would likely diminish the salience attributed to food related cues (Overton & Clark, 1997). Interestingly, insulin was more efficacious at reducing somatodendritic DA during stimulation at frequencies mimicking burst firing. Furthermore, while insulin did not disrupt hungry feeding, sated feeding (where the salience of the food would drive feeding behaviour) was attenuated. Taken together, insulin-mediated suppression of VTA DA may reduce the probability of burst firing in response to salient stimuli, resulting in a reduced consumption of food when sated.

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Abbreviations

aCSF	artificial cerebrospinal fluid
DA	dopamine

[DA]o	extracellular dopamine concentration
DAT	dopamine transporter
GABA	γ-aminobutyric-acid
mTOR	mammalian target of rapamycin
NE	norepinephrine
NET	norepinephrine transporter
PI3K	phosphatidylinositol 3 kinase
VTA	Ventral tegmental area

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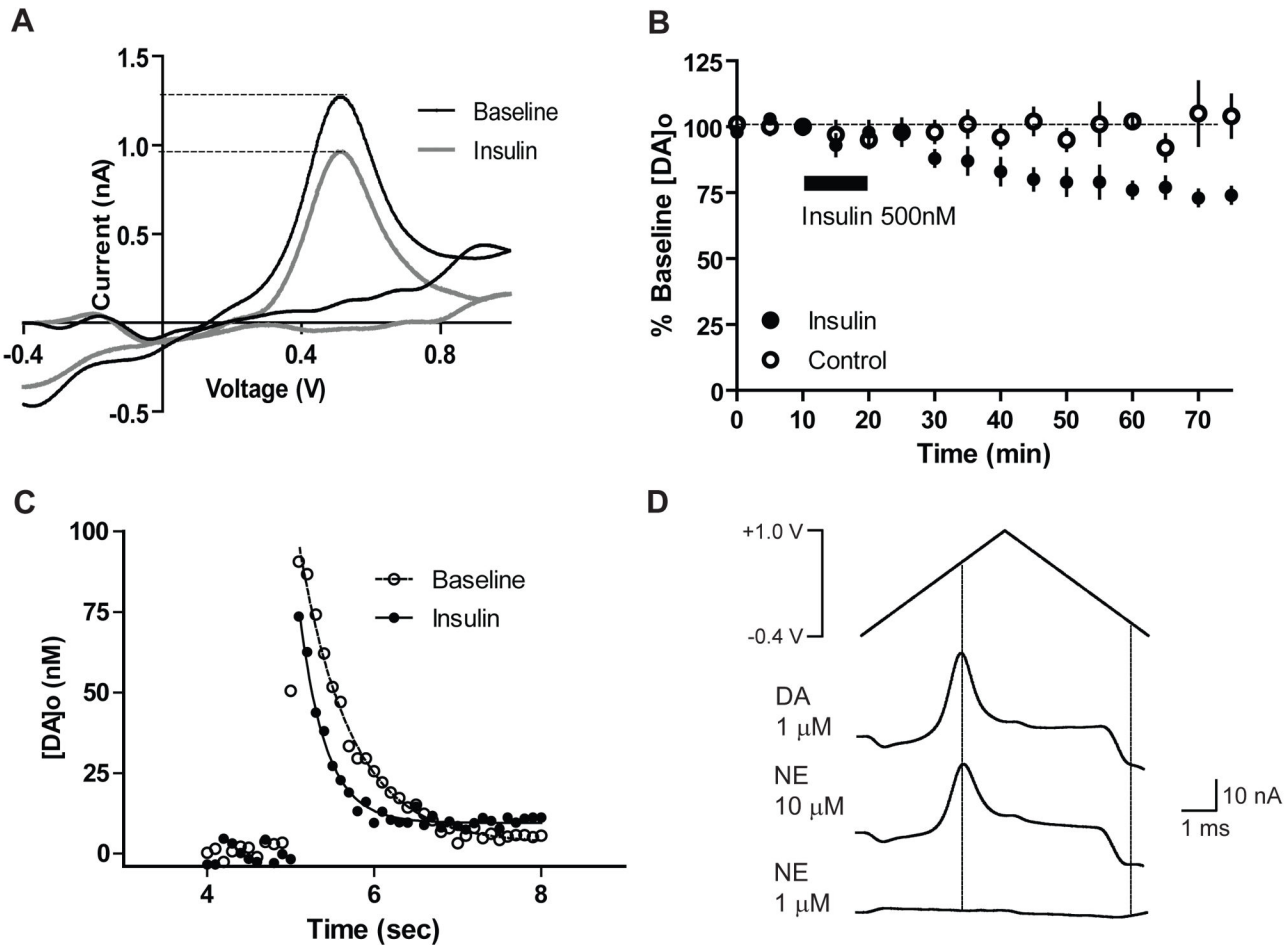

Figure 1. Insulin attenuates evoked somatodendritic DA

A. An example voltammogram from a single experiment for electrically evoked $[DA]_o$ before (black line) and 40 min after insulin (500 nM, grey line) application. **B.** Bath application of insulin (500 nM, 10 min, filled circles) inhibited evoked $[DA]_o$ in VTA slices (n=6) compared to control slices (n=6, open circles) ($P < 0.05$). **C.** A representative current-time plot from a single experiment showing DA evoked before (open circles) and 40 min after (filled circles) application of insulin (500 nM). The signals decay was fit with a one phase exponential curve to determine the rate of decay 5 min before insulin application (r^2=0.99) and 40 min after insulin application (r^2=0.98). **D.** A triangular waveform (−0.4 to 1.0 V vs Ag/AgCl at 400 V/s) was used to measure catecholamine concentrations with FSCV. An example experiment showing carbon fiber electrodes were 10 fold more sensitive to exogenously applied DA (1 μM) than norepinephrine (NE, 1, 10 μM). Symbols represent mean and bars represent SEM.

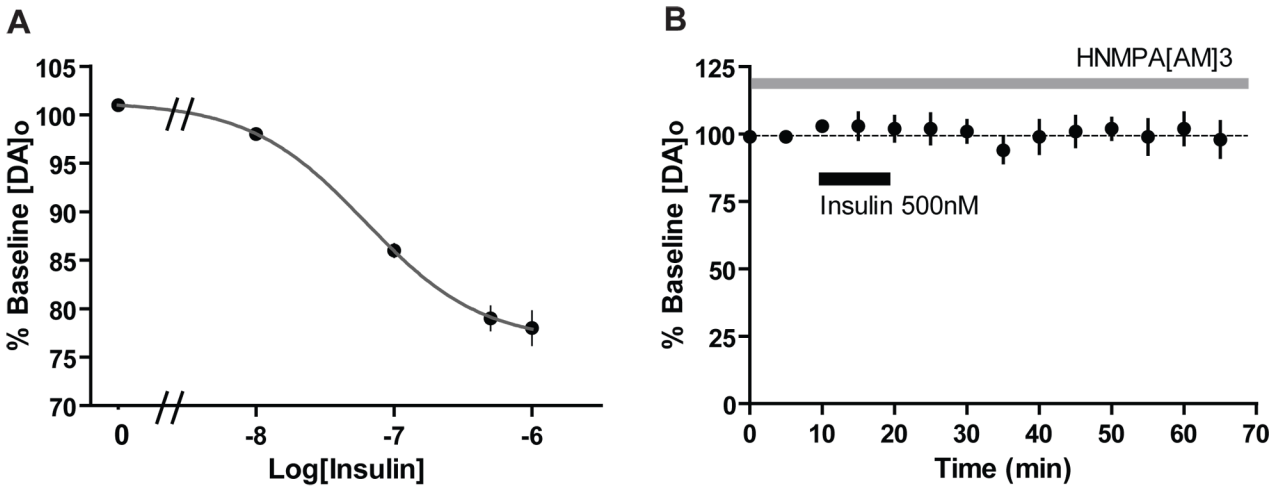

Figure 2. Insulin-mediated suppression of $[DA]_o$ is concentration-dependent

A. Insulin produced a concentration-dependent decrease of the evoked somatodendritic $[DA]_o$ (n=5 for 10nM, 100nM, and 1000nM; n=6 for 500nM). The maximal effect was measured 40 min after insulin application. **B.** Slices were pre-incubated with $HNMPA[AM]_3$ (300 μM), an inhibitor of insulin receptor tyrosine kinase, for 1 hour. $HNMPA[AM]_3$ blocked insulin-mediated suppression of somatodendritic $[DA]_o$ (n=7). Symbols represent mean and bars represent SEM.

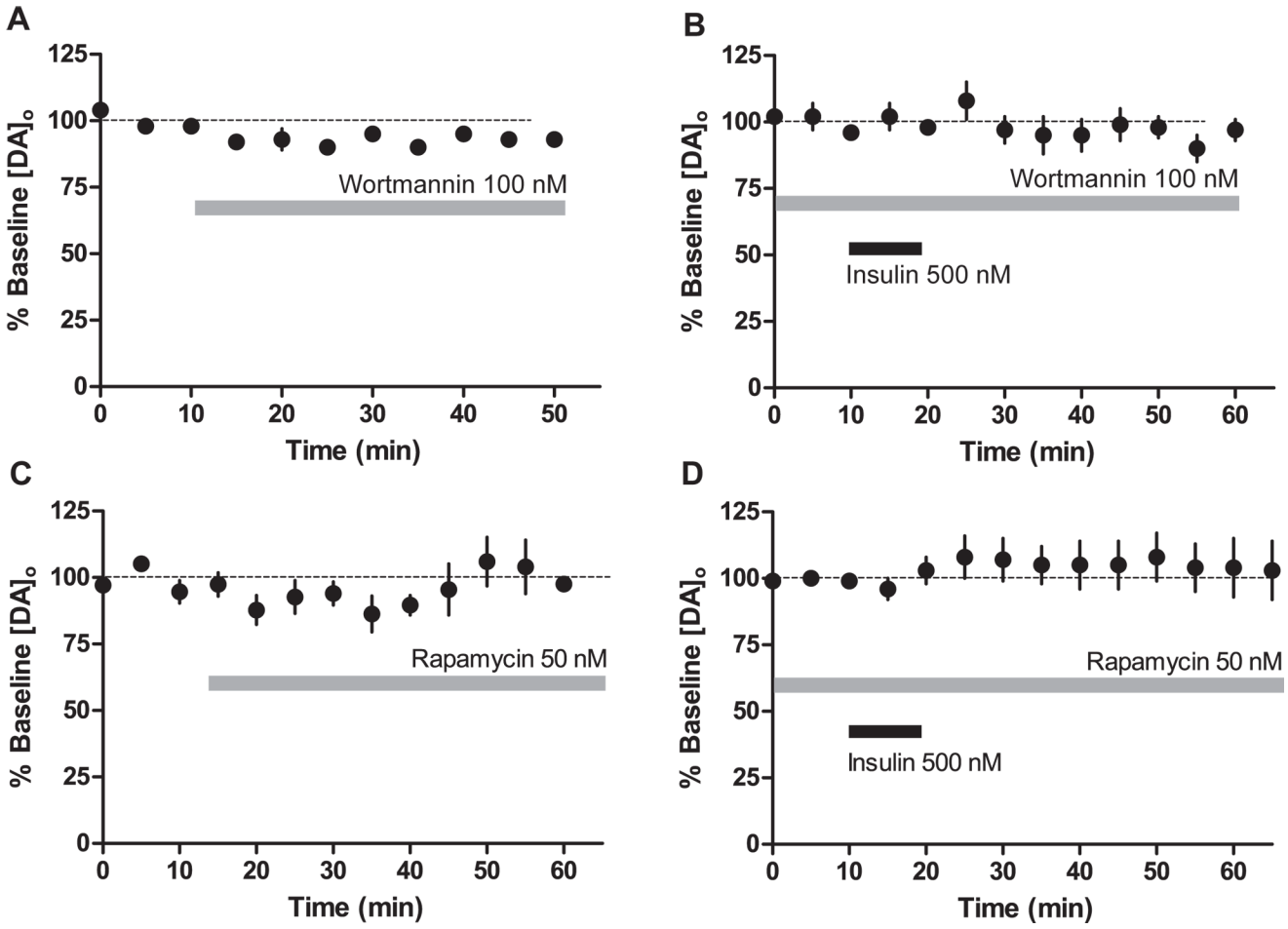

Figure 3. Insulin decreases evoked somatodendritic $[DA]_o$ via PI3K and mTOR

A. The PI3K inhibitor, wortmannin (100 nM) applied for 40 min to the VTA slices did alter evoked $[DA]_o$ (n=4). **B.** Slices were pre-incubated with wortmannin (100 nM) 30 min prior to and during recording. Insulin (500 nM, 10 min) did not inhibit evoked $[DA]_o$ in the presence of wortmannin (n=6). **C**. The mTOR blocker, rapamycin (50 nM) applied for 60 min to VTA slices did not produce significant changes in evoked $[DA]_o$ (n=5). **D.** Slices were incubated with rapamycin (50 nM) 20 min prior to and during recording. Insulin (500 nM, 10 min) did not inhibit evoked $[DA]_o$ in the presence of rapamycin (n=7). Symbols represent mean and bars represent SEM.

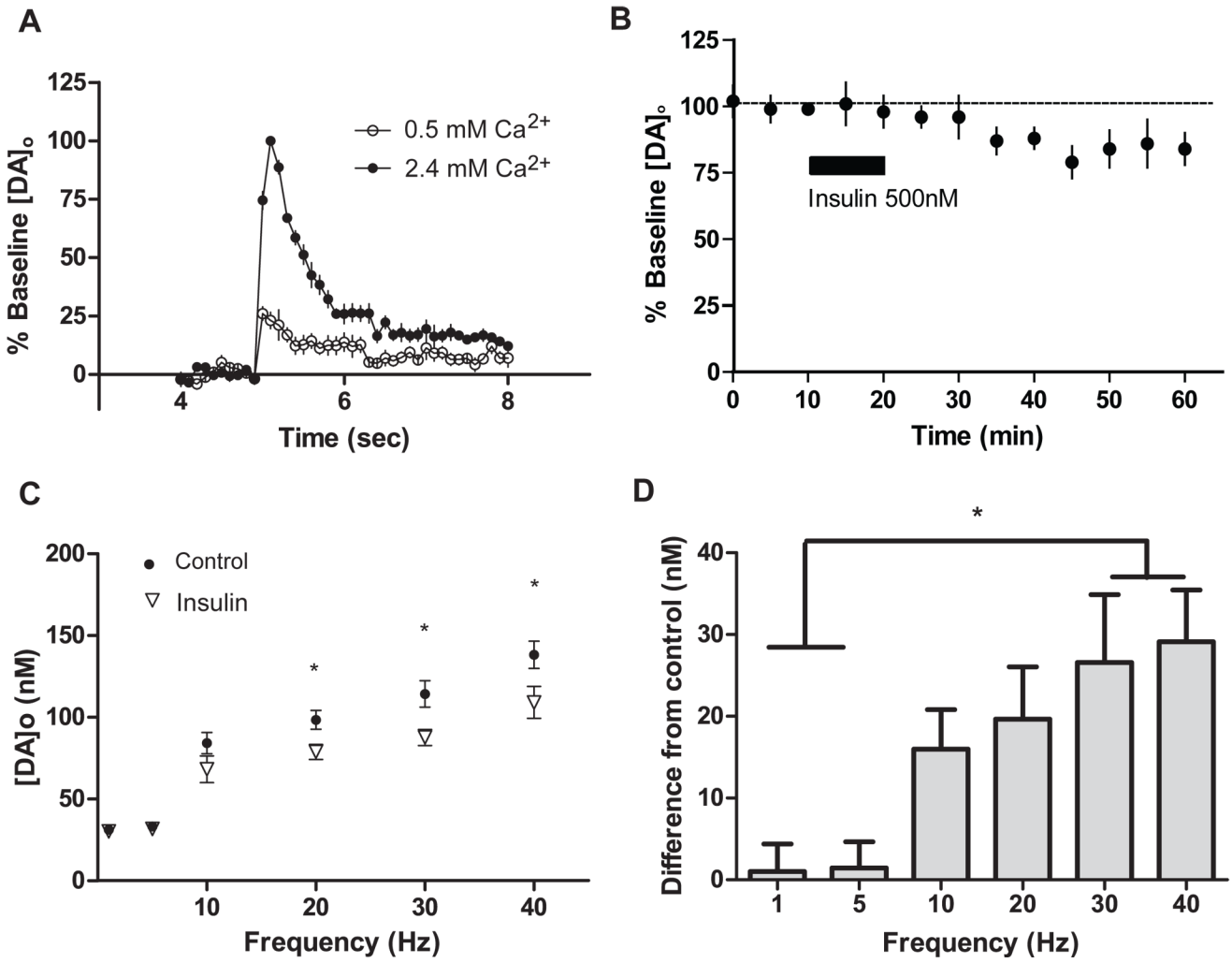

Figure 4. Insulin suppression of $[DA]_o$ is Ca^{2+}-independent and suppression is greater at higher stimulation frequencies

A. Current-time plot indicating that somatodendritic $[DA]_o$ was evoked in 0.5 mM external Ca^{2+} (open circles, n=4) or 2.4 mM external Ca^{2+} (filled circles, n=4). **B.** In the presence of 0.5 mM external Ca^{2+}, insulin (500 nM, 10 min) suppressed $[DA]_o$ (n=6). **C.** Somatodendritic $[DA]_o$ increased with increasing pulse frequency (filled triangles, n=4). The maximal effect of insulin (500 nM, 10 min) measured 40 min after application was greater at higher frequencies (open triangles; n=4). **D.** The difference between maximal-evoked $[DA]_o$ before and 40 min after insulin (500 nM, 10 min) observed at each frequency calculated based on data from C (*$P < 0.05$, one way ANOVA). Bars represent means and SEM from 4 slices.

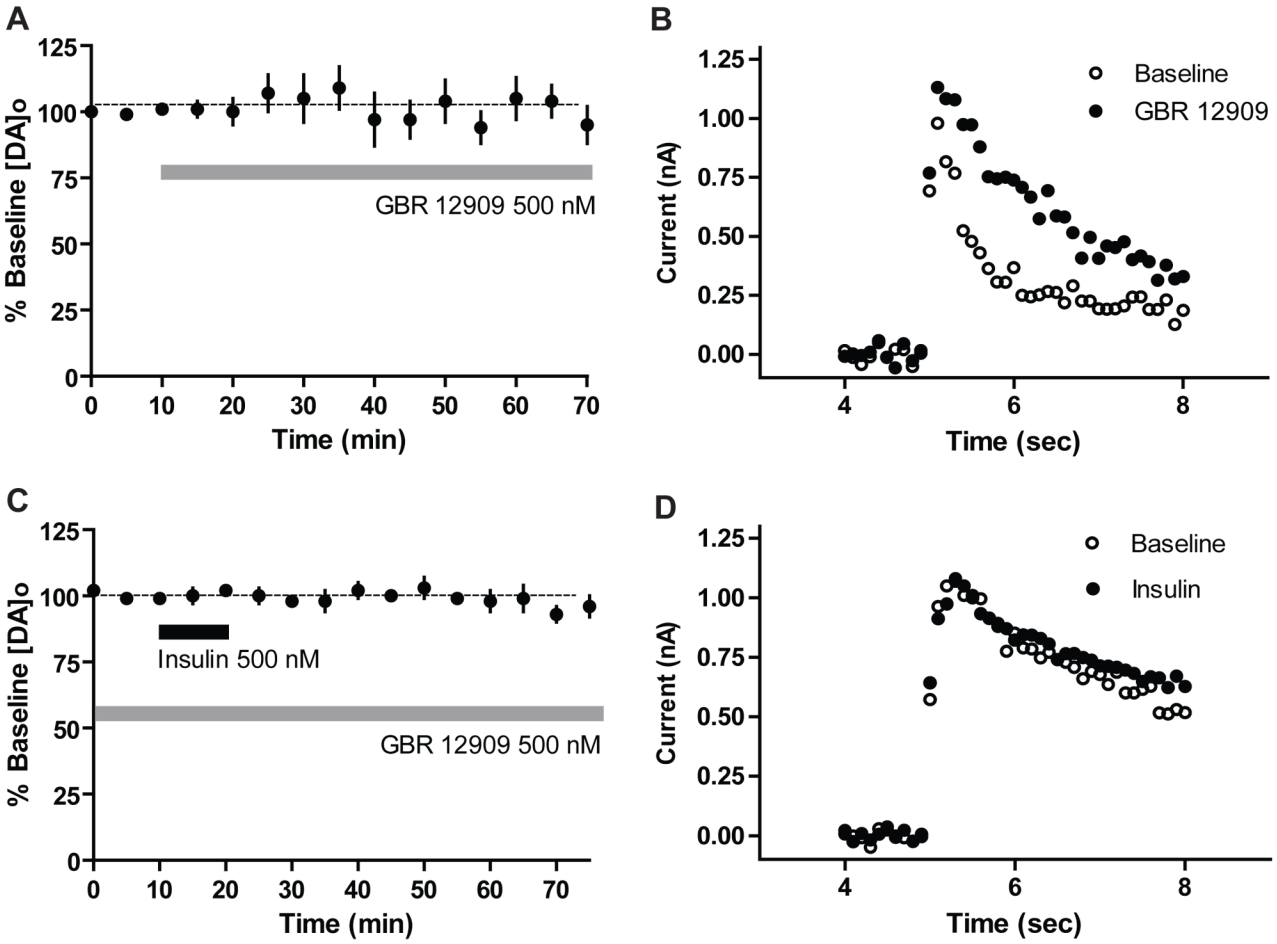

Figure 5. Insulin-suppression of somatodendritic $[DA]_o$ is absent in the present of a DA transporter (DAT) inhibitor

A. GBR 12909 (500 nM), a selective DAT blocker, did not alter the peak concentration of evoked DA (n=6). **B.** Representative current time plots of evoked $[DA]_o$ from a single slice show GBR 12909 (500 nM, filled circles) 50 min after application prolonged DA reuptake compared to $[DA]_o$ evoked prior to GBR 12909 application (open circles). **C.** VTA slices were preincubated with GBR 12909 (500 nM) 1 hr prior to and during recording. In the presence of GBR 12909, insulin-mediated suppression of $[DA]_o$ was abolished (n=5). **D**. An example current time plot demonstrating the reuptake of DA was not significantly different before (open circles) or after insulin (500 nM, filled circles) application in the presence of GBR 12909 (500 nM). Symbols represent mean, bars represent SEM.

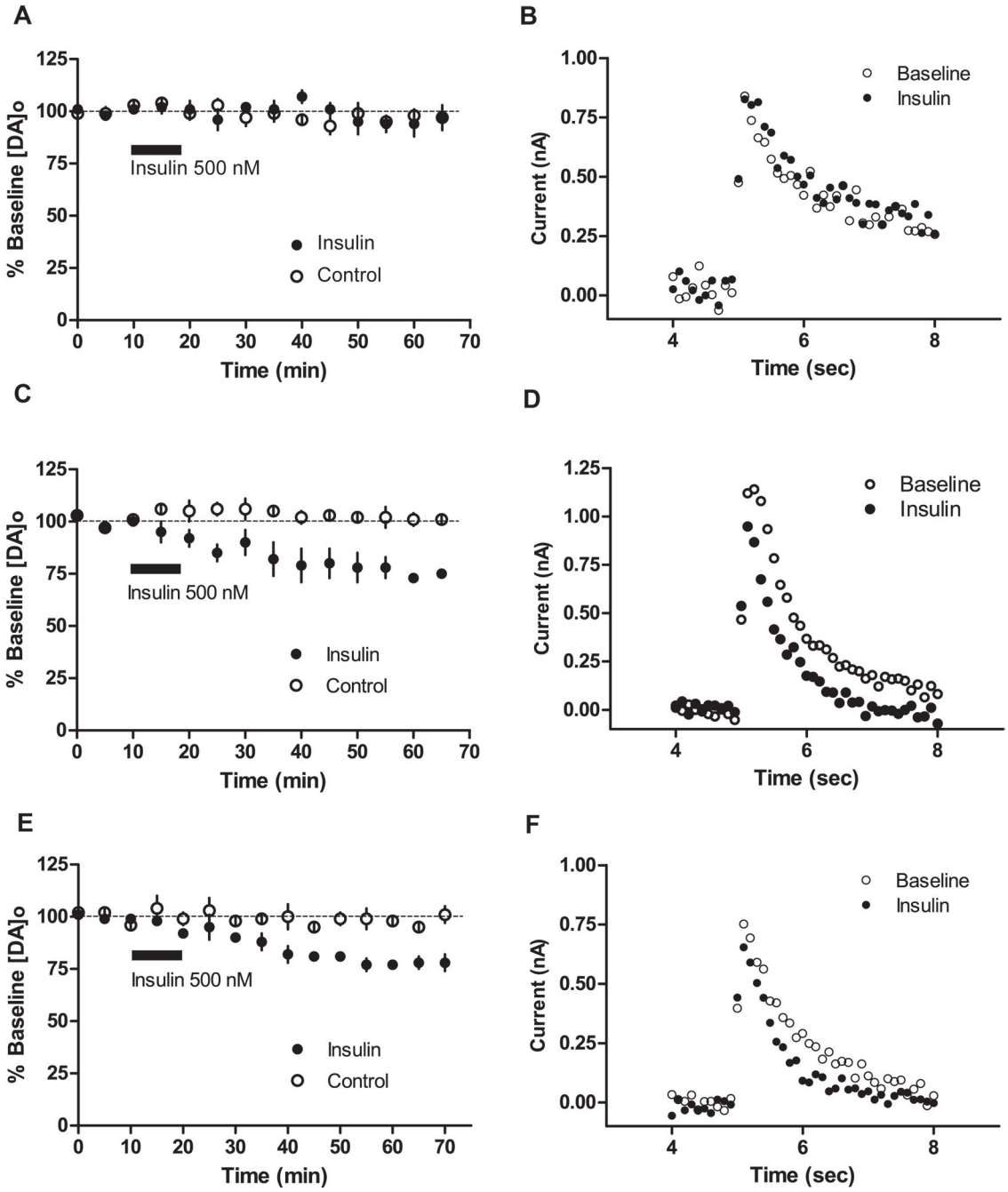

Figure 6. Insulin increases the reuptake of DA via DAT

A. Insulin (500 nM, 10 min, n=5) did not significantly alter evoked $[DA]_o$ compared to slices treated with vehicle (control; open circles, n=4) in VTA slices of DAT −/− mice ($P > 0.05$). **B**. A representative current-time plot of evoked $[DA]_o$ before (open circles) and 40 min after insulin (filled circles) application in VTA slices of DAT −/− mice. **C.** Insulin significantly inhibited evoked $[DA]_o$ (filled circles, n=5) compared to controls (open circles, n=4) in VTA slices of NET −/− KO mice ($P < 0.05$). **D.** A representative current-time plot before (open circles) and 40 min after insulin application (filled circles) to VTA slices of

NET −/− mice. **E**. Insulin significantly decreased evoked $[DA]_o$ (filled circles, n=6) compared to controls (open circles, n=3) in the wild-type mice ($P < 0.05$). **F.** A representative current-time plot showing DA release and reuptake before (open circles) and 40 min after insulin (500 nM) application to VTA slices of wild-type mice. Symbols represent mean, bars represent SEM.

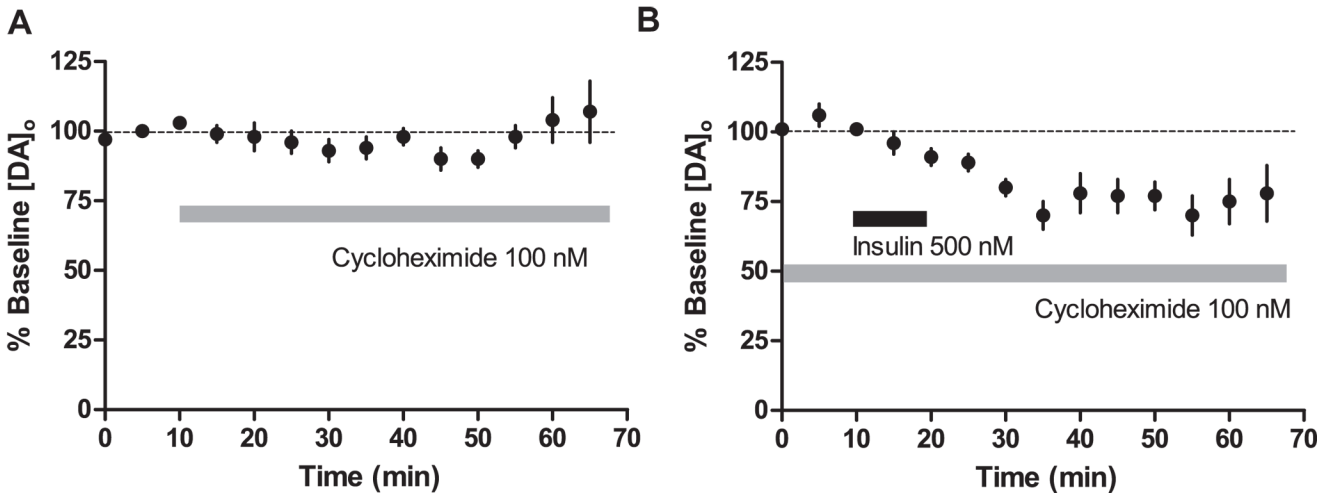

Figure 7. Insulin-mediated reduction of $[DA]_o$ is independent of protein synthesis

A. Protein synthesis inhibitor, cycloheximide (100 nM), applied for one hour to the VTA slices did not produce any significant changes in the evoked $[DA]_o$ (n=8). **B.** VTA slices were incubated with cycloheximide (100 nM) 20 min prior to and during recording. Insulin (500 nM, 10 min) suppressed evoked $[DA]_o$ in the presence of cycloheximide (n=8).

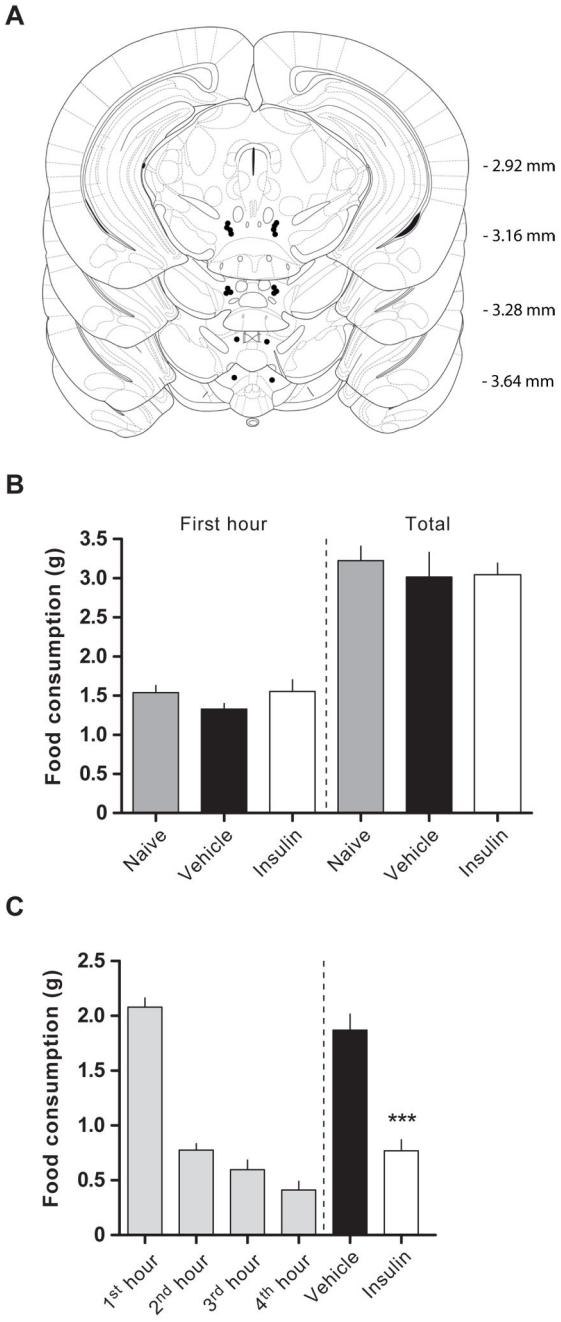

Figure 8. Intra-VTA microinfusion of insulin reduces hedonic feeding

Mice were entrained to consume their daily caloric needs within 4 hours of food access. **A.** Reconstructed injection sites in the VTA are shown in coronal sections. Distance from bregma is shown to the right of each section (in millimeters). **B.** Animals microinjected with vehicle (filled bars, n = 6) or insulin (open bars, n = 6) in VTA 10 min prior to food access did not consume significantly different amounts of regular chow during either the first hour (left) or total period of food access (right) compared to naive mice (shaded bars, n=6, $P <$ 0.05). **C.** Mice consumed at least 50 % of their daily food within the first hour of food

access and ate progressively less towards the end of the 4 hour food-access (left). Subsequent intra-VTA administration of insulin (open bars, n = 9) significantly decreased the 1 hour sated consumption of sweetened high-fat food compared to vehicle-treated mice (right; filled bars, n = 8, $P < 0.001$). Bars represent mean ± s.e.m. A one-way ANOVA followed by a Bonfferoni's multiple comparisons test was used.