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Interactions between insulin and diet on striatal dopamine uptake kinetics in rodent brain slices

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Abstract

Diet influences dopamine transmission in motor- and reward-related basal ganglia circuitry. In part, this reflects diet-dependent regulation of circulating and brain insulin levels. Activation of striatal insulin receptors amplifies axonal dopamine release in brain slices, and regulates food preference *in vivo*. The effect of insulin on dopamine release is indirect, and requires striatal cholinergic interneurons that express insulin receptors. However, insulin also acts directly on dopamine axons to increase dopamine uptake by promoting dopamine transporter (DAT) surface expression, counteracting enhanced dopamine release. Here we determined the functional consequences of acute insulin exposure and chronic diet-induced changes in insulin on DAT activity after evoked dopamine release in striatal slices from adult *ad-libitum* fed (AL) rats and mice, and food-restricted (FR) or high-fat/high-sugar obesogenic (OB) diet rats. Uptake kinetics were assessed by fitting evoked dopamine transients to the Michaelis-Menten equation and extracting C_{peak} and V_{max} . Insulin (30 nM) increased both parameters in the caudate putamen and

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and uptake in reward-related pathways (Mebel *et al.*, 2012; Labouebe *et al.*, 2013; Stouffer *et al.*, 2015; Fordahl *et al.*, 2017). Insulin receptors (InsRs) are expressed in midbrain DA neurons (Figlewicz *et al.*, 2003), and mediate the effects of insulin on DA neurons in the ventral tegmental area (VTA) to decrease feeding behavior (Mebel *et al.*, 2012; Labouebe *et al.*, 2013). In addition, physiological concentrations of insulin (i.e., 10 to 30 nM) that are typically reached in the CSF and brain after feeding (Strubbe *et al.*, 1988; Banks and Kastin, 1998; Woods *et al.*, 2003; Banks, 2004) enhance evoked $[DA]_o$ throughout the rat striatal complex with the greatest effect in the ventral striatum (Stouffer *et al.*, 2015; Fordahl *et al.*, 2017), which also has the highest levels of InsR expression (Werther *et al.*, 1987; Schulingkamp *et al.*, 2000).

The mechanism by which insulin enhances evoked increases in $[DA]_o$ in the striatum is indirect through InsRs on striatal cholinergic interneurons (ChIs); InsR activation increases ChI excitability and subsequently boosts DA release induced by nAChRs on DA axons (Stouffer *et al.*, 2015). Moreover, the sensitivity of evoked $[DA]_o$ to insulin is markedly enhanced with FR, in which plasma insulin levels are lower than in AL, whereas the response to insulin is absent in slices from rats on an OB diet in which plasma insulin levels are higher than in AL (Stouffer *et al.*, 2015). Thus, chronic diet-dependent changes in circulating insulin levels appear to alter acute insulin signaling in both motor and reward related pathways of the brain, although other nutritive factors including glucose and leptin that are also chronically altered with feeding state could play a role. Importantly, intact insulin signaling in the ventral striatum is required for the development of flavor preference, which has implications for food choice (Stouffer *et al.*, 2015; Woods *et al.*, 2016).

As well as enhancing striatal DA release, insulin also increases dopamine transporter (DAT) trafficking to the plasma membrane via a PI3-kinase (PI3K) and Akt signaling pathway, thereby increasing overall DAT activity and DA uptake (Carvelli *et al.*, 2002; Garcia *et al.*, 2005; Schoffelemeier *et al.*, 2011; Mebel *et al.*, 2012; Jones *et al.*, 2017). However, little is known about how opposite changes in circulating plasma insulin levels with FR *versus* OB diets *in vivo* influence striatal DAT activity in intact, *ex vivo* striatal slices and DAT responsiveness to acute insulin exposure. Elucidating these interactions is important because of the role of insulin in feeding behavior (Stouffer *et al.*, 2015; Woods *et al.*, 2016), and also because the DAT is instrumental in mediating the effects of psychostimulant drugs (e.g., Sulzer, 2011).

To assess DAT activity, we fitted the initial portion of the falling phase of evoked increases in $[DA]_o$ to the integrated form of the Michaelis-Menten equation to extract V_{max} (maximal uptake velocity, which is proportional to the number of functional DATs), as well as the accurate peak $[DA]_o$ (C_{peak}) for each record (*see* Li *et al.*, 2010). The primary data set analyzed were from Stouffer *et al.* (2015), in which data were presented mainly as measured peak evoked $[DA]_o$ normalized to the appropriate control for each condition. Here we focused on the effects of insulin and diet on DA uptake, and report unpublished V_{max} values and computed peak evoked $[DA]_o$ extracted from the original data set, using Michaelis-Menten analysis. For comparison, we have also illustrated the basic effect of insulin on V_{max} under control conditions using data that were previously reported in table form (Stouffer *et al.*, 2015). Overall, this new analysis allowed us to assess the role of the PI3-kinase/Akt

signaling pathway and feeding state on DAT-dependent uptake to complement our previous studies of the effects of insulin on DA release via activation of ChIs. Lastly, we compared DAT activity between AL-fed rats and mice, and assessed the influence of insulin on DA uptake in the absence of striatal acetylcholine (ACh).

Methods

Animals:

All animal procedures followed NIH guidelines and were approved by the NYU Langone Health Animal Care and Use Committee. Animals were kept on a 12 h light:dark cycle, with lights on from 06:00 to 18:00 local time. Male Sprague-Dawley Rats (Taconic) were normally housed in pairs but were singly housed for comparison of the effects of AL, FR and OB diets. Generation of mice lacking choline acetyltransferase (ChAT) and thus ACh in the forebrain (Nkx2.1^{Cre};ChAT^{flox/flox}) or non-mutant littermate controls (Nkx2.1^{Cre};ChAT^{flox/flox} or Nkx2.1^{Cre};ChAT^{flox/+}) was as described previously (Patel *et al.*, 2012); male mice were examined and were housed in groups of five or fewer.

Diets:

As described previously (Stouffer *et al.*, 2015), adult rats (8–10 weeks old) were assigned to AL, FR or OB diet regimens such that initial body weights were similar across groups. AL rats received both food (chow) and water *ad-libitum*. Rats on the FR diet received titrated amounts of chow to maintain body weight at 80% of initial weight but had free access to water. Rats on the OB diet were allowed free access to a moderately high-fat/high-sugar chocolate flavored liquid (Ensure), as well as chow and water. The data used for this study were taken from a subset of rats from each diet group for which plasma insulin values were available (n = 5 rats per group) (Stouffer *et al.*, 2015). Diets for these rats lasted a total of 21–32 days. FR rats exhibited significantly lower final body weights *versus* AL (one-way ANOVA, $F_{2,12} = 109.4$, with Dunnett's post hoc *vs.* AL; $P = 0.0001$) fed rats, whereas OB rats had higher final body weights than AL (one-way ANOVA, $F_{2,12} = 109.4$ with Dunnett's post hoc *vs.* AL; $P = 0.008$) fed rats (FR = 308 ± 3 g; AL = 456 ± 6 g; OB = 505 ± 15 g).

Plasma insulin:

At the time of decapitation for slice preparation (see below), trunk blood was collected in EDTA-containing tubes and centrifuged at $1500 \times g$ for 15 min. The plasma supernatant was then stored at -80°C until processing with an ALPCO Rat Insulin ELISA kit.

DA recordings:

Procedures for preparing *ex vivo* brain slices were as described in Patel *et al.* (2013) and Stouffer *et al.* (2015). All animals were deeply anesthetized with sodium pentobarbital (SleepAway) (50 mg/kg, i.p.) before decapitation and preparation of coronal striatal slices (400 μm thickness for rats, 300 μm for mice); these procedures were initiated between 08:00 and 10:00 each experimental day. Slices were allowed to recover at room temperature for exactly one hour in a HEPES-buffered aCSF containing (in mM): NaCl (120); KCl (5); NaHCO₃ (20); HEPES acid (6.7); HEPES sodium salt (3.3); MgSO₄ (2); glucose (10); CaCl₂ (2); equilibrated with 95% O₂/5% CO₂; two slices were then transferred to a

submersion recording chamber (Warner Instruments) superfused with a bicarbonate-buffered aCSF containing (in mM): NaCl (124); KCl (3.7); NaHCO₃ (26); MgSO₄ (1.3); KH₂PO₄ (1.3); glucose (10); CaCl₂ (2.4); and bovine serum albumin (0.1 mg/mL) equilibrated with 95% O₂/5% CO₂, and maintained at 32 °C. Slices were allowed to acclimatize to this environment for 30 min before recordings were initiated. [DA]_o evoked by local single-pulse electrical stimulation (0.1 ms duration) was monitored with 7 µm diameter carbon fiber electrodes and FSCV using a Millar Voltammeter scanning from –700 mV to +1300 mV to –700 mV at a rate of 800 V/s every 100 ms (Patel *et al.*, 2013; Patel, 2016). Initial recordings were made in three to five sites per region in the caudate putamen (CPu) and nucleus accumbens (NAc) core in each of two slices over 20 to 30 mins as an index of slice viability and to compare differences in DA release and uptake in feeding groups closer to the time of sacrifice. Immediately after this sampling, superfusion with either aCSF alone, aCSF plus insulin, aCSF plus test drug, or insulin plus test drug was begun; after 60 min, recording of evoked release in three to five sites per region was initiated in the continued presence of the superfusing solution. Responses were quantified by post-experiment calibration of carbon fiber electrodes with 1 µM DA in aCSF in the recording chamber at 32 °C. Release and uptake in the presence of insulin were compared to those obtained in the time-matched control experiments with aCSF alone or the test drug alone.

C_{peak} and V_{max} analysis:

The falling phase of single-pulse evoked DA transients detected by FSCV is governed by Michaelis-Menten uptake kinetics (Wightman *et al.* 1988; Wightman and Zimmerman, 1990; Wu *et al.*, 2001). To evaluate changes in DAT activity we used a MATLAB® script in which an integrated form of the Michaelis-Menten equation was solved and then fitted to the initial segment of the falling phase of single-pulse evoked [DA]_o curves (Figure 1) to extract V_{max} (maximal uptake velocity) as described previously (Li *et al.*, 2010). The value for K_m (which is inversely related to the affinity of the DAT for DA) was fixed at values obtained by prior FSCV studies which were 0.2 µM for rats (Wu *et al.*, 2001) or 0.9 µM for mice (Schmitz *et al.*, 2001). Previous studies have determined that K_m is similar across striatal subregions in a given species (Wu *et al.*, 2001). Importantly, K_m does not change with either FR or OB diets and is unaffected by insulin (Zhen *et al.*, 2006; Jones *et al.*, 2017). We also assumed that K_m was unaltered in ChAT-KO mice or with exogenous application of drugs used in these studies. During kinetic analysis, we found that the tail of some computed [DA]_o curves fell more rapidly than seen in the corresponding experimental record; this suggested that additional factors, including DA adsorption to the electrode, might play a role in prolonging the time course of experimental curves. To avoid this complication, we restricted the curve fitting to the initial portion of the falling phase (either three points for K_m = 0.2 µM or four points for K_m = 0.9 µM) after the peak (see Figure 1). It should also be noted that unlike some methods for evaluating V_{max} from FSCV data (e.g. Wu *et al.*, 2001), our method is not restricted to [DA]_o much greater than K_m.

A value for C_{peak} was also determined from the integrated Michaelis-Menten equation (Li *et al.*, 2010) because peak concentration is difficult to capture faithfully with 100 ms sampling intervals. These concentration values have not been reported previously. Goodness-of-fit and

coefficient of determination (R^2) values were estimated; R^2 values < 0.95 were rejected from the data sets reports here.

Drugs:

Salts for all buffers as well as insulin (from porcine pancreas), LY294002, and DA were purchased from Sigma Aldrich (St. Louis, MO, USA). Membrane permeable HNMPA (hydroxyl-2-naphthalenylmethylphosphonic acid trisacetoxymethyl ester) was from Enzo Life Sciences (Farmingdale, NY, USA), and picropodophyllotoxin (PPP) was from Tocris Bioscience (Bio-Techne Cooperation, Minneapolis, MN, USA).

Statistics:

Statistical analyses were performed using GraphPad Prism version 7.0. for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Data are expressed as means $\pm$ SEM or as individual measurements with means $\pm$ SEM, where n denotes the number of DA recording sites from three to five rats or mice per group. Significance of differences was assessed by unpaired Student's t -tests, with or without Welch correction for unequal variance if appropriate, for comparison of two groups, or by one-way ANOVA, as indicated; significance was considered to be $P < 0.05$.

Results

Insulin increases DA release and DAT activity

Using Michaelis-Menten kinetics, we calculated C_{peak} and V_{max} for DA release and uptake from evoked DA release transients monitored by FSCV. In the striatum of AL rats, average C_{peak} for single-pulse evoked $[DA]_o$ obtained in the absence and presence of insulin showed significant differences (one-way ANOVA, $F_{3,226} = 33.15$, $P < 0.0001$) as did V_{max} (one-way ANOVA, $F_{3,226} = 73.47$, $P < 0.0001$). C_{peak} was significantly higher in the CPu than in the NAc core, as was V_{max} (Figure 2). These data reflect the well documented decreasing dorsoventral gradient of DA axon innervation and DAT density (Ciliax *et al.* 1995; Gonzalez-Hernandez *et al.*, 2004; Marshall *et al.* 1990). Consistent with our previous report of increased evoked $[DA]_o$ with insulin (Stouffer *et al.*, 2015), the present Michaelis-Menten analysis shows a significant increase (unpaired two-tailed t -tests) in C_{peak} with insulin (30 nM) in both CPu ($P = 0.0003$) and NAc ($P < 0.0001$) (Figure 2A,B), despite the corresponding increase in V_{max} in both regions (CPu, $P = 0.0368$; NAc core, $P = 0.0020$) (Figure 2C) previously published in Table 1 of Stouffer *et al.*, 2015. Thus, the influence of insulin on DAT mediated DA uptake, which alone would be expected to decrease evoked $[DA]_o$, is outcompeted by enhanced DA release. Notably, this physiological concentration of insulin appears to be optimal for modulation of DA release and DA uptake since higher concentrations of insulin are less effective at augmenting FSCV-detected peak $[DA]_o$ or V_{max} (Stouffer *et al.*, 2015).

The effect of insulin on DAT activity and DA release requires InsRs and PI3-kinase

Our previous study demonstrated that the effect of insulin on evoked increases in $[DA]_o$ occurred via InsRs rather than signaling through insulin-like growth factor receptors (IGF-1Rs) (Stouffer *et al.*, 2015). It is also important to establish whether the effect of

insulin on $V_{\max}$ is mediated by InsRs, as well. Application of HNMPA (5 μM), an intracellular inhibitor of InsRs, had no effect on either $V_{\max}$ or C_{peak} (Figure 3). However, this InsR inhibitor blocked the effect of insulin on $V_{\max}$ and C_{peak} in both the CPu (unpaired one-tailed t-tests, $P = 0.2685$ for $V_{\max}$ and $P = 0.1098$ for C_{peak}) and NAc core (unpaired one-tailed t-tests, $P = 0.3192$ for $V_{\max}$ and $P = 0.4903$ for C_{peak}) (Figure 3). By contrast, insulin-induced increases in $V_{\max}$ (unpaired one-tailed t-tests, $P = 0.0378$ for CPu and $P = 0.0080$ for NAc core) and C_{peak} (unpaired one-tailed t-tests, $P = 0.0008$ for CPu and $P = 0.0001$ for NAc core) persisted in the presence of a selective IGF-1R inhibitor, PPP (1 μM) (Figure 3). Interestingly, PPP alone significantly decreased C_{peak} (CPu: one-way ANOVA, $F_{6,205} = 3.69$, $P = 0.0017$, with Bonferroni's post hoc, $P = 0.0033$; NAc core one-way ANOVA, $F_{6,255} = 5.092$, $P = 0.0017$, with Bonferroni's post hoc, $P = 0.0178$), but not $V_{\max}$ in both striatal subregions (Figure 3), implying that tonic activity at IGF-1Rs, presumably on ChIs, may play a part in regulating DA release.

The enhancement of DAT activity induced by insulin is in keeping with the established role of insulin in promoting surface expression of monoamine transporters, including DAT (Carvelli *et al.*, 2002; Garcia *et al.*, 2005; Jones *et al.*, 2017). This has been shown to involve activation of PI3-kinase and the Akt pathway (Carvelli *et al.*, 2002; Garcia *et al.*, 2005; Robertson *et al.*, 2010). Inhibiting PI3-kinase activity with LY249002 (1 μM) completely prevented insulin-induced increases in $V_{\max}$ and C_{peak} in both the CPu and NAc, without changing either parameter when applied alone (Figure 3). In fact, $V_{\max}$ was significantly lower when insulin was applied in the presence of LY249002 (unpaired one-tailed t-tests, $P = 0.0114$) (Figure 3), suggesting that insulin may also activate another as yet unknown mechanism/pathway that opposes DAT insertion and is only revealed when the PI3-kinase/Akt pathway is blocked.

Insulin's effect on DA release but not DAT activity is via ChIs

Our previous study revealed that the effect of insulin on evoked DA release was indirect by activating InsRs on striatal ChIs, increasing ChI excitability, and enhancing facilitation of DA release via nAChRs on DA axons (Stouffer *et al.*, 2015). Of course, DA axons, as well as ChIs express InsRs (Stouffer *et al.*, 2015), but whether the effect of insulin on DA uptake reflects a direct action or some indirect response to cholinergic activation has not been established. Because pharmacological nAChR agents can alter K_m and potentially confound data interpretation (Middleton *et al.*, 2007), we examined the effect of insulin on $V_{\max}$ and C_{peak} in mice that lack the ACh synthesizing enzyme ChAT in the forebrain (ChAT-KO) and therefore lack ACh release from otherwise intact ChIs (Patel *et al.*, 2012). Our analysis assumed no change in K_m for the DAT in ChAT-KO mice.

Interestingly, $V_{\max}$ was slightly lower (one-way ANOVA, $F_{3,107} = 12.26$, with Bonferroni's post hoc, $P = 0.0410$) in the CPu of ChAT-KO mice *versus* control mice (see Figure 4). Activation of nAChRs by nicotine has been shown to enhance DAT function, without increasing DAT surface expression (Middleton *et al.*, 2007). Consequently, lower $V_{\max}$ in KO mice could reflect loss of enhanced DA uptake by tonic nAChR activation that occurs in control mice. Importantly, the enhancing effect of insulin on both C_{peak} and $V_{\max}$ seen in AL rat CPu and NAc core was also seen in these regions in AL mice (Figure 4). Although

the effect of insulin on $V_{\max}$ persisted in ChAT-KO mice (unpaired one-tailed t-tests, $P=0.0035$ for CPu; $P<0.0001$ for NAc core), its enhancing effect on evoked DA release was lost in both CPu and NAc core (Figure 4). In fact, C_{peak} decreased significantly in the presence of insulin in both regions from ChAT-KO mice (unpaired one-tailed t-tests, $P=0.0003$ for CPu; $P=0.0179$ for NAc core), revealing the true consequence of insulin on increasing DAT activity when cholinergic transmission is absent (Figure 4). Thus, these data demonstrate that modulation of DAT activity by insulin is direct, in contrast to insulin regulation of DA release which is indirect via ChIs.

Striatal DA release and uptake kinetics in mice versus rats

We also took advantage of our FSCV data from AL mice and AL rats to compare C_{peak} and $V_{\max}$ in CPu and NAc core between these species obtained using the same methods. We found that C_{peak} was significantly greater in both striatal subregions from mice than from rats (one-way ANOVA, $F_{3,177}=32.4$, with Bonferroni's post hoc; CPu, $P<0.0001$, $1.63 \pm 0.09 \mu\text{M}$, $n=22$ sites from 3 mice vs. $1.25 \pm 0.03 \mu\text{M}$, $n=58$ recording sites from 9 rats; NAc core, $P=0.005$, $1.14 \pm 0.07 \mu\text{M}$, $n=28$ recording sites from 3 mice vs. $0.91 \pm 0.03 \mu\text{M}$, $n=73$ recording sites from 11 rats). Moreover, $V_{\max}$ values extracted from these same records were also significantly higher in mouse versus rat in both CPu and NAc core (one-way ANOVA, $F_{3,177}=164.0$ with Bonferroni's post hoc, $P<0.0001$ for both; CPu, $5.67 \pm 0.24 \mu\text{M/s}$ vs. $3.25 \pm 0.08 \mu\text{M/s}$; NAc core, $3.93 \pm 0.13 \mu\text{M/s}$ vs. $2.06 \pm 0.07 \mu\text{M/s}$). These data indicate greater DA release, as well as greater DAT activity in mice than in rats. In contrast, Calipari *et al.* (2012), also using FSCV, found no difference in $V_{\max}$ between rat and mouse, although lower $V_{\max}$ in monkey than in rodents. However this analysis used a single fixed value for K_m of $0.16 \mu\text{M}$ for all species. In the present study, C_{peak} and $V_{\max}$ were derived using species-dependent values of K_m . It is known that calculated K_m values can differ according to the methods used to obtain them as well as the tissue preparations examined (see Nicholson, 1995). The values used here were taken from two separate studies of evoked DA release transients using FSCV ($0.2 \mu\text{M}$ for rats from Wu *et al.*, 2001 and $0.9 \mu\text{M}$ for mice from Schmitz *et al.*, 2001). These values correlate extremely well with K_m values for DA inhibition of [^3H]-DA uptake obtained in cell lines expressing rat DAT ($K_m=0.32 \mu\text{M}$; Giros *et al.*, 1991) or mouse DAT ($K_m=0.93 \mu\text{M}$; Brüss *et al.*, 1999). Other studies in the literature find differences between K_m in rat versus human or bovine DAT (Lee *et al.*, 1996), possibly due to differences in N-linked glycosylation sites (Patel *et al.*, 1993), offering further support for the value of using species-dependent values for K_m .

FR and OB diets decrease DA release and DAT activity

We reported previously that single-pulse evoked $[\text{DA}]_o$ is significantly lower throughout the striatum of rats on either a FR or OB diet versus AL feeding, with no significant changes in DA tissue content among these diet groups (Stouffer *et al.*, 2015). Given that plasma insulin is positively correlated with body adiposity (Strubbe, 1988; Stouffer *et al.*, 2015; Woods *et al.*, 2016) and that brain insulin, which mirrors plasma insulin, modulates surface DAT expression, we hypothesized that diet might influence evoked $[\text{DA}]_o$ via changes in DAT activity. As expected, average plasma insulin in FR rats was significantly lower than ALs (one-way ANOVA, $F_{2,12}=9.518$, with Dunnett's post hoc vs. AL; $P=0.0252$), whereas plasma insulin with OB diet rats was generally higher, albeit not significantly ($P=0.3099$),

than ALs (FR: 0.36 ± 0.06 ; AL: 1.06 ± 0.19 ; OB: 1.40 ± 0.22 ng/mL, $n = 5$ rats per group). To assess the influence of diet and circulating insulin levels on DA release and uptake, FSCV recordings were made as soon as possible after preparation of brain slices (typically 90 min). It should be noted that C_{peak} in these early measurements are slightly lower than those made in fresh sites in slices that were in the recording chamber at 32°C for ~ 150 min for insulin and drug studies (compare C_{peak} values in Figure 5 with those in Figures 2 and 6). Higher levels in C_{peak} possibly reflect on-going DA synthesis in the recording chamber during prolonged incubation. *In vivo*, on-going DA neuron activity promotes DA release and thereby a DA tone at striatal D2 DA autoreceptors (Benoit-Marand *et al.*, 2001). By contrast, DA axons in striatal slices are no longer connected to DA neurons, so that on-going DA release is minimal, and slices lack a DA tone at D2 receptors (Patel *et al.*, 1992). Thus, minimal release coupled with lack of D2 feedback to dampen the rate of DA synthesis by tyrosine hydroxylase (Vulto & Fowler, 1986) would increase DA content. Although other factors could be involved, this is the most likely explanation for higher evoked $[\text{DA}]_o$ with increasing incubation time.

We found that the relationship between plasma insulin and C_{peak} or V_{max} values derived for individual evoked DA release transients in both the CPu and NAc core was an inverted U-shape that could be fitted to a second order polynomial (quadratic) (Figure 5A). Thus, with either low plasma insulin concentrations in FR rats or higher plasma insulin concentrations in OB rats, C_{peak} and V_{max} were lower than in AL-fed rats. Indeed, in both the CPu and NAc, average C_{peak} was significantly lower in rats on the FR diet compared to AL-fed rats (CPu: one-way ANOVA, $F_{2,106} = 28.03$, with Bonferroni's post hoc, $P < 0.0001$; NAc core: one-way ANOVA, $F_{2,118} = 12.94$, with Bonferroni's post hoc, $P < 0.0001$) and was also significantly lower in OB rats *versus* AL (CPu: one-way ANOVA, $F_{2,106} = 28.03$, with Bonferroni's post hoc, $P = 0.0001$; NAc core: one-way ANOVA, $F_{2,118} = 12.94$, with Bonferroni's post hoc, $P = 0.0065$) (Figure 5B). Notably, average V_{max} values were generally lower in CPu (CPu: one-way ANOVA, $F_{2,106} = 15.12$, $P < 0.0001$) and NAc core (NAc core: one-way ANOVA, $F_{2,118} = 6.505$, $P = 0.0021$) in both feeding groups (Figure 5B). Regardless of the cause, this observation eliminates enhanced DA uptake as a contributing factor to lower C_{peak} with either the FR or OB diet.

The effect of insulin on DAT activity is amplified in FR but blunted with OB diets

We next evaluated the effect of exogenous insulin application on peak evoked $[\text{DA}]_o$ and DAT-mediated uptake in these diet groups to test the hypothesis that InsR-dependent regulation of the DAT is chronically under-stimulated in FR rats but chronically over-stimulated and consequently insensitive in OB rats. In FR rats, insulin increased C_{peak} and V_{max} in both the CPu (C_{peak} : one-way ANOVA, $F_{3,107} = 38.08$ with Bonferroni's post hoc, $P < 0.0001$; V_{max} : one-way ANOVA, $F_{3,107} = 40.27$ with Bonferroni's post hoc, $P < 0.0001$) and NAc core (C_{peak} : one-way ANOVA, $F_{3,129} = 9.593$ with Bonferroni's post hoc, $P < 0.0001$; V_{max} : one-way ANOVA, $F_{3,129} = 10.6$ with Bonferroni's post hoc, $P < 0.0001$) (Figure 6A,B). Indeed, the effect of 30 nM insulin on both C_{peak} and V_{max} in FR rats was greater than in AL rats (*e.g.*, in CPu $\sim 60\%$ increase in C_{peak} in FR *vs.* $\sim 20\%$ in AL; and $\sim 50\%$ increase in V_{max} in FR *vs.* $\sim 16\%$ in AL). In contrast, the enhancing effects of insulin on C_{peak} and V_{max} were completely absent in OB rats in both the CPu (C_{peak} : one-way

ANOVA, $F_{3,107} = 38.08$ with Bonferroni's post hoc, $P > 0.9999$; $V_{\max}$: one-way ANOVA, $F_{3,107} = 40.27$ with Bonferroni's post hoc, $P = 0.2015$) and NAc core (C_{peak} : one-way ANOVA, $F_{3,129} = 9.593$ with Bonferroni's post hoc, $P > 0.9999$; $V_{\max}$: one-way ANOVA, $F_{3,129} = 10.6$ with Bonferroni's post hoc, $P = 0.3480$) (Figure 6A,B). Together, these data show that insulin-dependent regulation of DA uptake, as well as DA release (Stouffer *et al.*, 2015), is amplified by FR, but lost with an OB diet, reflecting consequences of dynamic changes in the sensitivity of InsR signaling pathways.

Discussion

We recently reported that exogenous insulin amplifies single-pulse evoked $[DA]_o$ by increasing ChI excitability and nAChR-dependent facilitation of DA release (Stouffer *et al.*, 2015). However, evoked $[DA]_o$ transients reflect a net balance between DA release and DA uptake. Given that insulin can promote DAT trafficking to the plasma membrane (Carvelli *et al.*, 2002; Garcia *et al.*, 2005; Schoffelmeer *et al.*, 2011; Mebel *et al.*, 2012; Jones *et al.*, 2017), we hypothesized that insulin-dependent regulation of $[DA]_o$ also includes changes in DAT-mediated DA uptake, and that dietary changes that chronically alter endogenous circulating, and therefore brain, insulin levels, should also affect DAT function and activity.

Our approach of fitting the initial part of the falling phase of evoked $[DA]_o$ to Michaelis-Menten kinetics allowed us to extract C_{peak} for DA release, as well as $V_{\max}$ for DA uptake. This analysis assumes that a relatively large volume of tissue surrounding the recording carbon-fiber microelectrode is at the same $[DA]_o$ so that diffusion does not contribute to DA clearance, as in previous models (Wightman *et al.* 1988; Wightman and Zimmerman, 1990; Wu *et al.*, 2001). The goodness of fit for the data presented here support the validity of this assumption; this is beneficial as the involvement of a diffusional component would require a much more complicated analysis (Nicholson, 1995). It should be noted that the C_{peak} values reported here are typically higher than peak evoked $[DA]_o$ obtained experimentally with FSCV, presumably because the 100 ms sampling interval used and imperfect response time of carbon-fiber microelectrodes prevent capturing true peak concentration. On the other hand, our reported $V_{\max}$ values should not be viewed as absolute $V_{\max}$ for the DAT, as this varies with experimental preparations and approaches, but rather as an index of DAT activity for the comparisons of conditions and diet groups evaluated using the same protocols.

Insulin enhances DAT $V_{\max}$ independently of effects on ChIs and ACh

Physiological concentrations of insulin that amplify evoked DA transients also enhance $V_{\max}$ for DA uptake (Stouffer *et al.*, 2015). We show here that this effect occurs through activation of InsRs rather than IGF-1Rs that also bind insulin. Inhibition of PI3-kinase had no effect on peak $[DA]_o$ or $V_{\max}$ alone, yet prevented insulin-induced increases in DAT function. These findings are consistent with PI3-kinase dependent trafficking of DAT to the surface of DA axons (Carvelli *et al.*, 2002; Garcia *et al.*, 2005; Schoffelmeer *et al.*, 2011; Mebel *et al.*, 2012; Jones *et al.*, 2017) as the likely mechanism for increased DA uptake with insulin.

Activation of striatal ChIs powerfully regulates DA release, with ChI stimulation able to trigger nAChR-dependent DA release in the absence of action-potential dependent

stimulation of DA axons (Threlfell *et al.*, 2012; Cachepe *et al.*, 2012). Moreover, pharmacological antagonism of nAChRs by application of nicotine at desensitizing concentrations (Rice & Cragg, 2003; Zhang & Sulzer, 2003; Koranda *et al.*, 2014), or genetic deletion of striatal ACh synthesis (Patel *et al.*, 2012) leads to enhanced frequency responsiveness of axonal DA release, thus enhancing signal-to-background DA signaling. Insulin acts at InsRs on ChIs to increase striatal ChI excitability, and thereby enhance evoked striatal DA release in an ACh and nAChR-dependent manner; a striking demonstration of this dependence is the complete absence of an effect of insulin on evoked DA release in striatal slices from ChAT-KO mice (Stouffer *et al.*, 2015). Here, we demonstrate that ChAT-KO striatum still responds to insulin, but through a direct effect on DAT function in DA axons. Indeed, a significant proportion of striatal InsRs are on DA axons (Stouffer *et al.*, 2015). In the absence of cholinergic transmission, the analyses reported here reveal a significant insulin-dependent decrease in net evoked $[DA]_o$ resulting from enhanced DA uptake. Thus, the effects of insulin on ChI-facilitated DA release will normally dominate over effects on DAT activity. However, in the absence of ChI activation, enhanced uptake could dominate, which would also enhance signal-to-background for striatal DA transmission.

FR and OB diets similarly decrease baseline evoked DA release and DAT activity, but oppositely affect sensitivity to insulin

Insulin levels in both plasma and CSF increase quickly after peripheral glucose elevation, with increased insulin detected in striatum in less than 5 min of an increase in plasma insulin elevation (Strubbe *et al.*, 1988; Banks *et al.*, 1997; Banks and Kastin, 1998), albeit with brain levels ~25% lower than in plasma (Gray *et al.*, 2014). Are the brain levels functional? The answer is yes; Woods *et al.* (2016) recently demonstrated that InsRs and Akt-pathways in the NAc can be stimulated within 7 minutes of glucose ingestion. Moreover, insulin in the NAc also influence food choices, as indicated by loss of development of preference for the flavor of a glucose-containing solution when paired with NAc infusion of antibodies to insulin which disrupts normal InsR signaling (Stouffer *et al.*, 2015). These rapid effects in a key brain reward center add a new dimension to the established role of insulin in the hypothalamus, where it provides a satiety signal to end further food consumption (Baskin *et al.*, 1999; Porte *et al.*, 2002).

Given that circulating insulin levels correlate with body adiposity (Strubbe *et al.* 1988; Stouffer *et al.*, 2015; Woods *et al.*, 2016), an opposing change in InsR sensitivity provides the most likely explanation for the enhanced insulin sensitivity of striatal DA release with FR and loss of responsiveness with OB (Stouffer *et al.*, 2015). In support of this, using the same diets, Woods *et al.* (2016) showed enhanced glucose-induced InsR phosphorylation in the NAc with FR relative to AL, with the absence of InsR phosphorylation in OB rats. Data reported here extend these previous results by showing that FR and OB diets decrease baseline striatal DA release and DA uptake. The seemingly paradoxical observation that dramatically different diets have the same negative effect on C_{peak} and V_{max} can also be explained by chronic, diet-induced changes in striatal InsR sensitivity. As already discussed, acute elevation of physiological levels of insulin leads to increased ChI excitability and nAChR-dependent DA release, as well as an increase in DAT surface expression and V_{max} .

In FR, the opposite conditions prevail, with chronically decreased insulin levels predicted to decrease ChI excitability and C_{peak} , and well as decrease surface DAT and V_{max} , as seen (Figure 5). Analogously, loss of striatal InsR sensitivity and insulin responsiveness with an OB diet (Stouffer *et al.*, 2015; Woods *et al.* 2016) should also decrease ChI excitability and DA release, as well as impair surface DAT expression and lower V_{max} from loss of tonic effects of insulin on these parameters. Although absolute values for V_{max} differ among experimental paradigms, the 25–30% decreases reported for CPu and NAc with these diets parallel decreases obtained under non-depolarizing conditions using synaptosomes and B_{max} (maximal binding) for surface DAT expression with either shorter food deprivation (Patterson *et al.* 1998) or the same FR and OB diets examined here (Zhen *et al.*, 2006; Jones *et al.*, 2017). Notably, a lower V_{max} was also seen in FSCV studies in striatal slices from mice on a high-fat only diet (normal sugar), although the decrease was <20% from control, and not accompanied by a change in peak evoked $[DA]_o$ (Fordahl & Jones, 2017). Whether these slight differences from the present results reflect diet or species differences has not been addressed. In this light, mice with global KO of brain InsRs show the opposite effect on DA uptake monitored in striatal slices with amperometry, with a 40% narrowing of the width of evoked $[DA]_o$ transients *versus* control suggesting enhanced uptake (Kleinridders *et al.*, 2015). However, these mice were 14 months old at the time of study, with other consequences of InsR KO, including increased monoamine oxidase activity and oxidative stress that could indirectly alter DA transmission (Kleinridders *et al.*, 2015).

We show here that the effect of acute exogenous insulin on DAT V_{max} , like the previously reported effects on DA release and InsR signaling in striatal slices (Stouffer *et al.*, 2015; Woods *et al.*, 2016), is amplified by FR and blunted by OB. These data mirror the influence of these diets on DAT-mediated $^3[H]$ -DA uptake in striatal synaptosomes (Jones *et al.*, 2017). The enhancing effect of insulin on DA release and uptake in mouse striatal slices is also lost with a chronic high-fat diet (Fordahl & Jones, 2017). Together, these results support the notion that diet-regulated levels of brain insulin set the tone for acute InsR-pathway mediated DA signaling. Loss of insulin sensitivity with a high-fat diet appears to be a consequence of impaired efficacy of downstream InsR-substrates, rather than a decrease in the number of InsRs *per se*, as it can be reversed by inhibiting tyrosine phosphatase 1B, which preserves phosphorylation at tyrosine residues on InsR-substrates (Fordahl and Jones, 2017). Similarly, restoring phosphorylation of the downstream target Akt selectively in DA neurons by striatal viral-injection can also rescue impairments in DAT surface expression and function (Speed *et al.*, 2011).

Implications for reward signaling and conclusions

Reward prediction and reward presentation information is encoded in the basal ganglia by phasic increases in midbrain DA neuronal activity that correlates with phasic striatal DA release transients (Schultz, 1998; Matsumoto & Hikosaka, 2009; Sombers *et al.*, 2009). Further regulation of striatal DA signaling typically occurs via local factors including locally released transmitters and neuromodulators (Rice *et al.*, 2011; Sulzer *et al.*, 2016). Here, we demonstrate that evoked increases in $[DA]_o$ are shaped by systemic changes in a circulating neuromodulator that crosses the blood-brain barrier, namely insulin. Altered responsiveness of striatal DAT function following phasic DA release with chronic changes in insulin

signaling in different feeding states could have implications for behavioral responses to both natural rewards and the rewarding effects of abused drugs, particularly psychostimulants that preferentially target the DAT. In general, hypoinsulinemic FR diets tend to enhance the rewarding effects of drugs (Carroll and Meisch, 1984; Pothos *et al.* 1995; Bell *et al.* 1997). For example, the FR regime used here decreases brain reward thresholds and basal locomotor activity but increases self-administration of psychostimulant drugs, as well as enhancing cocaine conditioned place preference (Cabeza de Vaca and Carr, 1998, Carr, 2007; Carr, 2011; Zheng *et al.* 2012). By contrast, hyperinsulinemic OB diets dampen brain reward systems (Davis *et al.*, 2008; Geiger *et al.*, 2009; Speed *et al.*, 2011; Hryhorczuk *et al.*, 2016) and lead to increased sensitization to psychostimulants (McGuire *et al.*, 2011; Baladi *et al.*, 2012; Fordahl *et al.*, 2016). Importantly, responsiveness to food preference is also altered with feeding state, with FR rats displaying enhanced flavor-nutrient preference *versus* AL rats, but hyperinsulinemic OB rats losing their ability to acquire significant flavor-nutrient preference (Woods *et al.*, 2016). Given that DA operates as a subsecond modulator for food seeking (Roitman *et al.*, 2004), incorrect valuation of nutrients by altered insulin-induce DA signaling could exacerbate maladaptive eating patterns such as those seen with anorexia nervosa or with obesity. Indeed, animals on a high-fat diet have increased motivation for food consumption (Speed *et al.*, 2011; Narayanaswami *et al.*, 2013).

Overall, the data presented here reveal that acute and chronic insulin dynamically regulate striatal DA release and uptake, which could contribute to the influence of diet and insulin on regulation of the rewarding effects of food and drugs.

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Abbreviations

ACh	acetylcholine
AL	ad libitum
ChAT	choline acetyltransferase
DA	dopamine
[DA]_o	extracellular dopamine concentration
DAT	dopamine transporter
FR	food restricted
HNMPA	hydroxyl-2-naphthalenylmethylphosphonic acid trisacetoxymethyl ester

InsR	insulin receptor
KO	knockout
nAChR	nicotinic acetylcholine receptor
OB	obesogenic
PPP	picropodophyllotoxin; $V_{\max}$, maximum uptake velocity
C_{peak}	extrapolated peak concentration

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Figure 1. Derivation of C_{peak} and V_{max} from MM-analysis of evoked $[DA]_o$ transients detected using FSCV.

(A) Representative evoked $[DA]_o$ plot showing the experimental data point for peak $[DA]_o$ (green cross), and the three data points used for curve fitting (red circles) to extract peak $[DA]_o$ (C_{peak}) and V_{max} in the CPU of an AL fed rat. (B) Fit of representative data to Michaelis-Menten (MM) equation with a fixed K_m of 0.2 μM gave $C_{\text{peak}} = 1.25 \mu\text{M}$ (blue circle), $V_{\text{max}} = 3.27 \mu\text{M/s}$ and $R^2 = 0.997$.

Figure 2. Insulin enhances DA release and DAT activity in AL.

(A) Average single-pulse (1p) evoked $[DA]_o$ transients recorded in CPu and NAc core in the absence and presence of insulin (30 nM). Error bars have been omitted for clarity. (B) Insulin (Ins) significantly enhanced C_{peak} derived from fitting individual $[DA]_o$ records to extract Michaelis-Menten parameters in both CPu and NAc core. (C) Insulin also increased average V_{max} for DA uptake in both striatal regions. Data are means $\pm$ s.e.m. In CPu, $n = 58$ recording sites from 9 rats for control and $n = 39$ recording sites from 5 rats for insulin. In NAc core, $n = 73$ sites from 11 rats for control and $n = 60$ sites from 9 rats for insulin. One-way-ANOVA with Bonferroni's multiple comparisons test of selected pairs was used to compare CPu *versus* NAc core under control conditions. Unpaired-two-tailed t-tests were used to compare each striatal subregion with and without insulin. ##### $p < 0.0001$ *versus* CPu; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ *versus* respective control. Data in (C) are taken from Table 1, Stouffer *et al.*, (2015).

Figure 3. Insulin enhances DA release and DAT activity via InsRs and PI3-kinase.

(A) In both the CPu and NAc core of AL rats the average insulin-induced enhancement of C_{peak} was prevented by the insulin receptor (InsR) antagonist HNMPA (5 μM) and the PI3-kinase inhibitor LY29002 (1 μM) but not by the insulin-like growth factor-1 receptor (IGF-1R) antagonist PPP (1 μM). (B) In both CPu and NAc core the insulin-induced increases in V_{max} were prevented by HNMPA but not by PPP. Insulin caused a decrease in V_{max} in the presence of LY29002. Data are means $\pm$ s.e.m. For control n numbers see Figure 2. For HNMPA, CPu n = 31 recording sites, NAc core n = 38 sites (4 rats). For HNMPA + Ins CPu, n = 30 sites, NAc, n = 33 sites (4 rats). For PPP, CPu, n = 22 sites, NAc core n = 29 sites (3 rats). For PPP + Ins, CPu n = 23 sites, NAc core n = 29 sites (3 rats). For LY29002 or LY29002 + Ins, CPu n = 24 sites, NAc core n = 30 sites (3 rats). One-way-ANOVA with Bonferroni's multiple comparisons test of selected pairs was used to compare each drug alone *versus* control. Unpaired one-tailed t-tests were used to compare each drug with and without Ins. n.s. = not significant ($P > 0.05$); # $P < 0.05$; ## $P < 0.01$ *versus* control; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 4. Insulin-enhanced DA release requires ACh, but increased DAT activity does not. (A) In both CPu and NAc core insulin (Ins; 30 nM) increased average C_{peak} in ChAT control (ChAT-con) mice, but decreased C_{peak} in ChAT-KO mice. (B) In both CPu and NAc core insulin increased V_{max} in ChAT-con and ChAT-KO mice. Data are means $\pm$ s.e.m. For ChAT-con CPu, $n = 22$ recording sites, NAc core $n = 28$ sites (3 mice). For ChAT-con + Ins, CPu, $n = 25$ sites, NAc core $n = 27$ sites (3 mice). For ChAT-KO CPu, $n = 25$ sites, NAc core $n = 28$ sites (3 mice). For ChAT-KO + Ins CPu, $n = 23$ sites, NAc core $n = 28$ sites (3 mice). One-way-ANOVA with Bonferroni's multiple comparisons test of selected pairs was used to compare ChAT-con *versus* ChAT-KO mice under control conditions. Unpaired one-tailed t-tests were used to compare each genotype with and without insulin. n.s. = not significant; # $P < 0.05$ *versus* ChAT-con; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.001$ *versus* respective control.

Figure 5. DA release and DAT-mediated uptake are decreased by FR and OB diets.

(A) Inverted-U relationship between individual C_{peak} values (upper panel) or V_{max} (lower panel) in CPu and NAc core with plasma insulin levels from rats on a food restricted (FR), *ad libitum* (AL) or obesogenic (OB) diet. (B) In both CPu and NAc core average C_{peak} (upper panel) was lower with FR and OB diets *versus* AL diet. Average V_{max} (lower panel) in CPu was lower with FR and OB diets and was also lower with FR *versus* an AL diet in NAc. Data are either individual values (A) or means $\pm$ s.e.m (B). For FR CPu, $n = 36$ recording sites, NAc core $n = 40$ sites (5 rats). For AL, CPu, $n = 35$ sites, NAc, $n = 41$ sites (5 rats). For OB, CPu, $n = 38$ sites, NAc core $n = 40$ sites (5 rats). A second order polynomial curve fit (Graphpad Prism) was used for C_{peak} or V_{max} *versus* plasma insulin data. One-way-ANOVA with Bonferroni's multiple comparisons test of selected pairs was used to compare average C_{peak} or V_{max} for each feeding group to AL; n.s. = not significant ($P > 0.05$); ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Figure 6. Effects of insulin on DA release and DAT activity are enhanced by FR diet, but blunted by OB.

(A) In both CPu and NAc core insulin (Ins, 30 nM) increased average C_{peak} in slices from FR but not from OB rats. (B) In both the CPu and NAc core insulin increased V_{max} in FR rats but failed to increase V_{max} in the CPu or NAc core of OB fed rats. Data are means $\pm$ s.e.m. For FR, CPu $n = 39$ recording sites, NAc core $n = 45$ sites (5 rats). For FR + Ins, CPu $n = 25$ sites, NAc core $n = 30$ sites (3 rats). For OB, CPu $n = 23$ sites, NAc core $n = 28$ sites (3 rats). For OB + Ins CPu, $n = 24$ sites, NAc core $n = 30$ sites (3 rats). One-way-ANOVA with Bonferroni's multiple comparisons test of selected pairs was used to compare each diet group with and without Ins; n.s. = not significant ($P > 0.05$); **** $P < 0.0001$.