ACS Chemical Neuroscience

Research Article

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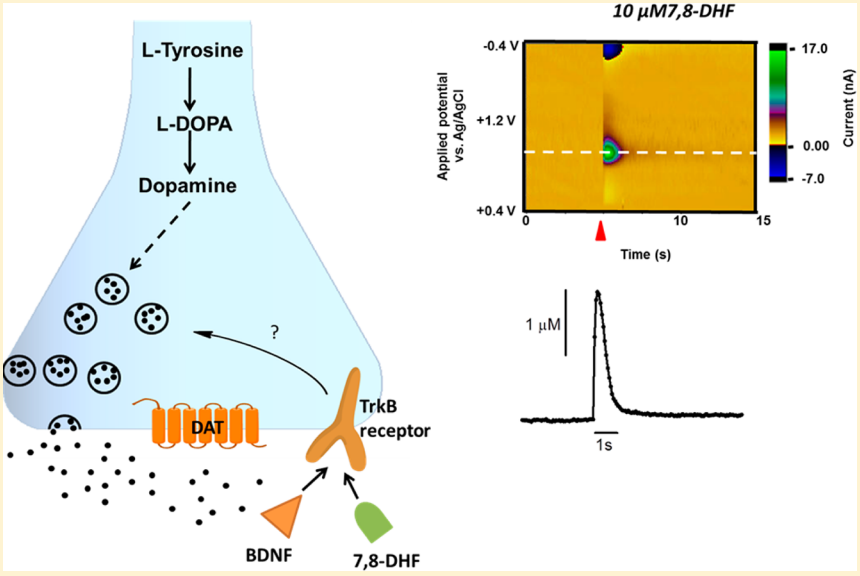
Probing the Ability of Presynaptic Tyrosine Kinase Receptors to Regulate Striatal Dopamine Dynamics

Aaron K. Apawu,[†] Francis K. Maina,[†] James R. Taylor, and Tiffany A. Mathews*

Department of Chemistry, Wayne State University, Detroit, Michigan 48202, United States



ABSTRACT: Brain-derived neurotrophic factor (BDNF) modulates the synaptic transmission of several monoaminergic neuronal systems. Molecular techniques using synapatosomes in previous studies have suggested that BDNF's receptor, tyrosine kinases (Trk), can quickly regulate dopamine release and transporter dynamics. Our main objective in this study is to determine whether slice fast scan cyclic voltammetry can be used to investigate the role of the TrkB receptor on dopamine release and uptake processes in the caudate-putamen. Fast scan cyclic voltammetry measured dopamine release and uptake rates in the presence of BDNF, or its agonist 7,8-dihydroxyflavone, or a TrkB inhibitor K252a. Superfusion of BDNF led to partial recovery of the electrically stimulated dopamine release response in BDNF$^{+/-}$ mice which is blunted compared to wildtype mice, with no effect in wildtype mice. Conversely, infusion of 7,8-dihydroxyflavone increased electrically stimulated dopamine release in wildtype mice with no difference in BDNF$^{+/-}$ mice. Overall, BDNF and 7,8-dihydroxyflavone had no effect on dopamine uptake rates. Concentrations greater than 3 μM 7,8-dihydroxyflavone affected dopamine uptake rates in BDNF$^{+/-}$ mice only. To demonstrate that BDNF and 7,8-dihydroxyflavone modulate dopamine release by activating the TrkB receptor, both genotypes were pretreated with K252a. K252a was able to block BDNF and 7,8-DHF induced increases during stimulated dopamine release in BDNF$^{+/-}$ and wildtype mice, respectively. Fast scan cyclic voltammetry demonstrates that acute TrkB activation potentiates dopamine release in both genotypes.

KEYWORDS: *Caudate-putamen, TrkB, brain-derived neurotrophic factor, 7,8-dihdyroxyflavone, K252a, fast-scan cyclic voltammetry*

Neurotrophic factors are endogenous soluble proteins that regulate the cell cycle, growth, differentiation, and survival of neurons.[1] Members of the neurotrophic family include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), all of which mediate their functions through tyrosine kinase (Trk) receptors. The Trk receptors are glycoproteins that have a molecular weight in the range of 140−145 kDa. Each neurotrophin appears to bind to a unique isoform of the Trk receptors. For example, NGF has a greater specificity to bind to the TrkA receptor, NT-3 interacts with TrkC, and both BDNF and NT-4 bind to TrkB.[2,3] When BDNF is expressed, it is transported to nerve terminals through the axon and undergoes exocytotic release from presynaptic vesicles.[4−6] Extracellular BDNF binds to TrkB receptors and causes receptor dimerization, which leads to phosphorylation of tyrosine residues within the cytoplasm, and activates kinases.[7]

Special Issue: Monitoring Molecules in Neuroscience

Received: March 25, 2013
Accepted: May 3, 2013
Published: May 3, 2013

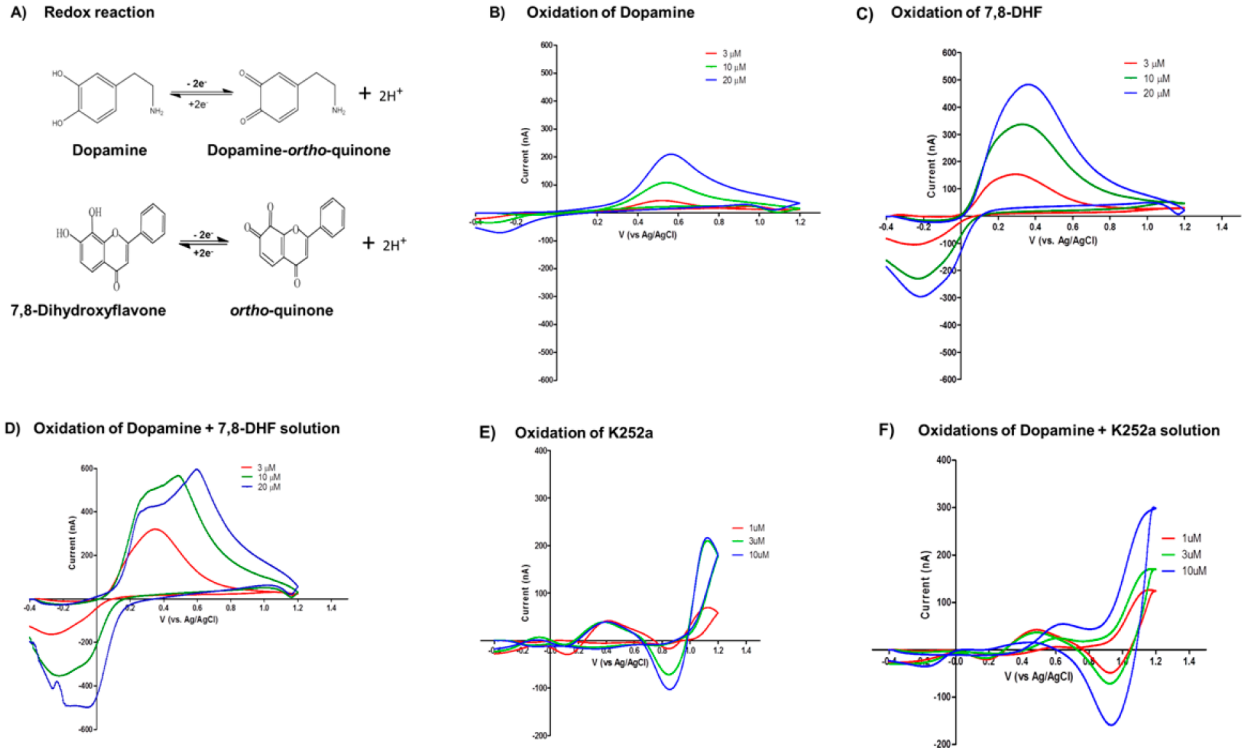


Figure 1. Cyclic voltammograms and redox reactions showing electroactive properties of DA, 7,8-dihydroxyflavone and K252a. Data was obtained using flow injection analysis of varying concentrations of each molecule. (A) Oxidation−reduction reactions of DA and 7,8-DHF. Both molecules oxidize in a similar electron transfer reaction to form ortho-quinones during the forward scan of the applied voltage. In the reverse scan, the quinones are reduced back to DA and 7,8-DHF. (B) Cyclic voltammogram shows oxidation peak of DA at ∼0.6 V and reduction peak at ∼ −0.2 V. (C) Cyclic voltammogram of 7,8-DHF. Oxidation peak of 7,8-DHF occurs at ∼0.4 V, whereas the reduction peak appears at ∼ −0.2 V. (D) A solution of both DA and 7,8-DHF demonstrates the characteristic oxidation peaks of the two compounds only at higher concentrations as depicted by their voltammograms. (E) Cyclic voltammogram showing oxidation and reduction peaks of K252a. (F) Cyclic voltammograms showing oxidation and reduction of DA-K252a solution.

Phosphorylated tyrosine residues recruit specific proteins, such as GBR2, SHC, and SOS, which in turn activate Raf, a serine/threonine protein kinase (Akt).[8] Trk activation induces numerous signaling cascades through three known pathways: the Ras/mitogen activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K), and phospholipase C, γ (PLCγ). Activating these signaling cascades regulate the transcription factor, cAMP response element binding (CREB), and leads to attenuation or potentiation in gene expression. Continuous activation or inhibition of these signaling events regulate synaptic plasticity, synaptic transmission, neurotransmitter release, neurogenesis, and cell survival.[3,6,9−11]

There is considerable evidence of reciprocal interactions between BDNF and the dopamine (DA) system, although the exact mechanism of how BDNF regulates DA dynamics is unknown.[11−13] BDNF regulates striatal function directly by activating TrkB receptors.[14] When BDNF is infused directly into the brain it influences the survival and function of DA neurons, affects the turnover ratio between DA and its metabolite DOPAC, and potentiates the activity-dependent release of DA.[11−13] Evidence suggests that BDNF expression can augment DA transmission in the reward pathway of the ventral tegmental-nucleus accumbens (NAc) circuit.[15] Mouse models with reduced BDNF expression exhibit a variety of alterations in the DA system, which indicate that BDNF has some influence on this system. Studies have shown that mice lacking one copy of the BDNF gene (BDNF$^{+/-}$) have higher tissue DA concentrations in the striatum, and decreased DA release in superfused striatal tissue fragments.[16,17] BDNF knockout (BDNF$^{-/-}$) and BDNF$^{+/-}$ mice have reduced expression and density of DA D3 receptors in the caudate-putamen (CPu), NAc core and shell, and in the Islands of Calleja.[18−20] Our laboratory has shown that BDNF$^{+/-}$ mice have an ∼2.5-fold increase in extracellular DA levels in the CPu compared to wildtype mice as measured by zero net flux with a concomitant decrease in evoked DA release and DA clearance measured by fast-scan cyclic voltammetry (FSCV).[21] Our initial neurochemical analysis on CPu DA dynamics suggests that endogenous BDNF influences DA system homeostasis primarily by regulating release leading to adaptions in the DA uptake function.[21] All of these studies suggest that BDNF can augment striatal DA functioning in a region-specific manner, but the mechanism of how BDNF modulates DA function still remains elusive.

The present study uses FSCV to characterize the functional effects of TrkB agonists and inhibitors on DA dynamics in the CPu. By using striatal slices, it eliminates contributions from postsynaptic TrkB receptors allowing us to better understand how presynaptic TrkB receptors contribute to modulating DA dynamics. We hypothesized that a life-long reduction in BDNF would result in compensatory alterations in DA dynamics, which may be mediated by presynaptic TrkB activation. DA release and uptake have been evaluated in the striatum by using the endogenous ligand BDNF, the commercially available TrkB agonist 7,8-dihydroxyflavone (7,8-DHF), and the inhibitor K252a. This is the first reported study that examines the effect

dx.doi.org/10.1021/cn4000742 | ACS Chem. Neurosci. 2013, 4, 895−904

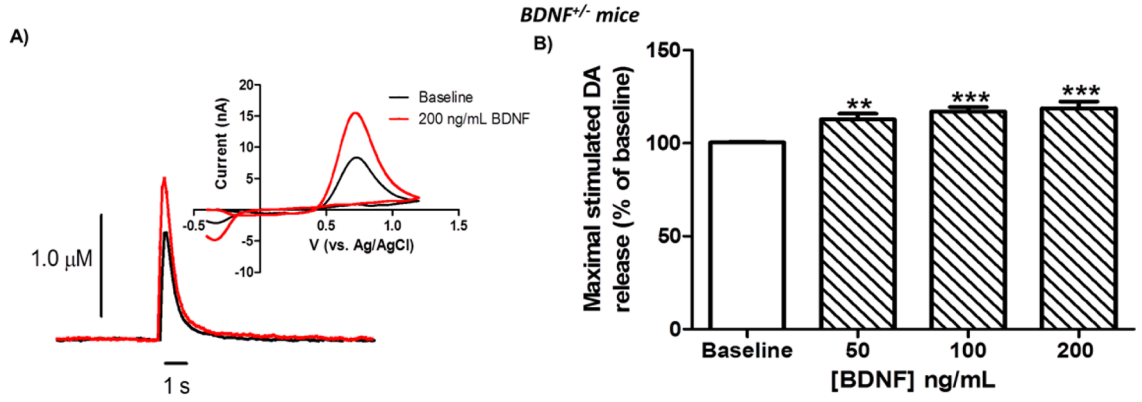


Figure 2. Infusion of BDNF dose dependently increases electrically stimulated DA release in the caudate-putamen of BDNF$^{+/-}$ mice. (A) Current versus time trace of baseline DA signal with corresponding cyclic voltammogram (inset) compared to current versus time trace of electrically evoked DA release after a 30 min perfusion of 200 ng/mL BDNF (red line) with corresponding cyclic voltammogram (red line, inset). (B) Effect of varying concentrations of exogenous BDNF on DA release expressed as a percentage of predrug DA concentration. Data are means ± SEMs (n = 5 mice). One-way ANOVA ($F_{3,36}$ = 9.42; P < 0.0001, n = 5) followed by Dunnett's post-test revealed that each concentration of BDNF increased DA release significantly. **P < 0.01, ***P < 0.0001.

of TrkB agonist and an inhibitor on presynaptic DA dynamics using carbon fiber microelectrodes. While BDNF or a TrkB agonist increases DA release with no difference in DA clearance, a TrkB inhibitor decreases DA uptake with no effect on DA release. FSCV has the ability to assess the effect of non-DA receptors like the TrkB on striatal DA dynamics, and will help to provide a better understanding of how other systems contribute to the strength of synaptic DA transmission.

RESULTS AND DISCUSSION

Characterization of the Electrochemical Properties of 7,8-Dihydroxyflavone and K252a. The structure of 7,8-DHF is similar to that of DA insofar as both molecules contain the catechol moiety. Although K252a does not contain the catechol moiety, it does have a single hydroxide group that could be easily oxidized like tyramine and octopamine.[22] To delineate the electrochemical properties of these molecules, we used a flow cell apparatus to evaluate their oxidation and reduction potentials. Similar to DA, 7,8-DHF has a single oxidation and reduction peak occurring at ∼0.4 V and at −0.2 V, respectively (Figure 1B). The single oxidation and reduction peak for 7,8-DHF suggests that it is a reversible electrochemical reaction. It is our hypothesis that the hydroxide groups on the catechol are oxidized in much the same way as it is in DA and other catecholamine molecules. A notable difference in 7,8-DHF and DA cyclic voltammograms is that 7,8-DHF oxidizes at ∼ +0.2 V less than DA. We believe this difference is due to the second ring structure attached to the catechol moiety providing additional stability/resonance to the molecule. K252a has a very distinct cyclic voltammogram with oxidation peaks at ∼ +0.4 and +1.1 V, and a reduction peak at ∼ +0.9 V (Figure 1E). When DA and K252a are combined, there appears to be no overlap with oxidation peaks or negative influence on DA oxidation (Figure 1F). Furthermore, by using background subtraction FSCV, the DA signal (current) and oxidation potential are no different in the presence of these compounds (e.g., Figure 5). This strongly supports the notion that background subtraction eliminates any possible electrochemical confounds associated with these treatments.

Effect of Exogenous BDNF on Electrically Evoked Dopamine Release in BDNF$^{+/-}$ Mice. Numerous reports suggest that exogenously applied BDNF enhances both DA release and uptake.[11,12,14,23] To date, no one method has been used to simultaneously analyze both release and uptake parameters. To evaluate the functional effects of how exogenous BDNF influences presynaptic DA dynamics directly in the CPu, electrically evoked DA release (Figure 2) and uptake rates (Table 1) were monitored every 5 min in BDNF$^{+/-}$ mice. Direct application of cumulative concentrations of exogenous BDNF (50, 100, and 200 ng/mL) was applied to brain slices for 30 min. BDNF$^{+/-}$ mice were only evaluated with BDNF perfusion because we have previously shown that DA release and uptake rates are no different in wildtype mice.[24] BDNF$^{+/-}$ mice showed a concentration-dependent increase in electrically stimulated DA release after BDNF was applied to striatal brain slices compared to their predrug controls (50 ng/mL BDNF, ∼12%; 100 ng/mL BDNF, ∼17%; 200 ng/mL BDNF, ∼18%; Figure 2A). However, no difference in V_{max} was observed after BDNF perfusion (Table 1). We have extended and confirmed our previous findings[24] that exogenous application of BDNF from 50 to 200 ng/mL elevates evoked-DA release with no effect on DA uptake rates in BDNF$^{+/-}$ mice.

Table 1. Effect of TrkB Agonist on DA Uptake Rates

agonist table			
genotype	agonist	[agonist]	V_{max} (μM/s) (mean ± SEM)
BDNF$^{+/-}$	BDNF (ng/mL)	predrug	2.2 ± 0.2
	BDNF (ng/mL)	50	2.2 ± 0.2
	BDNF (ng/mL)	100	2.2 ± 0.2
	BDNF (ng/mL)	200	2.3 ± 0.2
	7,8-DHF (μM)	predrug	3.0 ± 0.03
	7,8-DHF (μM)	0.010	3.0 ± 0.02
	7,8-DHF (μM)	1.00	2.7 ± 0.1
	7,8-DHF (μM)	30.0	2.4 ± 0.2[a]
wildtype	7,8-DHF (μM)	predrug	4.0 ± 0.01
	7,8-DHF (μM)	0.010	4.1 ± 0.12
	7,8-DHF (μM)	1.00	4.0 ± 0.1
	7,8-DHF (μM)	30.0	3.9 ± 0.2

[a]Significantly different from predrug control, **P < 0.01.

dx.doi.org/10.1021/cn4000742 | ACS Chem. Neurosci. 2013, 4, 895−904

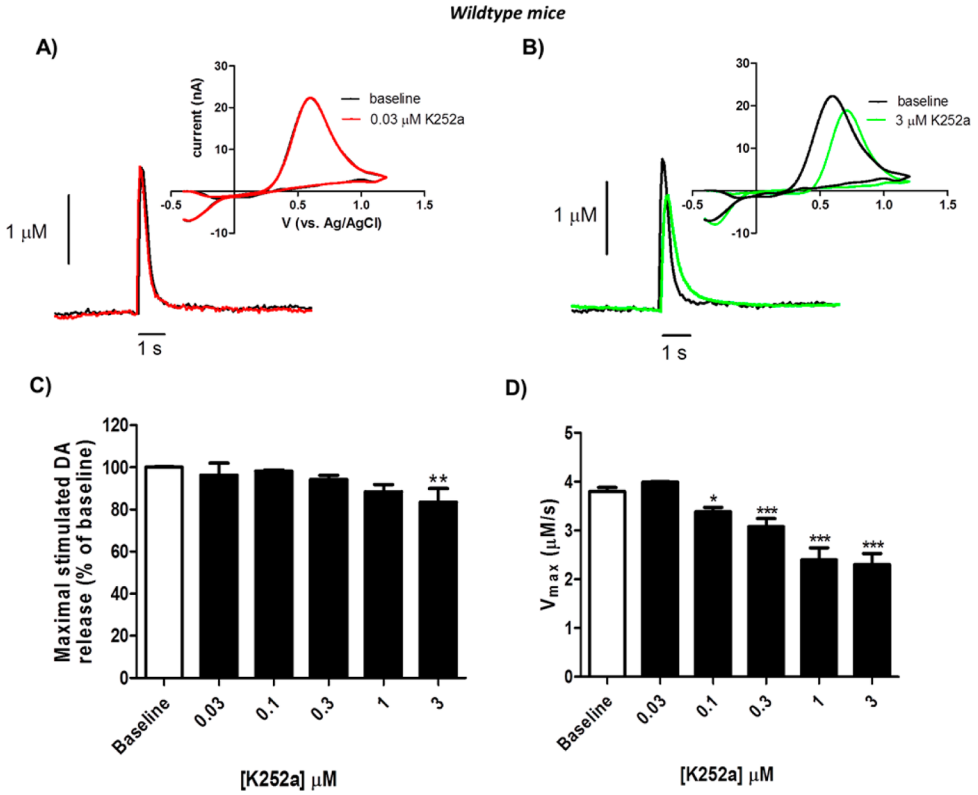


Figure 3. Effect of TrkB inhibitor, K252a, on stimulated DA release and uptake in the caudate-putamen of wildtype mice. (A) Representative DA release and uptake after a 30 min perfusion of 0.03 μM K252a compared to predrug DA signal shown by their representative current versus times traces with corresponding cyclic voltammograms (inset). (B) Effect of 3 μM K252a on DA release and uptake represented in terms of concentration versus time trace and cyclic voltammogram (inset). (C) K252a produced a significant effect on stimulated DA release as determined by a one-way ANOVA ($F_{5,61}$ = 3.05; P < 0.05, n = 4–7). A Dunnett's posthoc test confirmed that only 3 μM K252a perfusion attenuated stimulated DA release. (D) One-way ANOVA ($F_{4,25}$ = 14.93; P < 0.0001, n = 4–7) followed by Dunnett's post-test revealed that K252a concentrations greater than 0.01 μM K252a significantly decreased V_{max}. Data are means ± SEMs (n = 4–7 mice). *P < 0.05, **P < 0.001, ***P < 0.0001.

Effect of K252a on Presynaptic DA Dynamics in Wildtype Mice. BDNF signaling is mediated by the TrkB receptor.[2,3] However, it is unclear whether the TrkB receptor activation effects DA release and uptake. To find the answer, we used the potent, nonselective Trk receptor inhibitor, K252a.[25] K252a was perfused over a brain slice for 30 min per dose, and its effects were monitored every 5 min (Figure 3A). Dunnett's post-test revealed a significant (P < 0.05) reduction in stimulated DA release only at the highest K252a concentration (3 μM). Increasing the concentration of K252a from 0.01 to 3 μM reduced V_{max} in a concentration-dependent manner (Figure 3B and D).

Our results from the CPu of wildtype mouse brain slices show that concentrations less than 3 μM K252a alone have no effect on electrically stimulated DA release. These results agree with previous studies showing that concentrations of K252a less than 1 μM have no effect on stimulated DA release.[14,26] Only the highest concentration of K252a applied to brain slices reduced electrically stimulated DA release. We cannot rule out the possibility that K252a reduced the amount of stimulated DA release by acting at other Trk receptors. Although K252a is used to selectively block BDNF-TrkB signaling, it is also a nonspecific inhibitor of tyrosine protein kinase activity including the TrkA and TrkC receptor subtypes.[27] Such actions could contribute to the decrease in electrically evoked DA when K252a is applied at the highest concentration.

This is the first report demonstrating the rapid reduction in DA transporter kinetics in the CPu of wildtype mice when K252a concentrations are greater than 0.1 μM and is in agreement with the previous work by Hoover et al., where inhibition of tyrosine kinases by genistein (a nonspecific Trk inhibitor) or tyrphostin 23 (a selective Trk inhibitor) resulted in rapid (5 − 15 min), dose-dependent decreases in [^{3}H]DA uptake rates in a dorsal striatal synaptosomal preparation.[23] It may appear inconsistent that activation of TrkB with BDNF increases stimulated DA release, while inhibition of this receptor reduces DA uptake, but Trk receptors activate multiple signaling pathways. Considerable evidence in the literature suggests a divergent role for TrkB where BDNF activation increases neurotransmission,[13,14,24,26] while Trk inhibitors reduce DA uptake. [23] The exact mechanism causing this divergent response is unknown, but it is hypothesized that inhibition of the Trk receptor blocks autophosphorylation, which reduces activity in numerous signaling cascades such as PI3K, MAPK, and ERK1/2, all of which have been linked to decreases in DA transporter activity by decreasing V_{max}.[23,28−32] The results of this study suggest that inhibition of the TrkB receptor reduces the DA transporter function, but more studies are required to determine the specific Trk-signaling pathways that may be involved in regulating DA transporter kinetics.

K252a Inhibits BDNF's Ability to Acutely Modulate Striatal Dopamine Release in BDNF$^{+/-}$ Mice. To demonstrate that exogenously applied BDNF modulates presynaptic DA dynamics via the TrkB receptor electrically evoked DA release, and uptake rates were monitored every 5 min following direct application of BDNF (100 ng/mL) only,

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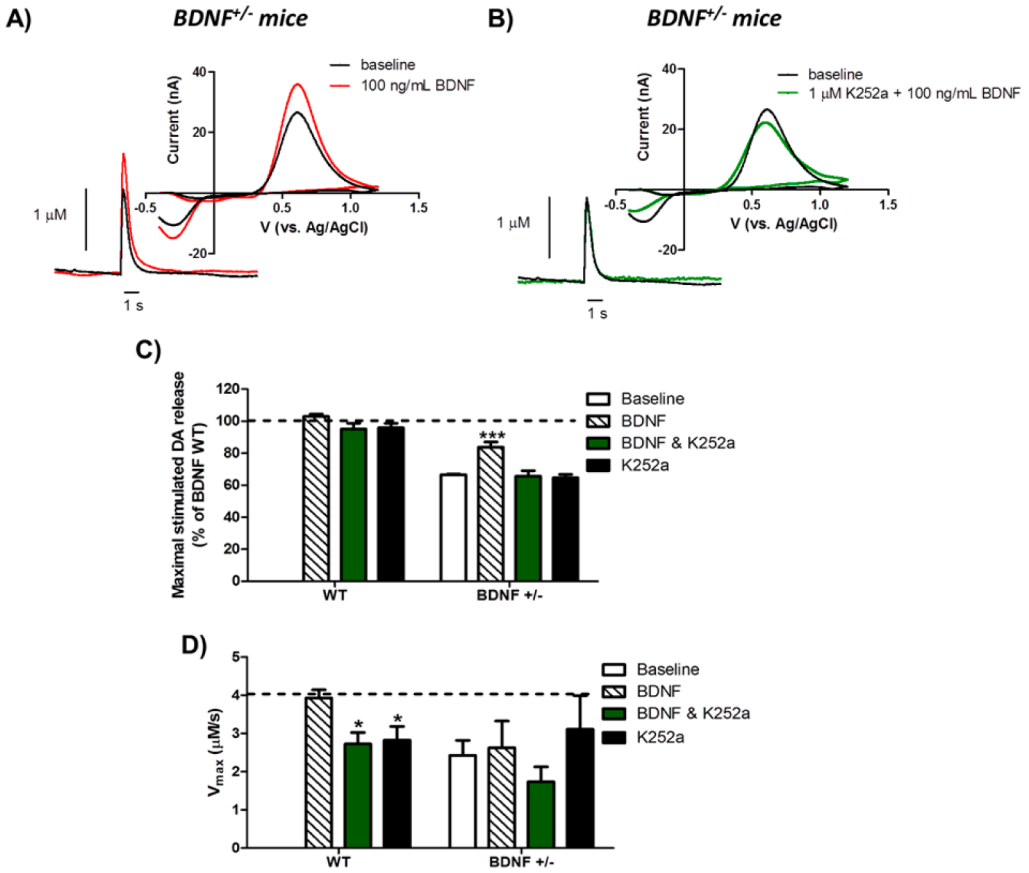

Figure 4. Effect of exogenous application of BDNF (100 ng/mL) on DA release and uptake rates in the caudate putamen of wildtype (WT) and $BDNF^{+/-}$ mice. (A) Representative current versus times traces with corresponding cyclic voltammograms (inset) from $BDNF^{+/-}$ mice before and after BDNF infusion. (B) K252a blocked the ability of BDNF to potentiate DA release in the CPu of $BDNF^{+/-}$ mice as shown in representative current versus times traces with corresponding cyclic voltammograms (inset). (C) Normalized single pulse, electrically evoked DA release represented as % of WT baseline, wildtype mice are represented by the dashed line. Two-way ANOVA of electrically stimulated DA release in response to drug application (Figure 4) showed a main effect of treatment [$F_{3,74} = 10.37$, $P < 0.0001$], genotype [$F_{1,74} = 216.9$, $P < 0.0001$], while there was no treatment X genotype interaction [$F_{3,74} = 2.612$, $P = 0.056$]. (D) DA uptake rates in WT and $BDNF^{+/-}$ mice before and after 30 min perfusion of either 100 ng/mL BDNF, 1 μM K252a, or both in the caudate-putamen. A two-way ANOVA evaluating DA uptake kinetics indicated there was no difference in the main effect of treatment [$F_{3,82} = 2.09$, $P = 0.11$] and treatment X genotype interaction [$F_{3,82} = 1.45$, $P = 0.23$]. There was a significant main effect for genotype [$F_{1,82} = 7.38$, $P < 0.01$], corroborating that the kinetics of the DA transporter are reduced in $BDNF^{+/-}$ mice versus WT mice ($P < 0.01$). Data are means ± SEMs ($n = 4 - 5$ mice per treatment group). ***$P < 0.001$ compared to untreated $BDNF^{+/-}$ mice (two-way ANOVA). *$P < 0.05$ as compared to WT mice baseline (one-way ANOVA).

K252a (1 μM) only, or pretreatment with K252a prior to BDNF administration to a slice for 30 min. In the absence of exogenous applications of BDNF or K252a, electrically evoked DA from the CPu of $BDNF^{+/-}$ mice (referred to as "baseline" in Figure 4) was reduced by ~40% compared to their wildtype littermates, which is delineated as the dashed line at 100% (Figure 4). Exogenous application of BDNF significantly potentiated stimulated DA release by ~16% in $BDNF^{+/-}$ mice ($P < 0.0001$), while there was no difference in wildtype mice. Pretreatment of the striatal $BDNF^{+/-}$ slice with K252a abolished this modulatory effect of BDNF, suggesting that the increased DA release may be mediated by presynaptic TrkB receptors. There was no difference on electrically evoked DA release in wildtype mice in the presence of BDNF, K252a, or the combination of the two.

The ability of K252a to block the BDNF-induced increase in DA release in the CPu of $BDNF^{+/-}$ mice suggests that the TrkB receptor can mediate DA release. Our findings are in agreement with previous results that have shown BDNF-induced stimulated DA release is blocked in the presence of K252a.[14,16] An issue with examining monoamine output after BDNF infusion is identifying the specific signal transduction cascade(s) involved. For example, the acute BDNF-potentiation of DA release is suggested to be dependent on PI3 kinase and Ras-MEK activation only.[14] However, the exact mechanism of how BDNF mediates DA release has remained elusive.

No Effect of Exogenous Application of BDNF in Wildtype Mice. Neurotrophic factors, such as BDNF, are typically thought of as promoting long-term effects on neurons such as synaptic plasticity, neuronal survival, and differentiation. Our results demonstrate that the acute effects of TrkB activation via BDNF lead to a rapid increase in DA release in the CPu of $BDNF^{+/-}$ mice, while there is no difference in stimulated DA release with BDNF perfusion in our wildtype mice (Figure 4C). Previous reports using wildtype mice/rats established that acutely, exogenously applied BDNF enhances synaptic events such as DA release both in vivo and in vitro.[11−13] We hypothesize that this difference in BDNF-induced DA release between our wildtype mice and those from previous studies is a result of methodological differences between slices and synpatosomes. For example, all synaptosomal preparations required the coincubation of BDNF and high

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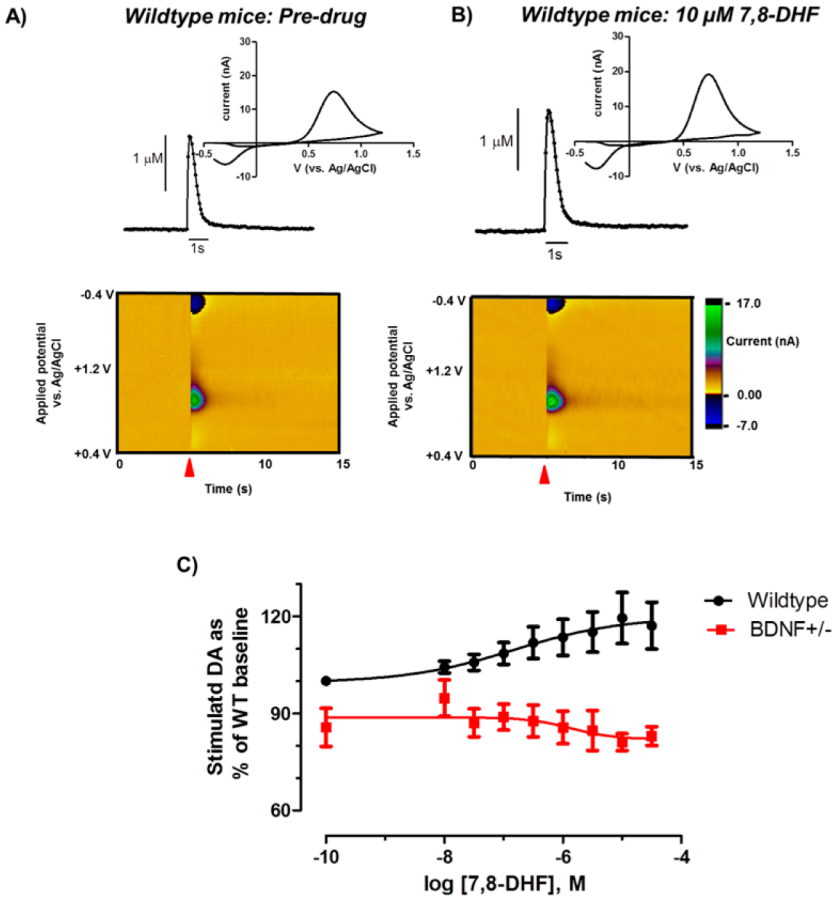

Figure 5. Effect of cumulative dose of TrkB agonist, 7,8-DHF, on electrically evoked DA release in the caudate-putamen of wildtype and BDNF$^{+/-}$ mice. (A) Current versus time trace of predrug DA signal with corresponding false color plot and cyclic voltammogram (inset). The false color plots present data in terms of time (*x*-axis) versus voltage (*y*-axis) versus current (color), where the green and blue colors denote oxidation and reduction current, respectively. The red triangle represents the point of electrical stimulation. (B) Current versus time trace of electrically evoked DA release after a 30 min perfusion of 10 μM 7,8-DHF with corresponding false color plot and cyclic voltammogram (inset). (C) Dose response curve of the effect of cumulative dose of 7,8-DHF on electrically evoked DA release in WT and BDNF$^{+/-}$ mice, which is normalized to percent of wildtype baseline stimulated DA. In the wildtype mice, there was a significant main effect of 7,8-DHF on stimulated DA release ($F_{8,204} = 0.18$, $P < 0.05$) as determined by one-way ANOVA. A Dunnett's post-test analysis indicated that 10 μM 7,8-DHF significantly increased stimulated DA release ($P < 0.05$) in wildtype mice. The cumulative dose of 7,8-DHF did not alter electrically evoked DA release in BDNF$^{+/-}$ mice ($n = 3-4$). Data are means ± SEMs.

K^+ artificial cerebral spinal fluid (aCSF) over a time course of minutes to observe BDNF-potentiated DA release.[11−14] Furthermore, DA release was measured as an accumulation of exogenously applied ^{3}[H]-DA, while our wildtype slices received a one-millisecond electrical stimulation that recruited endogenous DA. Taken together, an acute perfusion of BDNF can rapidly potentiate DA release in a system with low endogenous BDNF levels, but in a normal system, like that of wildtype mice, stronger stimulation parameters may be required.

K252a Does Not Modulate Striatal DA Transporter Kinetics in BDNF$^{+/-}$ Mice. Studies have also suggested a role for Trk receptors in modulation of DA uptake evidenced by the use of Trk inhibitors, which have been shown to alter DA transporter function, expression and/or kinetics.[23] An advantage of slice FSCV is that both presynaptic DA release and uptake are simultaneously evaluated. The objective was to use FSCV to determine if an exogenous application of BDNF influences DA transporter kinetics as it does with DA release. Exogenous BDNF applications only potentiate evoked DA release with no difference in DA uptake rates in BDNF$^{+/-}$ mice (Figure 4D). Therefore, we have hypothesized that the 50% reduction in DA transporter function is the compensatory response to having a life-long reduction in BDNF levels since this parameter was unaltered.[24]

There was no effect of K252a on DA V_{max} in BDNF$^{+/-}$ mice. With respect to DA transporter expression in BDNF$^{+/-}$ mice, when the mice are less than 6 months of age there is no difference in DA transporter expression or activity.[33,34] But little research has focused on evaluating whether TrkB inhibition influences DA transporter function or expression in BDNF$^{+/-}$ mice. There is no difference in striatal TrkB and pTrkB levels between the genotypes.[35] However, BDNF$^{+/-}$ mice show an increase in the ratio of striatal pTrkB/TrkB with a concomitant potentiation in striatal pERK/tERK2 levels.[35] In order to better understand *if and how* tyrosine kinase receptors modulate DA transporter expression or activity, future studies should evaluate specific intracellular cascades that may mediate the interactions between TrkB and the DA transporter.

K252a Modulates Striatal DA Transporter Kinetics in the Presence or Absence of BDNF in Wildtype Mice. Interestingly, perfusion of K252a in the presence or absence of

dx.doi.org/10.1021/cn4000742 | ACS Chem. Neurosci. 2013, 4, 895−904

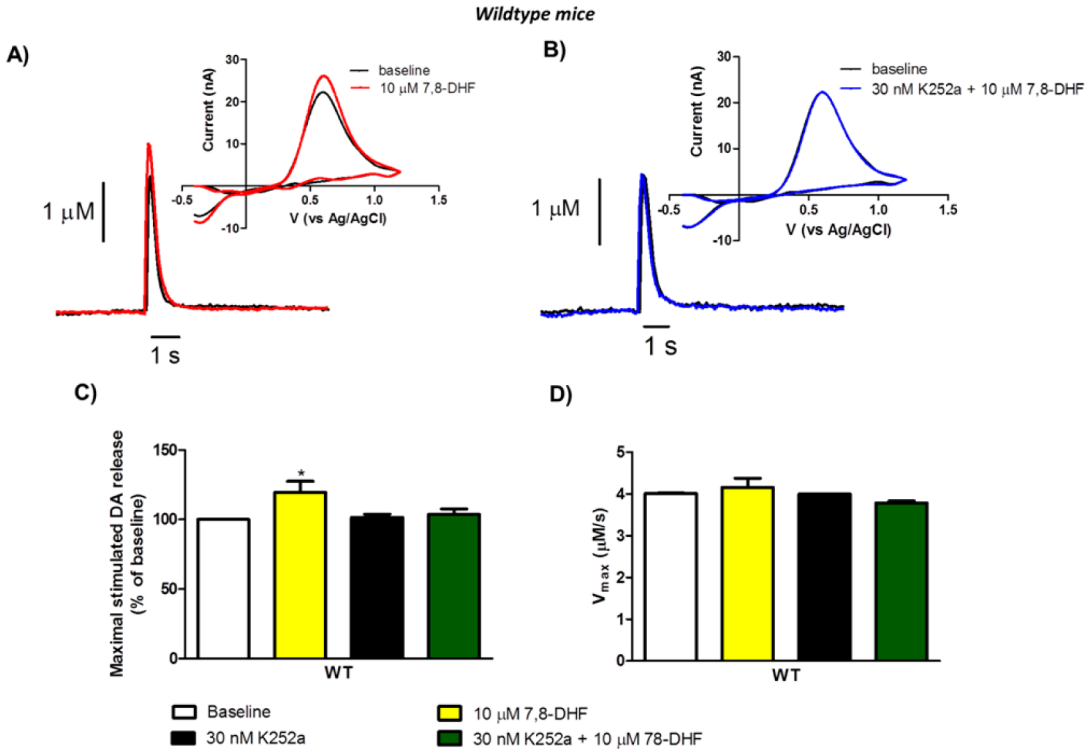

Figure 6. K252a blocks the effect of 7,8-DHF in the caudate-putamen of WT mice. (A) Representative current verse time traces with corresponding cyclic voltammograms (inset) showing perfusion of 7,8-DHF potentiates DA release in the wildtype mice. (B) Blocking the TrkB receptor with K252a inhibits the 7,8-DHF mediated increase in DA release shown by representative current versus time traces and cyclic voltamograms (inset). (C) Perfusion of 10 μM 7,8-DHF significantly increases electrically stimulated DA release in the wildtype mice. Evoked DA release mediated by the TrkB agonist 7,8-DHF is blocked in presence of 30 nM K252a. (D) Neither 30 nM K252a nor 10 μM 7,8-DHF altered DA uptake rate in wildtype mice. Data are means ± SEMs (n = 4−8 mice). Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparison test; *P < 0.05.

BDNF significantly attenuated DA uptake rates in wildtype mice (analyzed by Dunnett's post-test P < 0.05). These results show that Trk inhibition in wildtype slices has the ability to modify/regulate DA transporter kinetics given the reduction seen upon application of K252a alone. Although K252a has been shown to have no effect on DA V_{max} when applied to rat dorsal striatal synpatosomes, the same cannot be said about nonselective tyrosine kinase inhibitors.[23] When the nonselective tyrosine kinase inhibitors genistein and tyrphostin-23 were acutely applied, a significant decrease of striatal DA transporter surface expression and V_{max} were observed, but K_m was not different.[23] Western blotting suggested that this mechanism of decreasing DA transporter V_{max} and surface expression was mediated via MAPK p42 and p44 isoforms as these phosphorylated levels were decreased after inhibition.[23] The difference between the present results and those obtained by Hoover et al. with respect to K252a, could be due to the 10-fold less concentration of K252a (10 vs 1000 nM, respectively) than what we used. In the present study, the high concentration of K252a could be nonselectively binding to other tyrosine kinase receptors such as the TrkA and TrkC subtypes, which, in turn, could be inducing a reduction of striatal DA uptake rates. Furthermore, there are numerous methodological differences between our FSCV results and the results obtained via the synpatosome preps that Hoover et al. used. First, there is the species difference, rat versus mouse. Second, different methods were used to analyze uptake rates and their respective temporal resolution. Slice FSCV measures millisecond stimulated endogenous DA to evaluate DA release and uptake rates (V_{max}), where K_m is fixed, while synpatosome preparations require minutes to collect enough DA to analyze uptake parameters.

7,8-DHF Potentiates Electrically Evoked Dopamine in the CPu of Wildtype Mice Only. 7,8-DHF has been reported to be neuro-protective when administered prior to a DA-neurotoxic treatment using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).[36] To examine the ability of 7,8-DHF to modulate presynaptic DA dynamics, a cumulative dose (0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, and 30 μM) of 7,8-DHF was applied to striatal brain slices following a stable, electrically evoked DA signal. The effect of 7,8-DHF on DA release and uptake processes was measured every 5 min for 30 min per dose in both wildtype and BDNF$^{+/-}$ mice. A dose response curve for the effect of cumulative dose of 7,8-DHF on electrically evoked DA release in wildtype mice was normalized to percent of baseline stimulated DA. In the wildtype mice, 10 μM 7,8-DHF significantly increased stimulated DA release, where the half maximal effect (EC_{50} value) of 7,8-DHF is at ∼150 ± 12 nM (n = 8, Figure 5C).

With respect to BDNF$^{+/-}$ mice, there was no difference in DA release after perfusion of 7,8-DHF ($F_{8,90}$ = 0.014, P = 0.89). Stimulated DA release data from BDNF$^{+/-}$ mice was normalized with respect to wildtype mice since stimulated DA release levels are different under basal conditions (Figure 5C). Concentrations of 7,8-DHF greater than 1 μM show an ∼10% further reduction in stimulated DA release levels in BDNF$^{+/-}$ mice. These results in the BDNF$^{+/-}$ mice are surprising, since we initially predicted that BDNF$^{+/-}$ mice

dx.doi.org/10.1021/cn4000742 | ACS Chem. Neurosci. 2013, 4, 895−904

would have a leftward shift in their dose response curve based on their results with BDNF perfusion. However, future studies are required to better understand how 7,8-DHF induces DA release.

7,8-DHF Has No Effect on DA Transporter Kinetics in the CPu Across the Genotypes. There was no difference in DA transporter V_{max} rates in wildtype mice ($F_{8,207} = 0.32$, $P = 0.99$, Table 1). While a one-way ANOVA revealed a significant main effect of 7,8-DHF on the DA uptake rate in BDNF$^{+/-}$ mice ($F_{8,89} = 3.19$; $P < 0.01$, Table 1). Only concentrations 3.0 and 30 μM 7,8-DHF had a significantly decreased DA V_{max} rates in BDNF$^{+/-}$ mice as analyzed by a Dunnett's post-test analysis ($P < 0.05$).

Similar to BDNF, acutely applied 7,8-DHF does not affect striatal DA transporter kinetics in the wildtype mice. However, it is unknown what higher BDNF concentrations (>200 ng/mL) will do given its prohibitive cost. Thus, it is difficult to know if higher concentrations of BDNF would have a similar effect on DA transporter kinetics as 7,8-DHF. Although it is possible that these high concentrations of 7,8-DHF (>3.0 μM) are nonspecific and activating other Trk receptor subtypes such as TrkA and TrkC, we have not evaluated these receptor subtypes nor their ability to regulate presynaptic DA dynamics, and cannot rule out their contribution to presynaptic DA dynamics especially at these higher doses. Overall, the primary acute effect of 7,8-DHF does not appear to regulate striatal DA transporter kinetics in wildtype mice.

K252a Blocks the Ability of 7,8-DHF to Increase Electrically Evoked DA Release in Wildtype Mice. To demonstrate that 7,8-DHF is mediating its effect through the TrkB receptor, K252a (30 nM) was applied to the brain slice prior to agonist application. The objective was to block the 7,8-DHF mediated increases in electrically evoked DA in wildtype mice. A concentration of 10 μM 7,8-DHF was applied to the slices; this dose was chosen because it potentiated stimulated DA release in wildtype mice with no effect on DA transporter kinetics. A one-way ANOVA showed a main effect that wildtype mice respond differently depending on TrkB treatment ($F_{3,68} = 3.29$; $P < 0.05$, Figure 6C). Wildtype mice showed an approximate 20% increase in stimulated DA release after 7,8-DHF ($P < 0.05$) only. While there was no difference in stimulated DA release after K252a only.

When wildtype striatal slices were pretreated with K252a followed by 7,8-DHF applications, stimulated DA release was blocked. These results with K252a and 7,8-DHF demonstrate that the potentiated stimulated DA release in wildtype mice is mediated via the TrkB receptor. Numerous reports have indicated that 7,8-DHF is an agonist for the TrkB receptor,[36−43] and our results further confirm these previous findings. 7,8-DHF, like exogenous BDNF applications, can quickly regulate trophic factor pathways leading to activation and/or recruitment of DA release mechanisms. These results further highlight the hypothesis that enhancement of trophic factors or their agonist not only regulate long-term synaptic events such as synaptic plasticity, neuronal differentiation, or survival, but that there is an immediate response to trophic factors. Although with FSCV we have only evaluated DA in the CPu after 7,8-DHF, others have shown that this acute TrkB activation via BDNF is not exclusive to DA or monoamines like serotonin, but influences other systems such as GABA and glutamate.[13,14,26]

K252a and 7,8-DHF Have No Effect on DA Transporter Kinetics in Wildtype Mice. To confirm that 7,8-DHF applications is exclusive to DA release dynamics, DA uptake rates were evaluated. A one-way ANOVA showed no effect of TrkB treatment on V_{max} ($F_{3,80} = 1.18$, $P = 0.32$, Figure 6B) in wildtype mice. By decreasing the amount of K252a that is bathed over the slice, we demonstrated that this lower dose could still inhibit TrkB release without effecting DA transporter kinetics in wildtype mice. Together, these results from wildtype mice confirm the hypothesis that 7,8-DHF is mediating DA release via the TrkB receptor in wildtype mice with no effect on DA uptake kinetics.

■ CONCLUSIONS

A long-term goal in neuropharmacology has been to find small molecules that could mimic trophic factors and be easily administered where the small molecules could pass through the blood-brain barrier. With the recent discovery that 7,8-DHF is an agonist for TrkB receptors, numerous in vivo studies have shown that subacute applications of 7,8-DHF lead to many of the same benefits as that of a direct application of BDNF.[36,38,40,41] The results from this study highlight the utility of FSCV to probe acute, striatal BDNF/TrkB receptor mediated DA release and uptake. An advantage of using FSCV versus traditional synaptosomal models is that we can quickly measure both DA release and uptake in a more normal physiological state. FSCV has shown that a more "physiological" stimulation (1 ms) potentiates stimulated DA release in the presence of 7,8-DHF and BDNF in wildtype and BDNF$^{+/-}$ mice, respectively. This divergent role between 7,8-DHF and BDNF activating DA release may be a result of using a mouse model with a 50% reduction in BDNF protein levels, where there are not only intrinsic alterations in the BDNF/TrkB system but other neuroadaptions throughout the brain that are still unaccounted for. Our FSCV results are not limited to the evaluation of a TrkB agonist, but we also examined a TrkB inhibitor. K252a did not have any effect on stimulated DA release except at the highest dose (3 μM), while concentrations greater than 1 μM altered DA transporter kinetics. Since DA uptake V_{max} was altered in both genotypes at concentrations greater than 1 μM of K252a, this suggests a promiscuous response. Although K252a is often described as a TrkB inhibitor, it is a nonselective protein kinase inhibitor that inhibits PKC, Ca^{2+}/calmodulin-stimulated phosphodiesterases (IC_{50} = 1.3−2.9 μM from Tocris online catalog), MLCK (K_i = 20 nM from Tocris online catalog), and receptor tyrosine kinases, suggesting that TrkB or some other signaling system that K252a inhibits is influencing DA transporter uptake rates. By using FSCV, we were able to delineate a more comprehensive understanding of how TrkB receptor activation can modulate presynaptic DA dynamics using BDNF, a TrkB receptor agonist, or an inhibitor.

■ METHODS

Animals. Wildtype and BDNF$^{+/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME), and offspring were raised as a colony in-house in a certified vivarium (Association for Assessment and Accreditation of Laboratory Animal Care; AAALAC) at Wayne State University. The Institutional Animal Care and Use Committee at Wayne State University approved all animal procedures. Genotype identification was performed using PCR analysis of tail DNA as previously described.[24]

Slice FSCV. Mouse brain slices were obtained as previously described.[44,45] Voltammetric recordings were made using a homemade carbon fiber microelectrode with a length between 50 and 200 μm and a 7 μm diameter. The microelectrode was placed approximately 75 μm

dx.doi.org/10.1021/cn4000742 | ACS Chem. Neurosci. 2013, 4, 895−904

below the surface of the brain slice and approximately 100−200 μm away from a bipolar stimulating tungsten electrode placed directly on the slice. A triangular waveform was applied to the microelectrode with an initial potential of −0.4 V. The potential was ramped up to +1.2 V at a scan rate of 400 V/s at 10 Hz and then back to −0.4 V. DA release was electrically evoked from a brain slice every 5 min using a one pulse electrical stimulation (monophasic, 350 μA, 60 Hz, and 4 ms pulse width) generated by a Neurolog stimulator (Digitmeter, Hertfordshire, England) delivered through the stimulating electrode. The carbon fiber and stimulating electrodes were connected to the head stage of a low noise ChemClamp potentiostat (Dagan Corporation, Minneapolis, MN), and were controlled by TH software (ESA Inc., Chlemsford, MA). Current generated at the electrode surface as a result of the redox reaction of DA was subtracted from the background current. The peak oxidation current for DA was converted into concentration based on the postcalibration of 3 μM DA.

Chemicals. Chemicals used in the preparation of aCSF for voltammetric solutions were purchased from either Sigma-Aldrich (St. Louis, MO), Fisher Scientific Co. (Fairlawn, NJ), or EMD Chemicals, Inc. (Gibbstown, NJ) unless otherwise noted. The following compounds were purchased from specific vendors: 7,8-DHF from Tokyo Chemical Industry Co. Ltd. (Portland, OR), BDNF from PeproTech (Rocky Hill, NJ), and K252a from LC Laboratories (A Division of PKC Pharmaceuticals Inc., Woburn, MA). In all slice experiments, the pharmacological agents were dissolved in ultrapure (18 MΩ cm) water or DMSO, unless otherwise stated, and then diluted in oxygenated aCSF (composition in mM: 0.4 ascorbic acid, 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 11 D-glucose, and pH 7.4). All chemicals used in calibration experiments, including the verification of the redox capability of the drugs, were dissolved in DMSO and then diluted in a modified calibration aCSF buffer (composition in mM: 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, and pH 7.4)

Data Analysis. All voltammetric data were analyzed using LabVIEW National Instruments software (National Instruments, Austin, TX). Current versus time trace were fitted to a nonlinear regression.[46,47] A Michaelis−Menten based kinetic model was used to evaluate stimulated presynaptic DA release ([DA]p) and DA uptake kinetics (maximum velocity, V_{max}) and affinity of DA for its transporter (apparent K_m) by fitting DA current versus time traces.[44,48] In all voltammetric analysis, K_m values were set to 0.16 μM, which permits a nonlinear fit of DA release and uptake. Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA) during which statistical significance was determined by one-way ANOVA with Dunnett's multiple comparison test. The criterion for the statistical significance of DA analysis was set to $P < 0.05$. All data are reported as mean ± standard errors of the means (SEMs).

AUTHOR INFORMATION

Corresponding Author

*Mailing address: Department of Chemistry, 5101 Cass Ave., Detroit, MI 48202. Tel: 313-577-8660. Fax: 313-577- 8822. E-mail: tmathews@chem.wayne.edu.

Author Contributions

†A.K.A. and F.K.M. have contributed equally to this work and are co-first authors.

Author Contributions

A.K.A., F.K.M., and T.A.M. conceived and designed the experiments. A.K.A., F.K.M., and J.R.T. performed the experiments. A.K.A., F.K.M., J.R.T., and T.A.M. analyzed the data. A.K.A., F.K.M., and T.A.M. wrote the paper.

Funding

Funding supported this work provided by the National Institute on Alcohol Abuse and Alcoholism (NIAAA; AA-016967, T.A.M.) and Start-Up funds from Wayne State University. The content is solely the responsibility of the authors and does not represent the official views of NIAAA or National Institutes of Health.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Christopher Rogalla, Michelle L. Colombo, and Brooke D. Newman for genotyping the wildtype and $BDNF^{+/-}$ mice. The authors would like to thank Johnna A. Birbeck, Brooke D. Newman, and Bill H. Mathews for their insightful comments to the manuscript.

ABBREVIATIONS

CPu, caudate-putamen; NAc, nucleus accumbens; DA, dopamine; FSCV, fast-scan cyclic voltammetry; BDNF, brain-derived neurotrophic factor; TrkB, Tyrosine kinase receptor B; 7,8-DHF, 7,8-dihydroxyflavone; MAPK, mitogen activated protein kinase; PI3K, phosphatidylinositol-3-kinase; PLCγ, phospholipase C, γ, and; CREB, cAMP response element binding

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