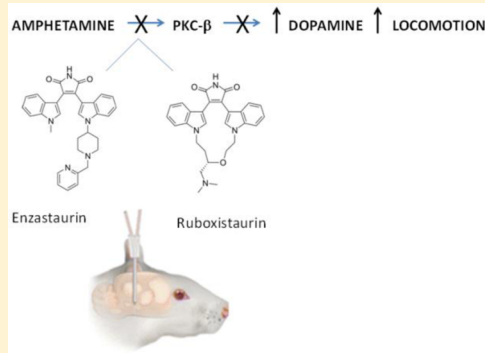


PKC $\beta$  Inhibitors Attenuate Amphetamine-Stimulated Dopamine EffluxAlexander G. Zestos,<sup>†,‡</sup> Sarah R. Mikelman,<sup>†</sup> Robert T. Kennedy,<sup>†,‡</sup> and Margaret E. Gnegy<sup>\*,†</sup><sup>†</sup>Department of Pharmacology, University of Michigan, 2301 MSRB III, 1150 W. Medical Center Drive, Ann Arbor, Michigan 48109-5632, United States<sup>‡</sup>Department of Chemistry, University of Michigan, 9300 North University Avenue, Ann Arbor, Michigan 48105, United States

## Supporting Information

**ABSTRACT:** Amphetamine abuse afflicts over 13 million people, and there is currently no universally accepted treatment for amphetamine addiction. Amphetamine serves as a substrate for the dopamine transporter and reverses the transporter to cause an increase in extracellular dopamine. Activation of the beta subunit of protein kinase C (PKC $\beta$ ) enhances extracellular dopamine in the presence of amphetamine by facilitating the reverse transport of dopamine and internalizing the D2 autoreceptor. We previously demonstrated that PKC $\beta$  inhibitors block amphetamine-stimulated dopamine efflux in synaptosomes from rat striatum *in vitro*. In this study, we utilized *in vivo* microdialysis in live, behaving rats to assess the effect of the PKC $\beta$  inhibitors, enzastaurin and ruboxistaurin, on amphetamine-stimulated locomotion and increases in monoamines and their metabolites. A 30 min perfusion of the nucleus accumbens core with 1  $\mu$ M enzastaurin or 1  $\mu$ M ruboxistaurin reduced efflux of dopamine and its metabolite 3-methoxytyramine induced by amphetamine by approximately 50%. The inhibitors also significantly reduced amphetamine-stimulated extracellular levels of norepinephrine. The stimulation of locomotor behavior by amphetamine, measured simultaneously with the analytes, was comparably reduced by the PKC $\beta$  inhibitors. Using a stable isotope label retrodialysis procedure, we determined that ruboxistaurin had no effect on basal levels of dopamine, norepinephrine, glutamate, or GABA. In addition, normal uptake function through the dopamine transporter was unaltered by the PKC $\beta$  inhibitors, as measured in rat synaptosomes. Our results support the utility of using PKC $\beta$  inhibitors to reduce the effects of amphetamine.



**KEYWORDS:** Protein kinase C, catecholamine, amphetamine, microdialysis, LCMS, dopamine

Amphetamine-type stimulants are the second most commonly abused drug in the world. However, there is no commonly accepted treatment for amphetamine abuse.<sup>1</sup> Effects of amphetamine include tachycardia, hyperactivity, psychosis, euphoria, and drug seeking,<sup>2</sup> which are a result of amphetamine increasing extracellular dopamine and norepinephrine.<sup>3,4</sup> As a transporter substrate, amphetamine is taken up into dopamine neurons. Thus, amphetamine competitively blocks the uptake of dopamine and elicits a reversal of the transporter, increasing extracellular dopamine.<sup>1</sup>

When amphetamine is taken up by the dopamine transporter, there is a release of intracellular Ca<sup>2+</sup><sup>5</sup> and an activation of protein kinase C (PKC).<sup>6–8</sup> This increase in PKC activity<sup>9</sup> has been shown to contribute to reverse transport of dopamine through the dopamine transporter.<sup>9,10</sup> PKC can phosphorylate N-terminal serines in the dopamine transporter,<sup>11</sup> and evidence suggests that phosphorylation of one or more N-terminal serines is essential for amphetamine-stimulated dopamine efflux.<sup>12</sup> Ten isozymes of PKC are known and are grouped according to their domain composition and activating cofactors.<sup>13</sup> Amphetamine-stimulated increases in extracellular dopamine require intracellular Ca<sup>2+</sup>, diacylglycerol, and a

phospholipid,<sup>14,5,15</sup> consistent with the involvement of a Ca<sup>2+</sup>-requiring conventional PKC isozyme. Studies have demonstrated that the Ca<sup>2+</sup>-requiring conventional isozyme of PKC, PKC $\beta$ , modulates dopamine transporter and amphetamine function.<sup>16</sup> Both inhibition and deletion of PKC $\beta$  reduce amphetamine-stimulated dopamine efflux *in vitro*.<sup>16,17</sup> Moreover, PKC $\beta$  is coexpressed with the dopamine transporter in midbrain neurons.<sup>18</sup> Such observations suggest that PKC $\beta$  is a target for modulating the effects of amphetamine.

Although a nonselective bisindolylmaleimide PKC inhibitor has been demonstrated to reduce amphetamine-stimulated dopamine release *in vivo* via microdialysis,<sup>19,20</sup> the effect of selective inhibition of the  $\beta$  isoform of PKC on amphetamine-stimulated increases in extracellular dopamine has not been demonstrated. Further, amphetamine stimulates the efflux of norepinephrine and serotonin, but the effect of PKC $\beta$  inhibition on reverse transport of these monoamines has not been examined. In this study, we test the effects of the selective

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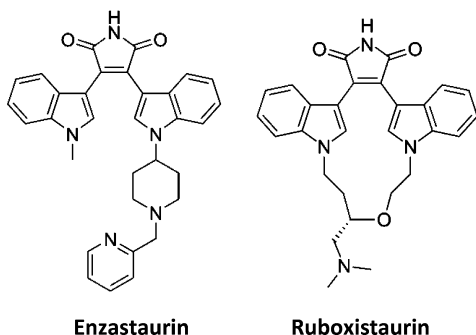
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PKC $\beta$  inhibitors, ruboxistaurin and enzastaurin, both bisindolylmaleimides, on amphetamine-stimulated extracellular neurotransmitter levels using retrodialysis in the nucleus accumbens. The bisindolylmaleimide moiety binds to and inhibits the active catalytic ATP-binding site of PKC, while the side chain of these drugs provides specificity to the PKC $\beta$  isoform. The  $\beta$  isoform of PKC is one of the few PKC isoforms for which relatively specific small molecular inhibitors exist. Through the sensitivity of our measurement technique, we are able to determine the effect of the PKC $\beta$  inhibitors on amphetamine-stimulated levels of monoamine neurochemicals and their metabolites.

We find that the PKC $\beta$  inhibitors attenuate amphetamine-stimulated overflow of dopamine in the nucleus accumbens without affecting basal levels of dopamine. In addition to dopamine overflow, the PKC $\beta$  inhibitors were effective in reducing the overflow of norepinephrine. The effect of the PKC $\beta$  inhibitors on serotonin efflux in the nucleus accumbens was less pronounced than that for dopamine and norepinephrine. Moreover, the PKC $\beta$  inhibitors, enzastaurin and ruboxistaurin, had no effect on the uptake of dopamine.

## RESULTS AND DISCUSSION

**Effect of Amphetamine.** The existence of selective small molecular inhibitors of PKC $\beta$  enabled us to determine the direct effect of PKC $\beta$  inhibition on amphetamine-stimulated dopamine overflow *in vivo*. The bisindolylmaleimide moieties contained in enzastaurin and ruboxistaurin (Figure 1) also form



**Figure 1.** Molecular structures of enzastaurin and ruboxistaurin. Enzastaurin and ruboxistaurin are both bisindolylmaleimides that bind to the catalytic ATP-binding site of PKC. The side chains make them specific to bind to the  $\beta$ -isoforms of PKC.

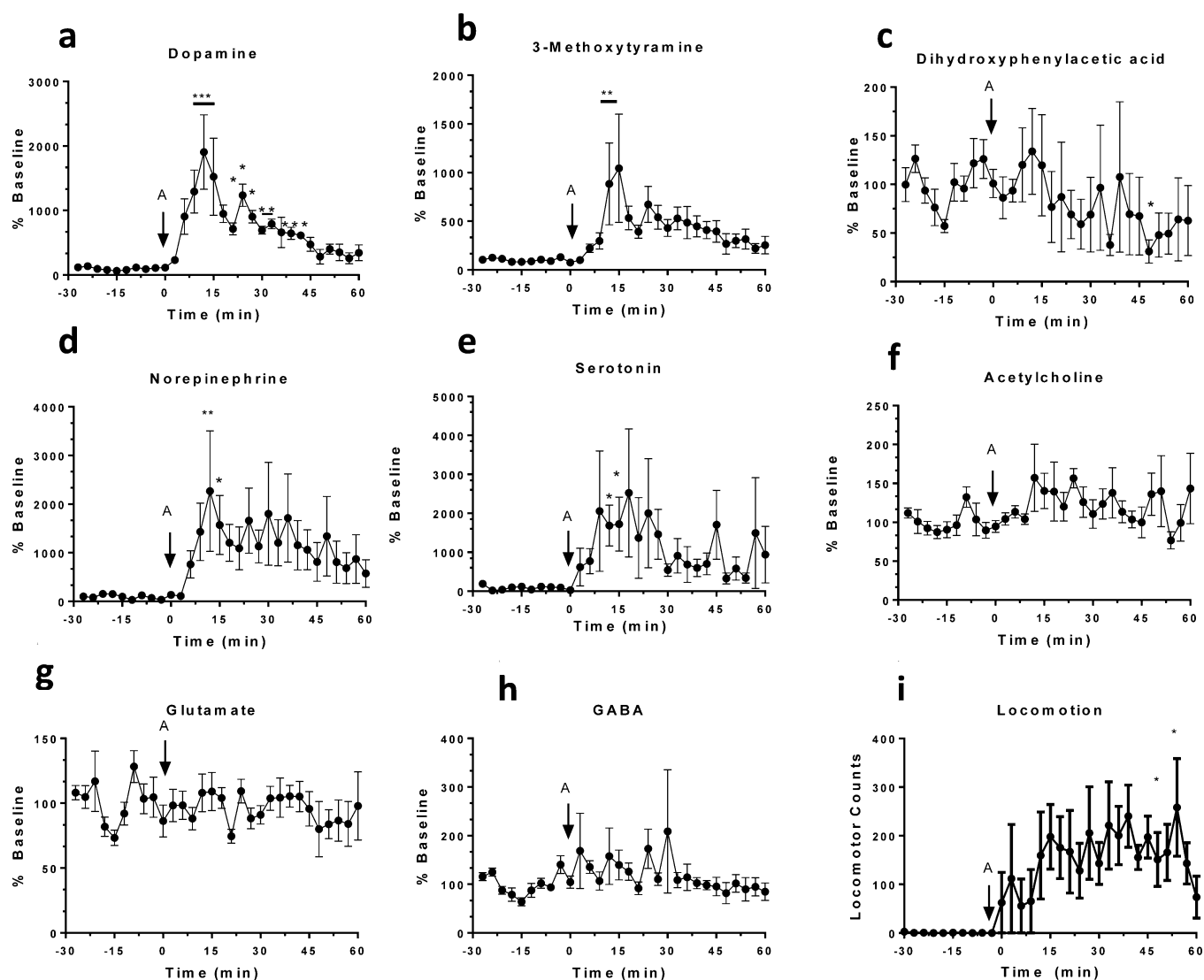
the core of a wide variety of biologically active compounds such as in tivantinib and bisindolylmaleimide I.<sup>21</sup> Bisindolylmaleimides inhibit PKC by binding to the active site.<sup>22,23</sup> Despite the similarities in the active site among the isozymes, bisindolylmaleimides with different side chains, such as ruboxistaurin and enzastaurin, have been developed that selectively inhibit PKC $\beta$ .<sup>22</sup>

We measured the effect of systemic amphetamine on the extracellular concentration of the monoamines dopamine, norepinephrine, and serotonin, and several monoamine metabolites such as dihydroxyphenylacetic acid, 3-methoxytyramine, and normetanephrine, and other key neurochemicals such as glutamate,  $\gamma$ -aminobutyric acid (GABA), and acetylcholine in the rat nucleus accumbens core (Supplemental Figure 2 and Table 1). As shown in Figure 2a, an intraperitoneal injection of 2.5 mg/kg amphetamine rapidly and robustly increased the concentration of dopamine in the microdialysate ( $p = 0.0001$  by RM one-way ANOVA,  $F(29,116) = 6.459$  for

treatment,  $n = 5$ ). There was a similar significant increase in the dopamine metabolite, 3-methoxytyramine (Figure 2b,  $p = 0.0001$  by one-way RM ANOVA,  $F(29,116) = 5.874$  for treatment,  $n = 5$ ). 3-Methoxytyramine levels should reflect those of extracellular dopamine since it is produced by the metabolism of extracellular dopamine by catechol-*O*-methyltransferase. In contrast, dihydroxyphenylacetic acid (Figure 2c) is produced from dopamine presynaptically by monoamine oxidase. As expected, dihydroxyphenylacetic acid decreases as dopamine is released from the cell upon amphetamine administration as shown in Figure 2. Dihydroxyphenylacetic acid and 3-methoxytyramine are further metabolized by catechol-*O*-methyltransferase and monoamine oxidase, respectively, to homovanillic acid. Concentrations of homovanillic acid did not change upon amphetamine administration (Supplemental Table 1). Amphetamine is also a substrate at the norepinephrine and serotonin transporters, with a potency range of norepinephrine > dopamine  $\gg$  serotonin transporters.<sup>24,25</sup> As expected, obvious increases in extracellular norepinephrine and serotonin resulted from the injection of amphetamine (Figure 2d,e), but only the increases in norepinephrine reached statistical significance ( $p = 0.001$  by RM one-way ANOVA;  $F(29,116) = 2.893$ ). For serotonin, significance by RM one-way ANOVA reached  $p = 0.07$ . Serotonin terminals synapsing with postsynaptic elements have been identified in both the nucleus accumbens core and shell.<sup>26</sup> Noradrenergic terminals have been identified in the nucleus accumbens shell but very few in the core.<sup>27</sup> Although we aimed for the nucleus accumbens core, it is possible that some of the probes sufficiently extended into the shell to allow for the detection of norepinephrine (Supplemental Table 1). The data of Figure 2f–h demonstrate that there was no change in efflux of acetylcholine (Figure 2f), glutamate (Figure 2g), or  $\gamma$ -aminobutyric acid (GABA) (Figure 2h) following amphetamine administration.

The basal levels of the monoamine analytes measured are given in the figure legends. There was no significant change in dialysate concentration of any other analyte measured in the nucleus accumbens in response to amphetamine (Supplemental Figure 2). It is possible that a different result could be attained if another brain area was measured. We focused on the nucleus accumbens because that area engenders locomotor activity and reinforcement in response to amphetamine.<sup>28</sup> We functionally assessed the effect of amphetamine by measuring the effect of the drug on locomotion concurrent with microdialysis as described in Methods. Amphetamine administration elicited a significant increase in locomotion ( $p = 0.0002$  by RM one-way ANOVA;  $F(29,87) = 2.706$ ) correlated with the increase in extracellular dopamine (Figure 2i). Locomotor counts are very close to 0 before amphetamine administration because the animal is usually sleeping, and the camera and Matlab software do not take into account normal movements such as breathing and whisker movement.

**Enzastaurin.** Having established the baseline effects of amphetamine, we next investigated the effect of the selective PKC $\beta$  inhibitors on amphetamine-stimulated efflux of the neurotransmitters. As analyzed by one-way RM ANOVA, in this experiment the amphetamine injection significantly increased the extracellular concentrations of dopamine ( $p < 0.0001$ ,  $F(39, 156) = 10.89$ ), 3-methoxytyramine ( $p < 0.0001$ ,  $F(39, 156) = 3.602$ ), norepinephrine ( $p = 0.0011$ ,  $F(35, 140) = 2.117$ ), serotonin ( $p = 0.021$ ,  $F(39, 156) = 1.620$ ), and locomotor activity ( $p < 0.0001$ ,  $F(40, 120) = 5.758$ ), and

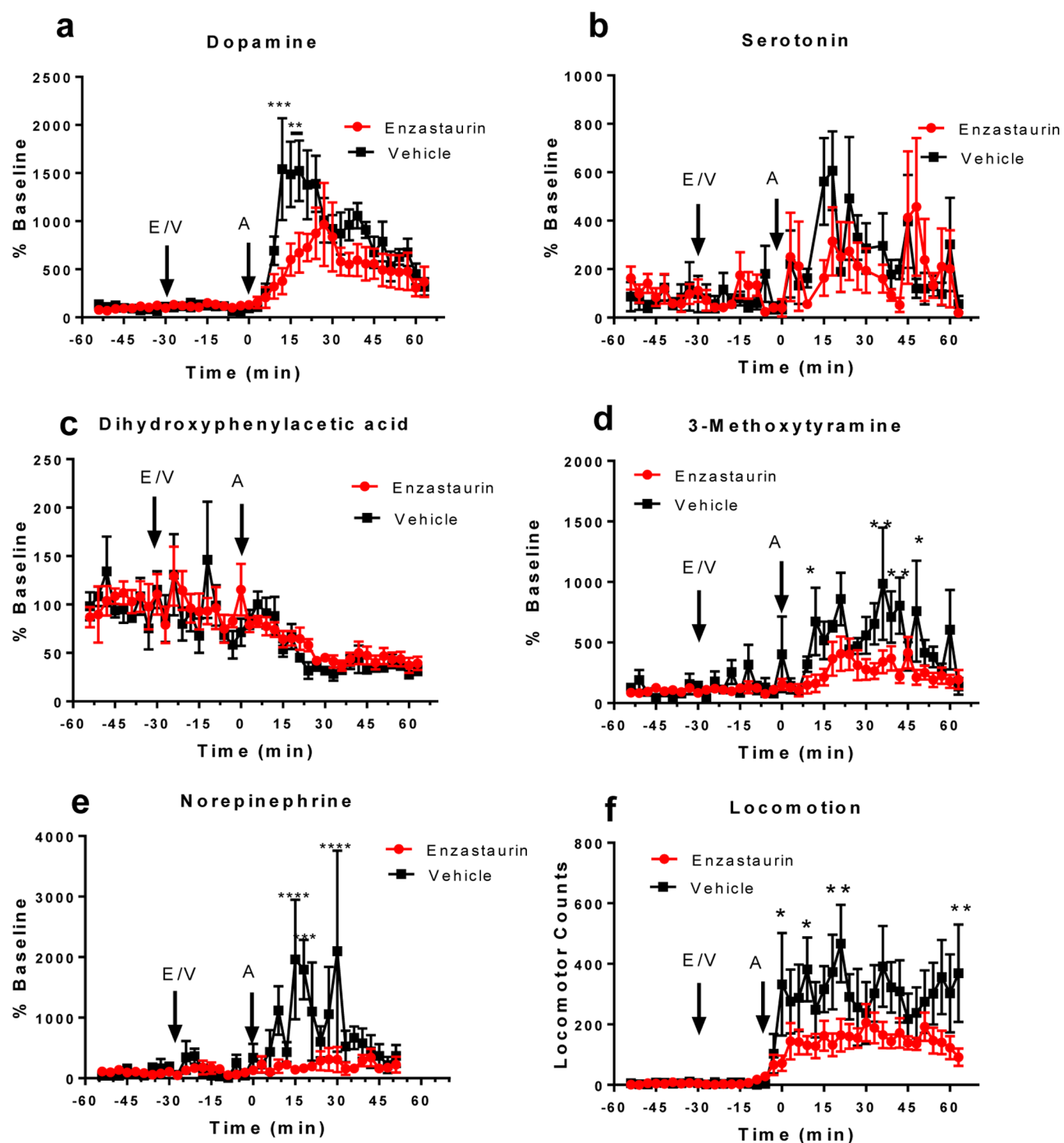


**Figure 2.** Effects of amphetamine administration (arrow, 2.5 mg/kg IP) on extracellular levels of (a) dopamine, (b) 3-methoxytyramine, (c) dihydroxyphenylacetic acid, (d) norepinephrine, (e) serotonin, (f) acetylcholine, (g) glutamate, (h) GABA, and (i) locomotion. Amphetamine increases levels of dopamine, norepinephrine, and serotonin over 2,000% of the baseline. It is a substrate for the dopamine, norepinephrine, and serotonin transporters. The rise in locomotor counts correlates with the increase in extracellular dopamine ( $n = 5$ ). Preamphetamine baseline values for the monoamines were (in nM) as follows: dopamine,  $1.95 \pm 0.74$ ; 3-methoxytyramine,  $1.11 \pm 1.40$ ; dihydroxyphenylacetic acid,  $676 \pm 76$ ; norepinephrine,  $0.43 \pm 1.39$ ; normetanephrine,  $0.27 \pm 0.17$ ; serotonin,  $0.07 \pm 0.21$ ; glutamate,  $729 \pm 263$ ; acetylcholine,  $0.3 \pm 0.14$ ; and GABA,  $24.7 \pm 8.1$ . (a) In the post hoc Dunnett's multiple comparison test,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ; (b) in the post hoc Dunnett's multiple comparison test,  $*p < 0.05$ . The arrows indicate the administration of amphetamine (A).

decreased dihydroxyphenylacetic acid ( $p < 0.0001$ ,  $F(39, 156) = 3.547$ ). Enzastaurin ( $1 \mu\text{M}$ , perfused for 30 min before amphetamine injection) had no effect on the basal concentration of any of the 18 compounds measured in the assay (Supplemental Table 1 and Figure 2); however, it significantly reduced amphetamine-stimulated (2.5 mg/kg) dopamine efflux (Figure 3a). In a one-way repeated measures (RM) ANOVA,  $p < 0.0001$  ( $F(39, 351) = 14.92$ ) for time,  $p = 0.126$  for drug ( $F(1, 10) = 2.791$ ), but  $p < 0.0001$  ( $F(39, 351) = 2.786$ ) ( $F(39, 390) = 2.264$ ) for interaction of time and drug. A similar reduction in amphetamine-stimulated extracellular 3-methoxytyramine occurred following the perfusion of enzastaurin (Figure 3d,  $p < 0.0001$  for time ( $F(39, 390) = 6.992$ ),  $p = 0.102$  ( $F(1, 10) = 3.250$ ) for drug but  $p < 0.0001$  ( $F(39, 390) = 2.315$ ) for the interaction of drug and time. Enzastaurin had no effect on extracellular dihydroxyphenylacetic acid (Figure 3c) or

homovanillic acid (Supplemental Table 1). In parallel with the inhibition of dopamine efflux, perfusion with enzastaurin reduced amphetamine-stimulated locomotion as compared with the vehicle (Figure 3f). In a one-way RM ANOVA,  $p < 0.0001$  ( $F(39, 351) = 14.92$ ) for time,  $p < 0.081$  ( $F(1, 9) = 3.874$ ) for drug, but  $p < 0.0001$  ( $F(39, 351) = 2.786$ ) for the interaction of time and drug. Our results are compatible with the concept that amphetamine activates  $\text{PKC}\beta$ ,<sup>29</sup> which then potentiates the reversal of the dopamine transporter and efflux of dopamine.<sup>7</sup> Direct activation of PKC has been demonstrated to increase dopamine efflux *in vitro*<sup>10</sup> and to enhance 3,4-methylenedioxymethamphetamine-stimulated dopamine efflux *in vivo*.<sup>20</sup>

Whereas enzastaurin only partially blocked the increase in dopamine in response to amphetamine, it nearly completely blocked the amphetamine-stimulated increase in extracellular

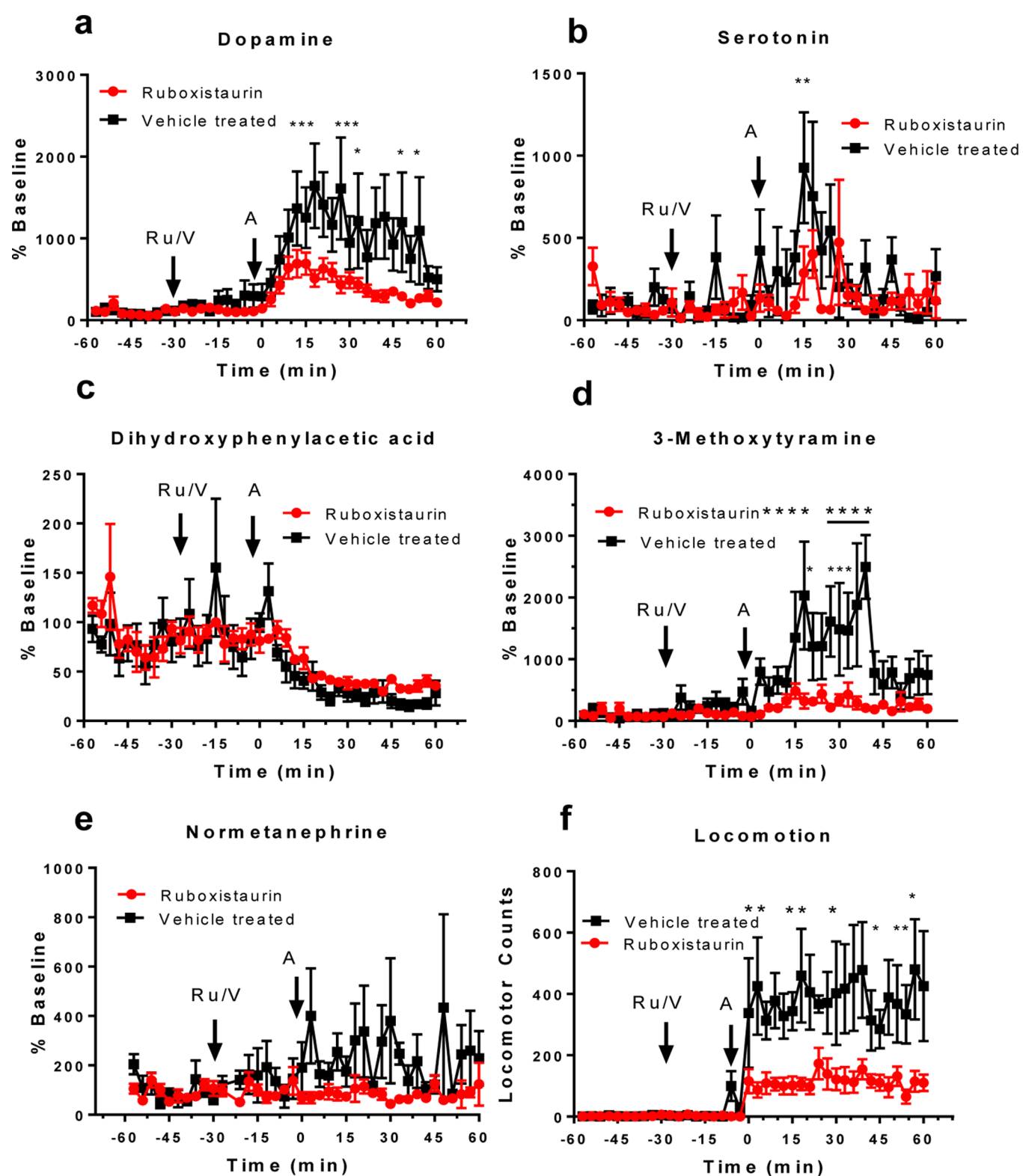


**Figure 3.** Enzastaurin attenuates amphetamine-stimulated catecholamine overflow and locomotor behavior. Shown are the effects of perfusing 1  $\mu$ M enzastaurin using retrodialysis on amphetamine-stimulated overflow of (a) dopamine, (b) serotonin, (c) dihydroxyphenylacetic acid, (d) 3-methoxytyramine, (e) norepinephrine, and (f) locomotion. The first arrow indicates the perfusion of enzastaurin or vehicle (E/V), while the second arrow is attributed to amphetamine (A) administration (2.5 mg/kg IP). Dopamine, 3-methoxytyramine, norepinephrine, normetanephrine, and locomotion are significantly decreased by enzastaurin. Serotonin and dihydroxyphenylacetic acid are unaffected.  $N = 7$  for enzastaurin and  $N = 5$  for the vehicle. Post hoc analysis was determined in Prism 6 using Sidak's multiple comparison test for a–g; \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , and \* $p < 0.05$ . Preamphetamine baseline values for the monoamines were (in nM) as follows: dopamine,  $3.1 \pm 0.32$ ; 3-methoxytyramine,  $1.04 \pm 0.10$ ; dihydroxyphenylacetic acid,  $931 \pm 92$ ; norepinephrine,  $0.40 \pm 0.1$ ; and serotonin,  $0.27 \pm 0.05$ .

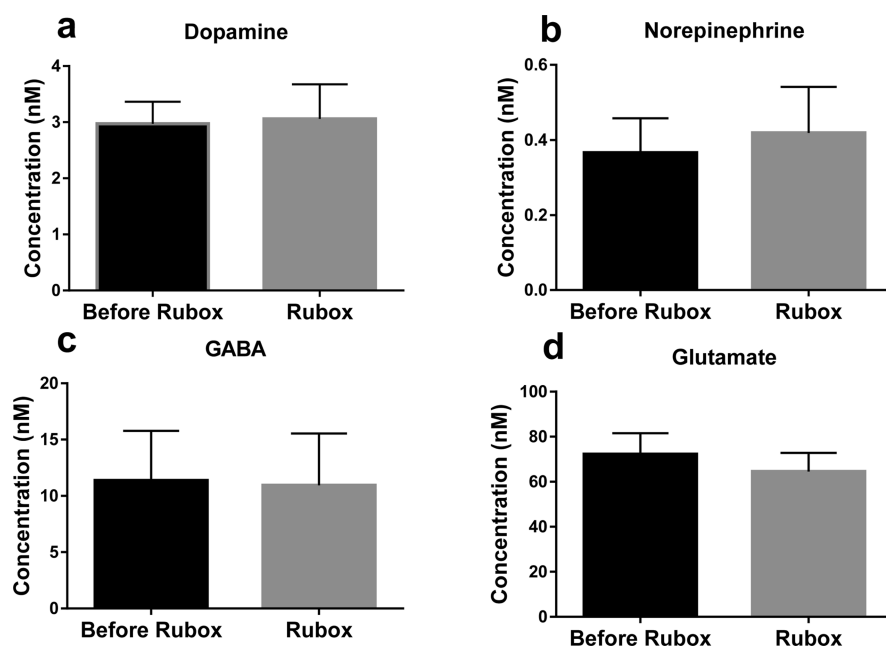
norepinephrine (Figure 3e; in one-way RM ANOVA,  $p < 0.0001$  for time,  $(F(35, 350) = 3.260)$ ,  $p < 0.093$  for drug and  $p < 0.0001$  for the interaction of time and drug  $(F(35, 350) = 2.498)$ ). There was no significant effect of enzastaurin on

serotonin efflux (Figure 3f). This result suggests that the PKC $\beta$  inhibitor, enzastaurin, acts more selectively on the catecholamine (dopamine and norepinephrine) than on serotonin transporters. The effect of selective PKC $\beta$  inhibitors on





**Figure 4.** Ruboxistaurin attenuates amphetamine-stimulated catecholamine efflux and locomotor behavior. Shown are the effects of perfusing  $1 \mu\text{M}$  ruboxistaurin using retrodialysis on amphetamine-stimulated efflux of (a) dopamine, (b) serotonin, (c) dihydroxyphenylacetic acid, (d) 3-methoxytyramine, (e) normetanephrine, and (f) locomotion. The first arrow indicates the perfusion of ruboxistaurin or vehicle (Ru/V), while the second arrow is attributed to amphetamine administration (A,  $2.0 \text{ mg/kg IP}$ ). Dopamine, 3-methoxytyramine, norepinephrine, and locomotion are significantly decreased by ruboxistaurin. Dihydroxyphenylacetic acid is unaffected. Post hoc analysis was determined in Prism 6 using Sidak's multiple comparison test for a–g; \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , and \* $p < 0.05$ . Preamphetamine baseline values for the monoamines were (in nM) as follows: dopamine,  $1.94 \pm 0.03$ ; 3-methoxytyramine,  $0.58 \pm 0.10$ ; dihydroxyphenylacetic acid,  $858 \pm 106$ ; normetanephrine,  $0.14 \pm 0.02$ ; and serotonin,  $0.20 \pm 0.04$ .



**Figure 5.** Stable isotope label. *In vivo* calibration of isotopically labeled (a) dopamine, (b) norepinephrine, (c) GABA, and (d) glutamate. The isotope of each neurochemical was perfused into the probe using retrodialysis. The percent recovery was used to calculate the extraction fraction, which was used to calculate the applied concentrations of neurochemicals *in vivo*. Glutamate is found in much larger concentrations than the other three neurochemicals. Perfusion with 1  $\mu$ M ruboxistaurin had no effect on the average basal levels of these four neurochemicals ( $n = 5$ ).

amphetamine-stimulated norepinephrine efflux has not previously been investigated since it is usually found in higher concentrations in other brain regions such as the prefrontal cortex. We have shown, however, that general PKC inhibitors will inhibit dopamine efflux through the norepinephrine transporter in pheochromocytoma PC12 cells.<sup>14</sup> Activation of PKC by serotonin transporter substrates, such as fenfluramine and 3,4-methylenedioxymethamphetamine, has been reported in the hippocampus<sup>30</sup> and cortex.<sup>31</sup> However, the consequences of inhibition of PKC in general, or PKC $\beta$  in particular, on serotonin transporter-mediated efflux have not been previously examined. It is possible that enzastaurin could decrease amphetamine-stimulated serotonin efflux more robustly in an area of the brain that has a higher concentration of serotonin, such as the hippocampus or frontal cortex. The serotonin transporter appears to be a substrate for PKC,<sup>32</sup> and activation of PKC in cells<sup>33</sup> and platelets<sup>34</sup> resulted in the internalization of the transporter.

**Ruboxistaurin.** Like enzastaurin, ruboxistaurin is also a bisindolylmaleimide that inhibits PKC $\beta$  by binding to its active site.<sup>35</sup> Although ruboxistaurin underwent phase III clinical trials to treat diabetic peripheral retinopathy, it does not cross the blood–brain barrier.<sup>36</sup> The effect of ruboxistaurin on amphetamine-stimulated neurochemical efflux and associated behavior was also measured to further delineate the efficacy of PKC $\beta$  inhibition. The different side chains on the bisindolylmaleimide moiety could elicit different effects.

Following 30 min of baseline value collection, 1  $\mu$ M ruboxistaurin in aCSF was perfused into the nucleus accumbens for 30 min. During this period, ruboxistaurin had no effect on the basal concentration of any of the chemicals measured (Figure 3 and Supplemental Table 1). After 1 h total, a 2.0 mg/kg dose IP injection of amphetamine was administered. Three minute fractions were collected for the baseline for the entire duration of the experiment (1.5 h). In contrast to the enzastaurin experiment, a lower dose of amphetamine (2.0

mg/kg from 2.5 mg/kg) was given to reduce the potential development of stereotypy, which was observed with the aforementioned higher dose of amphetamine (2.5 mg/kg). As analyzed by one-way RM ANOVA, in this experiment the amphetamine injection significantly increased the extracellular concentrations of dopamine ( $p < 0.0001$ ;  $F(39, 156) = 4.044$ ), 3-methoxytyramine ( $p < 0.0001$ ;  $F(39, 156) = 3.154$ ), serotonin ( $p < 0.0007$ ;  $F(39, 156) = 2.104$ ), and locomotor activity ( $p < 0.0001$ ;  $F(39, 156) = 6.799$ ), and decreased dihydroxyphenylacetic acid ( $p < 0.0001$ ;  $F(39, 156) = 3.556$ ). As shown in Figure 4a, similar to enzastaurin, perfusion of ruboxistaurin robustly decreased amphetamine-stimulated efflux of dopamine (Figure 4a) and 3-methoxytyramine (Figure 4d). A one-way RM ANOVA for dopamine produced  $p < 0.0001$  for time ( $F(39, 351) = 13.78$ ),  $p < 0.002$  for drug ( $F(1, 9) = 19.59$ ), and  $p < 0.0001$  for the interaction of drug and time ( $F(39, 351) = 4.972$ ). In a one-way RM ANOVA for 3-methoxytyramine (Figure 4d),  $p < 0.0001$  for time ( $F(39, 351) = 6.359$ ),  $p < 0.01$  for drug ( $F(1, 9) = 22.24$ ), and  $p < 0.0001$  for the interaction of time and drug ( $F(39, 351) = 3.966$ ). Ruboxistaurin had no effect on dihydroxyphenylacetic acid levels (Figure 4c). There was no change in basal locomotor activity during the 30 min of perfusion with ruboxistaurin, but concomitant with decreases in dopamine and 3-methoxytyramine, there was a significant reduction in locomotor counts after amphetamine injection in comparison to that of controls (Figure 4f, one-way RM ANOVA,  $p < 0.0001$  for time ( $F(39, 351) = 12.44$ ),  $p < 0.05$  for drug ( $F(1, 9) = 7.532$ ), and  $p < 0.0001$  for the interaction of drug and time ( $F(39, 351) = 3.901$ ).

In these experiments, the increases in norepinephrine and normetanephrine (Figure 4e) following amphetamine were highly variable, so significant changes in norepinephrine and normetanephrine concentrations due to ruboxistaurin could not be assessed. In contrast to the effective inhibition of extracellular dopamine, ruboxistaurin perfusion only partially reduced the amphetamine-stimulated efflux of serotonin

(Figure 4f) (in one-way RM ANOVA,  $p < 0.0001$  for time ( $F(39, 351) = 3.383$ ),  $p = 0.16$  for drug ( $F(1,9) = 0.16$ ), and  $p < 0.001$  for the interaction of drug and time ( $F(39, 351) = 2.017$ )). It is not clear why ruboxistaurin seemingly had a greater effect on serotonin efflux than enzastaurin. Both compounds have an  $IC_{50}$  of approximately 6 nM for PKC $\beta$  in cell-free assays and similar relative selectivity for PKC $\beta$  over other subtypes.<sup>22,37</sup> It is possible that small differences in the structures could elicit different effects in the neurons.

**Effect on Basal Concentrations.** As mentioned above, neither enzastaurin nor ruboxistaurin altered the dialysate concentration of neurotransmitters and metabolites that were measured suggesting no nonspecific or generalized effects of PKC $\beta$  inhibition. To further examine this issue, we used quantitative microdialysis based on the stable-isotope label method<sup>38</sup> to measure the effect of ruboxistaurin on the basal concentration of the monoamines, glutamate, and GABA. This method can account for changes in recovery *in vivo* and allows detection of more subtle changes. For these measurements, 15 min fractions were collected for 1.5 h to determine the baseline, then 1  $\mu$ M ruboxistaurin was perfused, and fractions were collected for another 1.5 h. Basal concentrations (nM) found with the stable isotope label were  $11.4 \pm 0.4$  for GABA,  $3.0 \pm 0.4$  for dopamine,  $0.37 \pm 0.01$  for norepinephrine, and  $72 \pm 9$  for glutamate ( $n = 5$ ). Upon perfusing with ruboxistaurin, there was no significant change in the concentrations of these compounds (Figure 5). These results provide evidence for the specificity of ruboxistaurin for interrupting amphetamine action on the dopamine transporter, rather than nonspecifically altering other neurotransmitter levels.

**Effect on Reuptake.** One explanation for the reduction of amphetamine-stimulated outward transport by enzastaurin and ruboxistaurin is that the drugs could also block inward transport. Consequently, [<sup>3</sup>H]dopamine uptake in response to enzastaurin and ruboxistaurin was measured in striatal synaptosomes. Concentrations of the PKC $\beta$  inhibitors were chosen to match the concentration of the total drug in the tissue following perfusion. While the concentration of drug perfused was 1  $\mu$ M, the estimated recovery through the probe was 40%, as measured in the dialysate using LC-MS. As shown in Table 1, neither drug altered [<sup>3</sup>H]dopamine uptake at any

**Table 1.** [<sup>3</sup>H]Dopamine Uptake in Rat Striatal Synaptosomes<sup>a</sup>

[drug]	pmol/3 min/mg protein $\pm$ SEM			
	vehicle	300 nM	560 nM	1000 nM
ruboxistaurin	17 $\pm$ 4	19 $\pm$ 5	17 $\pm$ 3	18 $\pm$ 5
enzastaurin	24 $\pm$ 3	22 $\pm$ 1	23 $\pm$ 3	23 $\pm$ 2

<sup>a</sup>Rat striatal synaptosomes were incubated with or without variable concentrations of ruboxistaurin and enzastaurin. Results are given as pmol of [<sup>3</sup>H]dopamine/3 min/mg protein  $\pm$  the standard error of the mean (SEM),  $n = 3$ . No significant difference in [<sup>3</sup>H]dopamine uptake was seen between drug-treated and vehicle-treated synaptosomes.

concentration up to 1  $\mu$ M. Therefore, both enzastaurin and ruboxistaurin decreased amphetamine-stimulated dopamine efflux without reducing the normal uptake function of the transporter. This conclusion may seem counterintuitive because the transporter functions as a pump that can work in both directions. However, there is evidence that amphetamine can act on a transporter oligomer and that phosphorylation of one of the subunits potentiates outward transport.<sup>39</sup> Therefore,

phosphorylation could affect the transporter asymmetrically. While it is possible that PKC $\beta$  could directly phosphorylate the dopamine transporter at N-terminal serines,<sup>11</sup> that possibility is less likely for the norepinephrine transporter because it does not contain the N-terminal serines that are PKC substrates in the human or rat dopamine transporter.<sup>11,40</sup> PKC-stimulated phosphorylation of the norepinephrine transporter (NET) at Thr-258 and Ser-259 is linked to PKC-stimulated internalization of NET.<sup>41</sup> It is possible that PKC $\beta$  activation leads to an interaction of another component/protein with the intracellular face of the transporters that may asymmetrically alter their function. A number of proteins bind to the dopamine transporter (DAT),<sup>42</sup> and PKC $\beta$  coimmunoprecipitates with the dopamine transporter.<sup>16,42</sup>

We have previously demonstrated that PKC $\beta$  is crucial for an interaction between the dopamine autoreceptor and the DAT.<sup>43</sup> Activation of the dopamine autoreceptor increases surface DAT through a PKC $\beta$ - and extracellular receptor kinase-dependent mechanism.<sup>44</sup> We have further demonstrated that inhibition of PKC $\beta$  potentiates dopamine autoreceptor function by retaining surface D2-like receptors.<sup>45</sup> Prior studies demonstrated that PKC activation can inhibit dopamine autoreceptor function<sup>46</sup> and cause the internalization of dopamine D2-like receptors.<sup>47</sup> Considering that inhibition of PKC $\beta$  can reduce extracellular dopamine both by potentiating autoreceptor function and by inhibiting the effect of amphetamine, it is possible that inhibition of PKC $\beta$  could be a useful strategy to reduce amphetamine abuse.

## CONCLUSIONS

We demonstrated that the PKC $\beta$ -selective inhibitors, enzastaurin and ruboxistaurin, robustly reduce amphetamine-stimulated dopamine and norepinephrine reverse transport through their respective transporters. This study is the first to utilize retrodialysis of specific PKC $\beta$  inhibitors into the nucleus accumbens *in vivo* with subsequent measurement of metabolites and other neurotransmitters. Our results demonstrate that the effect of these inhibitors is primarily on amphetamine-induced enhancement of catecholamine transport, at least in the nucleus accumbens. Perfusing ruboxistaurin, without amphetamine, into the nucleus accumbens does not alter the basal concentrations of dopamine, GABA, glutamate, and norepinephrine. Therefore, these experiments suggest that PKC $\beta$  inhibitors such as ruboxistaurin inhibit PKC $\beta$  specifically in response to amphetamine activation of monoamine concentrations in the nucleus accumbens. This study provides novel insight into the effects of PKC $\beta$  on the mechanism of amphetamine-stimulated dopamine efflux and suggests that PKC $\beta$  inhibitors could be potentially used therapeutically for amphetamine abuse.

## METHODS

**Materials.** All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Artificial cerebral spinal fluid (aCSF) comprised 145 mM NaCl, 2.68 mM KCl, 1.01 mM MgSO<sub>4</sub>, 1.22 mM CaCl<sub>2</sub>, 1.55 mM Na<sub>2</sub>HPO<sub>4</sub>, and 0.45 mM NaH<sub>2</sub>PO<sub>4</sub> (salts from Fisher Scientific, Pittsburgh, PA). Vehicle for PKC $\beta$  inhibitors was 0.05% dimethyl sulfoxide (DMSO) in ACSF. [<sup>13</sup>C<sub>6</sub>-Dopamine (<sup>13</sup>C<sub>6</sub>-DA) was purchased from CDN isotopes (Quebec, Canada). Amphetamine D-sulfate was purchased from the University of Michigan hospital (Ann Arbor, MI). Ruboxistaurin was obtained from NIDA-NIH (Bethesda, MD). Enzastaurin was obtained from Fisher Scientific (Milwaukee, WI).

**Microdialysis.** Probes were constructed as previously described.<sup>48</sup> Briefly, 75/150  $\mu$ m (i.d./o.d.) fused silica capillaries (Polymicro

Technologies, Phoenix, AZ) were glued together with a 2 mm offset with some variability in fabrication. The capillaries were ensheathed in a regenerated cellulose membrane with both ends sealed by polyimide resin (Grace, Deerfield, IL). Flow rate of perfusion fluid through the probe was 1  $\mu\text{L}/\text{min}$ .

Male Sprague–Dawley rats (approximately 2 months old and 250–300 g) were anesthetized with 5% isoflurane and placed in a stereotaxic frame. A burr hole was drilled where the probe was to be inserted (+1.7 A/P,  $\pm 1.4$  M/L, and  $-7.5$  D/V in reference to bregma) to sample from the nucleus accumbens core.<sup>49</sup> Probes were implanted bilaterally, and measurements for both probes in each animal were averaged for a single replicate. The probes were lowered 7.5 mm from the top of the skull. Additional burr holes were drilled for anchor screws, and dental cement (A-M systems Inc., Sequim, WA) was used to hold the probes in place. All animals were obtained from Harlan (Envigo). Procedures and housing were approved by the University Committee for the Use and Care of Animals at the University of Michigan.

Immediately after probe insertion, animals were allowed to recover and acclimate in the Return testing chamber (Bioanalytical Systems, Inc., West Lafayette, IN) for 24 h. The recovery of 24 h has previously been shown to result in dopamine concentrations that are sensitive to tetrodotoxin<sup>50</sup> and is commonly used. The animals were observed behaviorally for any residual effects of the surgery, while we observed consistent basal level concentrations of neurochemicals in properly performed surgeries. Prior to the beginning of the experiment, the probes were attached to inlet and outlet fluid lines. The rat was tethered to the balance arm of the Return (Bioanalytical Systems, Inc., West Lafayette, IN). The microdialysis probe was perfused with aCSF at a flow rate of 1  $\mu\text{L}/\text{min}$  for 1 h before the experiments began. Probe placements were checked with histology (see Supplemental Figure 1).

**Drug Administration.** Each microdialysis experiment was 120 min in duration with collection of 3 min fractions. Initially, 30 min of fractions (10, 3 min fractions collected, predrug) were collected to establish baseline concentrations. At 30 min, a vehicle or 1  $\mu\text{M}$  ruboxistaurin or 1  $\mu\text{M}$  enzastaurin was administered directly into the nucleus accumbens by retrodialysis, and perfusion continued for the remainder of the experiment. At 60 min, a 2.0 or 2.5 mg/kg intraperitoneal injection of amphetamine in saline was administered, and fractions were collected for an additional hour for a total experiment time of 2 h. The vehicle for PKC $\beta$  inhibitors was 0.05% dimethyl sulfoxide (DMSO) in ACSF.

**Locomotor Behavior Analysis.** While animals were undergoing microdialysis, their movement was recorded using Logitech webcams (Apples, Switzerland) as previously described.<sup>50,51</sup> Webcams were connected via USB ports to analysis PCs running Matlab 2009 (Mathworks, Natick, MA, USA) software. Using the image acquisition toolbox in Matlab, data were collected via a custom designed motion monitoring program (Mark Dow, University of Oregon). The threshold of motion detection software was set at 150. This threshold limit was selected to detect only larger motions (e.g., walking, running, and rearing) and not to detect small motions (e.g., breathing or whisker movement). Data were collected every 60 s and then binned into 3 min intervals to correlate with neurochemistry results. Data are expressed in terms of absolute locomotor activity as calculated by the detection software.

**Sample Derivatization.** Each dialysate or standard sample was derivatized with benzoyl chloride and analyzed as described previously.<sup>52</sup> Briefly, samples were sequentially mixed with 100 mM sodium carbonate, benzoyl chloride (2% in acetonitrile, v/v), and internal standard in a 2:1:1:1 volume ratio. Internal standard comprised 10  $\mu\text{M}$  3,4-dihydroxyphenylacetic acid, 3-methoxytyramine, homovanillic acid, and dopamine reacted with <sup>13</sup>C<sub>6</sub>-benzoyl chloride in 100 mM sodium tetraborate which was then diluted 1:100 in dimethyl sulfoxide and 1% formic acid (v/v). The samples were analyzed on a Thermo TSQ LCMS. Mobile phase A was 10 mM ammonium formate and 0.15% (v/v) formic acid in water. Mobile phase B was acetonitrile. The gradient was initial, 5% B; 0.1 min, 19% B; 1 min, 26% B; 1.5 min, 75% B; 2.50 min, 100% B; 3.0 min, 100% B; 3.1 min, 5% B; 3.5 min,

5% B; and 4.0 min, 0% B. The peak areas of each analyte were divided by the area of the internal standard for quantification.

**Quantitative Microdialysis of Dopamine Using Stable Isotopes.** For quantitative microdialysis of dopamine (DA), 200 nM <sup>13</sup>C<sub>6</sub>-dopamine (DA) was perfused through the probe. The <sup>13</sup>C<sub>6</sub>-DA lost from the probe allowed for the measurement of the extraction fraction ( $E_d$ ) as

$$E_d = 1 - \frac{C_{\text{out}}^{\text{SIL}}}{C_{\text{in}}^{\text{SIL}}} \quad (1)$$

where  $C_{\text{out}}^{\text{SIL}}$  is the concentration of stable-isotope labeled (SIL) dopamine that exits the outlet of the probe, and  $C_{\text{in}}^{\text{SIL}}$  is the concentration of <sup>13</sup>C<sub>6</sub>-DA that is infused into the probe.<sup>53–55</sup> The  $E_d$  is related to the apparent *in vivo* concentration ( $C_{\text{app}}$ ) by

$$C_{\text{app}} = \frac{C_{\text{measured}}^{\text{endogenous}}}{E_d^{\text{SIL}}} \quad (2)$$

where  $C_{\text{measured}}^{\text{endogenous}}$  is the concentration of endogenous dopamine recovered and measured in the dialysate. As shown previously, this method allows for the *in vivo* calibration of the probe for estimating *in vivo* concentration by accounting for differences in probes and tissue environment.<sup>53–55</sup>

**Measurement of [<sup>3</sup>H]Dopamine Uptake into Rat Synaptosomes.** Male Sprague–Dawley rats (weight, 250–300 g) were killed by decapitation, and the nucleus accumbens was dissected on ice using a brain-cutting block as described by Heffner et al.<sup>56</sup> The tissue was homogenized in 10 volumes of ice-cold homogenization solution containing 0.32 M sucrose, 0.25 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu\text{M}$  pepstatin A, and 10  $\mu\text{M}$  leupeptin at pH 7.4. Homogenate fractions were centrifuged at 1000g for 10 min. The pellet was washed, and the combined supernatants were centrifuged at 16,000g for 15 min. The P2 fraction was resuspended in 1.1 mL of Krebs Ringer buffer containing (in mM) 125 NaCl, 2.7 KCl, 1.0 MgCl<sub>2</sub>, 1.2 CaCl<sub>2</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 10 glucose, 24.9 NaHCO<sub>3</sub>, 0.25 ascorbic acid, and 10  $\mu\text{M}$  pargyline. The buffer had been oxygenated with 95% O<sub>2</sub>/5% CO<sub>2</sub> for 1 h and adjusted to a pH of 7.4. Synaptosomes were incubated for 30 min at 37 °C in a shaking water bath with or without a range of concentrations of ruboxistaurin or enzastaurin to correspond with the possible concentrations of drug achieved during retrodialysis. Ruboxistaurin and enzastaurin were dissolved in DMSO, and all assay mixtures had the same final concentration of DMSO (0.001% for ruboxistaurin and 0.01% for enzastaurin). After preincubation, 10 nM [<sup>3</sup>H]dopamine (33.3 Ci/mmol, PerkinElmer) supplemented by 300 nM unlabeled dopamine was added to the synaptosomes, and the incubation proceeded for 3 min. The reaction was terminated by the addition of 3 mL of ice-cold KRB followed by filtration on GF/C filters (Whatman, Maidstone, UK) and two additional KRB washes on a Hoefer filtration manifold. Filters were counted in ScintiVerse BD in a Beckman Instruments (Columbia, MD) LS 5800 Scintillation Counter.

**Statistics.** Data were analyzed for statistical significance by either repeated measures (RM) 1-way analysis of variants or two-way RM analysis of variants using Prism 6.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acchemneur-0.6b00028.

Microdialysis probe placements; table of neurochemical concentrations pre- and post-drug; and a total ion chromatogram of neurochemicals detected with a 4 min gradient (PDF)



## AUTHOR INFORMATION

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## Notes

The authors declare no competing financial interest.

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