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# Natural and Synthetic Corticosteroids Inhibit Uptake$_2$-Mediated Transport in CNS Neurons

Jonathan E. Hill
*Marquette University*

Khadijah Makky
*Marquette University*, khadijah.makky@marquette.edu

Lalita Shrestha
*Marquette University*

Cecilia J. Hillard
*Medical College of Wisconsin*

Paul J. Gasser
*Marquette University*, paul.gasser@marquette.edu

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Jonathan E. Hill
*Department of Biomedical Sciences, Marquette University*
*Milwaukee, WI*
Khadijah Makky
*Department of Biomedical Sciences, Marquette University*
*Milwaukee, WI*
Lalita Shrestha
*Department of Pharmacology & Toxicology, Medical College of Wisconsin*
*Milwaukee, WI*
Cecilia J. Hillard
*Department of Pharmacology & Toxicology, Medical College of Wisconsin*
*Milwaukee, WI*
Paul J. Gasser
*Department of Biomedical Sciences, Marquette University*
*Milwaukee, WI*

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**Abstract:** In addition to exerting actions via mineralocorticoid and glucocorticoid receptors, corticosteroids also act by inhibiting uptake$_2$, a high-capacity monoamine transport system originally described in peripheral tissues. Recent studies have demonstrated that uptake$_2$ transporters are expressed in the brain and play roles in monoamine clearance, suggesting that they mediate some corticosteroid effects on physiological and behavioral processes. However, the sensitivity of brain uptake$_2$ to many natural and synthetic corticosteroids has not been characterized. Cultured rat cerebellar granule neurons (CGNs) were previously shown to exhibit corticosterone-sensitive accumulation of the uptake$_2$ substrate1-methyl-4-phenylpyridinium ($MPP^+$). We examined the expression of uptake$_1$ and uptake$_2$ transporters in CGNs, and tested the effects of a variety of natural and synthetic corticosteroids on accumulation of [$^3$H]-$MPP^+$ by these cells. Cultured rat CGNs expressed mRNA for three uptake$_2$-like transporters: organic cation transporters 1 and 3, and the plasma membrane monoamine transporter. They did not express mRNA for the dopamine or norepinephrine transporters, and expressed very little mRNA for the serotonin reuptake transporter. Accumulation of [$^3$H]-$MPP^+$ by CGNs was dose-dependently inhibited by corticosterone and decynium-22, known inhibitors of uptake$_2$. Accumulation of $MPP^+$ was also dose-dependently inhibited, with varying efficacies, by aldosterone, 11-deoxycorticosterone, cortisol, and cortisone, and by the synthetic glucocorticoids betamethasone, dexamethasone and prednisolone, and the glucocorticoid receptor antagonist RU38486. These studies demonstrate that uptake$_2$ in the CNS is inhibited by a variety of natural and synthetic corticosteroids, and suggest that inhibition of uptake$_2$-mediated monoamine clearance may underlie some behavioral and physiological effects of these hormones.

**Keywords**: brain, glucocorticoid, mineralocorticoid, monoamine, transport, non-genomic

# 1. Introduction

Natural and synthetic corticosteroids are powerful modulators of neuronal physiology and behavior. By actions at a variety of cellular targets, they initiate both rapid and delayed effects on central nervous system (CNS) function. Recent studies suggest that, in addition to exerting actions via the mineralocorticoid and glucocorticoid receptors (MR and GR), corticosteroids also act by inhibiting monoamine clearance mediated by uptake$_2$, a high-capacity, low-affinity transport system for norepinephrine, epinephrine, dopamine, histamine and serotonin [1-4]. In contrast to uptake$_1$, which is mediated by a

combination of the specific transporters for norepinephrine (NET), dopamine (DAT), and serotonin (SERT), uptake$_2$ is a higher-capacity but lower affinity transport system, and is acutely inhibited by corticosterone and other steroids [5, 6]. Inhibition of uptake$_2$ in cardiac or smooth muscle tissue by acute bath application of corticosteroids enhances the contractile effects of exogenously applied epinephrine, norepinephrine, serotonin and histamine [7-12]. Recent studies have identified a small group of transporters that mediate uptake$_2$-like transport and have demonstrated their expression in the brain [3, 4, 13-15]. Thus, uptake$_2$ is a mechanism by which corticosteroids may act to enhance the actions of monoamines in the CNS as well as in peripheral targets.

Uptake$_2$ activity has been attributed to a group of broadly-specific organic cation transporters. These include the organic cation transporter (OCT) family: OCTs 1, 2 and 3, and the plasma membrane monoamine transporter (PMAT). Because OCT3 is the most sensitive of these transporters to inhibition by corticosterone, it has been described as the most important uptake$_2$ transporter[16-18]. However, all of the above transporters have uptake$_2$-like characteristics. All are broadly-specific organic cation transporters, capable of transporting, with varying efficiencies, norepinephrine, epinephrine, serotonin, dopamine and histamine, as well as the cationic neurotoxin 1-methyl-4-phenylpyridinium ($MPP^+$) [13, 16, 19, 20]. All are sensitive to inhibition by corticosterone, though their sensitivities differ widely [3, 13, 17, 18, 21, 22], and all are inhibited by the pseudoisocyanine compound 1, 1'-diethyl-2, 2'-cyanine iodide (decynium-22) [13, 23].

All of the uptake$_2$ transporters are expressed, at varying levels and with distinct distributions, in rodent and human brain [3, 4, 13-15], and recent studies suggest that they play important roles in monoamine clearance. Pharmacological inhibition of uptake$_2$ by direct application of decynium-22 decreases the rate of serotonin clearance in mouse hippocampus [1], and treatment of rats with the OCT3 inhibitor normetanephrine potentiates venlafaxine-induced increases in extracellular norepinephrine concentrations in rat prefrontal cortex [24]. Extracellular concentrations of dopamine are elevated in the striatum of OCT3 knockout mice [25]. These studies demonstrate that

uptake$_2$ transporters play important roles in regulating extracellular concentrations of monoamines in the rodent brain.

Each of the identified uptake$_2$-like transporters is inhibited by corticosterone, though they differ in their sensitivity to this corticosteroid. OCT3 is the most sensitive ($IC_{50}$ = 0.04 – 0.2 μM), followed by OCT2 ($IC_{50}$ = 4 μM), OCT1 ($IC_{50}$ = 150 μM) and PMAT ($K_i$ = 450 μM) [3, 13, 17, 18, 21, 22]. Studies in peripheral tissues suggest that uptake$_2$-mediated transport is also inhibited by a variety of natural and synthetic corticosteroids. In vascular smooth muscle, the mineralocorticoids aldosterone and 11-deoxycorticosterone enhance the contractile effects of epinephrine [26, 27], and OCT3-mediated norepinephrine clearance in bronchial smooth muscle is inhibited by the synthetic corticosteroids budesonide, methylprednisolone, and fluticasone [28]. These studies suggest that uptake$_2$ inhibition may play an important role in mediating the effects of both natural and synthetic corticosteroids on neuronal physiology and behavior.

Given the powerful behavioral effects attributed to a wide variety of both natural and synthetic corticosteroids, it is important to understand the potential contribution of uptake$_2$ inhibition to their actions in the CNS. However, the structure activity relationships among the natural and synthetic corticosteroids for inhibition of uptake$_2$ in neurons have not been determined. We recently demonstrated that rat cerebellar granule neurons (CGNs) in culture accumulate [$^3$H]-MPP$^+$ in a corticosterone-sensitive manner, and that they express the uptake$_2$ transporter OCT3, but not the uptake$_1$ transporter DAT [29]. In the present studies, we fully characterized the expression of uptake$_1$ and uptake$_2$ transporters in CGNs, and examined the sensitivity of uptake$_2$-mediated accumulation of [$^3$H]-MPP$^+$ to various natural and synthetic corticosteroid hormones. This information is important for a full understanding of the mechanisms by which corticosteroids influence CNS function.

# 2. Materials and methods

## *2.1. Materials*

All steroids were purchased from Steraloids (Newport, RI). Timed pregnant female Sprague-Dawley rats were purchased from Harlan (Madison, WI). 1-[$^3$H]-Methyl-4-phenylpyridinium ([$^3$H]-MPP$^+$) (specific activity of 86.4 Ci/mol) was purchased from Perkin Elmer (Waltham, MA). Primers for RT-PCR were synthesized by Invitrogen (Carlsbad, CA).

## *2.2. Culture of cerebellar granule neurons*

CGNs were prepared from 6-8-day-old rat pups of either sex as described previously [30] except that cerebellae were incubated in 40 rather than 20 U/mL of papain. Cells were plated onto 6-well culture plates (2 ml, 1x10$^6$ cells/mL) and were maintained in basal minimal Eagle's media (BME; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 25 mM KCl, 5 mM glutamine, and 0.01 mg/ml ampicillin. After 24 h, cytosine arabinoside (10 μM) was added to the cultures to inhibit glial proliferation. After 5 days in culture, 600 μl of the media was removed and replaced with BME supplemented as above, except that B-27 (Invitrogen) was substituted for fetal bovine serum.

## *2.3. [$^3$H]-MPP$^+$ uptake assay*

Uptake experiments were carried out at room temperature on CGNs that had been in culture for 6-8 days. Cells were washed and pre-incubated in 1.8 mL transport buffer (25 mM Tris Base, pH 8.5, 280 mM mannitol, 5.4 mM KCL, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose). To test the effects of putative uptake$_2$ inhibitors on MPP$^+$ accumulation, CGNs were incubated in the presence of vehicle or increasing concentrations of inhibitors for 5 minutes before the addition of 20 nM [$^3$H]-MPP$^+$. Uptake was terminated after two minutes by aspiration of transport buffer, followed by two washes with 1mL icecold transport buffer. CGNs were lysed by scraping in 500 μl water. Radioactivity in the cell lysate was determined by liquid scintillation

spectroscopy. Uptake experiments were repeated at least three times, except where noted. The effects of the following inhibitors were tested: 11-dehydrocorticosterone (10 nM – 5.0 μM); 11-deoxycorticosterone (10 nM- 1 μM); aldosterone (10 nM – 1 μM); betamethasone (10 nM – 1 μM); corticosterone (10 nM – 5 μM); cortisol (10 nM – 10 μM); cortisone (10 nM – 5 μM); decynium-22 (0.01 nM – 0.1 μM); dexamethasone (0.1 nM – 1 μM); prednisolone (10 nM – 5 μM); RU38486 (10 nM – 5 μM).

## *2.4. Reverse transcriptase-PCR*

After 7 days in culture, CGNs were washed twice with transport buffer and total RNA was isolated using 1 mL TRIzol Reagent (Invitrogen) per well according to the manufacturer's protocol. One microgram of the resulting total RNA was reverse transcribed in the presence and absence SuperScript™II Reverse Transcriptase (Invitrogen) using oligo$(dT)_{20}$ primers. Two microliters of the resulting cDNA were used as a template for PCR reactions using GoTaq Green Master Mix (Promega, Madison, WI) and gene specific primers (Invitrogen) at 1 μM. Sequences for gene-specific primers and the corresponding PCR cycling parameters are shown in Table 1.

**((TABLE 1 here))**

## *2.5. Data Analysis*

Results of uptake assays are expressed as means ± SEM from independent replicates. Uptake data were analyzed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA). Transport data were analyzed by fitting untransformed data to appropriate equations using iterative, least-squares curve-fitting techniques. $IC_{50}$ values for inhibitors were determined by fitting the pooled data from independent experiments to the one-site competition equation using non-linear regression.

# 3. Results

**((FIGURE 1 here))**

## *3.1. Expression of transporter mRNA in CGNs*

RT-PCR was used to determine the expression of mRNA for uptake$_1$ (DAT, NET and SERT) and uptake$_2$ (OCT1, OCT2, OCT3 and PMAT) transporters in CGNs. All primer pairs were tested for their ability to amplify the target genes from cDNAs obtained from tissues known to express each of the transporters. All primer pairs were able to amplify products of the expected size in cDNA from positive control tissue: midbrain for OCT3, OCT2, PMAT, DAT, NET and SERT; and kidney for OCT1(a and b) and OCT2; data not shown). As shown in Figure 1, mRNAs for OCT1, OCT3, and PMAT were clearly detected in CGNs, while mRNA for SERT was detected at very low levels, and mRNAs for OCT2, DAT, and NET were not detected. Because the results of the current OCT1 RT-PCR contradicted our previous results [29], we repeated the OCT1 PCR using either the primer pair that we used in the earlier publication (OCT1b), or the new primer pair (OCT1a). Using the OCT1a primer pair, we detected OCT1 mRNA in CGNs, as well as in forebrain, liver, kidney and lung. Using the OCT1b primer pair, we failed to detect OCT1 mRNA in CGNs or in rat forebrain, but did detect OCT1 mRNA in kidney, liver and lung tissue (data not shown).

**((FIGURE 2 here))**

**((FIGURE 3 here))**

## *3.2. [$^3$H]-MPP$^+$ uptake assays*

Previous studies demonstrated saturable accumulation of [$^3$H]-MPP$^+$ by cultured CGNs, and presented evidence that this accumulation was mediated by OCT3 (Shang et al, 2003). We tested the sensitivity of CGN accumulation of [$^3$H]-MPP$^+$ to the potent uptake$_2$ inhibitor decynium-22, and to a variety of natural and synthetic corticosteroids. Uptake of [$^3$H]-MPP$^+$ was inhibited by both decynium-22 and corticosterone in a dose dependent manner, with decynium-22

inhibiting a larger fraction of $MPP^+$ accumulation than did corticosterone (Figure 2, Table 2). Accumulation of [$^3$H]-$MPP^+$ was also partially inhibited by other naturally occurring corticosteroids and their metabolites, including aldosterone, cortisol, 11-deoxycorticosterone (11-DOC), and cortisone (Figure 3-4, Table 2). These steroids inhibited [$^3$H]-$MPP^+$ uptake with efficacies similar to that of corticosterone (Table 2). 11-Dehydrocorticosterone had only a slight inhibitory effect on [$^3$H]-$MPP^+$ uptake (Figure 4, Table 2). Synthetic corticosteroids inhibited [$^3$H]-$MPP^+$ uptake with varying efficacies. Betamethasone inhibited uptake with an efficacy similar to that of corticosterone, while dexamethasone and prednisolone were less effective (Figure 5, Table 2). The glucocorticoid receptor antagonist RU38486 inhibited [$^3$H]-$MPP^+$ uptake, but was less effective than corticosterone (Figure 5,Table 2). Decynium-22 inhibited a larger fraction of uptake than did any of the steroids examined (Figure 2, Table 2).

**((TABLE 2 here))**

# 4. Discussion

This study is the most detailed characterization of the corticosteroid sensitivity of uptake$_2$-mediated transport, and the first in CNS cells. The data from expression and functional studies indicate that uptake$_2$ is the primary monoamine transport system in CGNs, and that, as in peripheral tissue, uptake$_2$ activity in the CNS is broadly sensitive to inhibition by both natural and synthetic corticosteroid hormones. These data are consistent with the hypothesis that natural and synthetic corticosteroids exert modulatory actions in the CNS via inhibition of uptake$_2$ -mediated clearance of monoamines, and that inhibition of uptake$_2$ -mediated monoamine clearance may underlie some of the effects of these hormones on neuronal physiology and behavior.

**((FIGURE 4 here))**

The data from RT-PCR studies indicate that [$^3$H]-$MPP^+$ accumulation by CGNs is mediated primarily by three uptake$_2$ transporters: OCT1, OCT3 and PMAT. These data are consistent with previous studies demonstrating expression of OCT3, OCT1 and PMAT

protein or mRNA expression in the cerebellar granule layer [4, 14, 31]. However, our detection of OCT1 mRNA expression in CGNs is not consistent with our previous report that OCT1 mRNA is not expressed in these cells [29]. This discrepancy may be due to the use of different primer sets in the two studies. In the present study, we used a primer set (OCT1a) that targets the 3' end of the OCT1 cDNA, whereas in the previous study, we used a primer set (OCT1b) that targets a region in the middle of the OCT1 cDNA. Both primer sets detected OCT1 mRNA in cDNA from kidney, raising the possibility that kidney and brain OCT1 mRNAs represent alternatively spliced forms of the gene, as has been reported for human OCT1 [32]. To address this discrepancy, we performed parallel PCR using each of the OCT1 primer pairs to amplify product from CGN and kidney cDNAs. Using the OCT1b primers (from our earlier publication), we again detected OCT1 mRNA in kidney, but not in CGN cDNA. Using the OCT1a primers, we detected OCT1 mRNA in both kidney and CGN. These results suggest that the OCT1 mRNA we detected in CGNs represents an alternatively spliced form of the gene. Further studies are required to test this hypothesis.

The data from RT-PCR studies further demonstrate that CGNs do not express DAT and NET, and that they express only very low levels of SERT. These results are consistent with previous studies examining DAT and SERT mRNA expression in CGNs [29, 33]. Our data on the expression of these uptake$_1$ transporters seem to contradict previous studies demonstrating the expression of DAT, NET and SERT in the cerebellar granule layer. However, these studies measured radioligand binding in intact brain sections, or uptake of substrates by acutely-prepared synaptosomes [34-38]. Data obtained using these techniques likely reflect transporters expressed on monoaminergic terminals in the cerebellar granule layer [39-41], rather than specifically in CGNs. Our data reflect transporter expression almost exclusively in CGNs.

**((FIGURE 5 here))**

Our functional studies are also consistent with a primary role of uptake$_2$ in CGN transport. Two well-characterized uptake$_2$ inhibitors, decynium-22 and corticosterone, each inhibited substantial fractions of $MPP^+$ uptake in the present studies. All of the transporters we detected in CGNs are inhibited by decynium-22, though their sensitivities are

not equivalent. OCT3 and PMAT are the most sensitive to decynium-22 inhibition ($IC_{50}$ = 9-100 nM for OCT3, 100 nM for PMAT), while OCT1 is significantly less sensitive ($IC_{50}$ = 0.5-1μM) [13, 23, 42-44]. Some reports have suggested that, at very high concentrations, decynium-22 can inhibit NET and SERT [45, 46]. In the present studies, decynium-22 inhibited approximately 80% of [$^3$H]-$MPP^+$ accumulation with the same potency ($IC_{50}$ = 3.4 nM) as reported in our previous studies [29]. As the concentrations of decynium-22 used in the current study (0.01 nM – 0.1 μM) did not reach the previously reported $IC_{50}$s for OCT1, the data suggest that the decynium-22-sensitive fraction of $MPP^+$ accumulation reported here is mediated primarily by PMAT and OCT3, and that the remaining fraction of $MPP^+$ accumulation is mediated by OCT1, with a possible contribution of SERT.

While all of the $uptake_2$ transporters expressed in CGNs are inhibited by corticosterone, they differ greatly in their sensitivity. OCT3 is the most corticosterone-sensitive, followed by OCT1 and PMAT [3, 13, 16-18, 47]. The concentrations of corticosterone used in the current study did not reach the previously-reported $IC_{50}$s for inhibition of OCT1 (150 μM) or PMAT (450 μM). Thus, the *corticosterone*-sensitive $MPP^+$ uptake (50-60% of total uptake) observed in the present study is likely mediated primarily by OCT3, with the corticosterone-insensitive fraction (40-50% of total uptake) mediated by PMAT and OCT1. Given the high levels of PMAT mRNA we detected in relation to OCT3, it is surprising that the corticosterone-insensitive fraction of $MPP^+$ accumulation in CGNs is not larger. This may be due to differences in the efficiencies with which OCT3 and PMAT transport $MPP^+$. In support, recently published studies demonstrated that the $V_{max}$ for OCT3-mediated $MPP^+$ uptake is twofold higher than that of PMAT [18].

Previous studies demonstrated that corticosterone-induced inhibition of OCT3-mediated transport is a non-genomic, GR-independent phenomenon [7]. The present studies are consistent with a non-genomic mechanism, in that inhibition of $MPP^+$ uptake by all tested corticosteroids was evident with only 5 minutes of steroid exposure, and that the GR antagonist RU38486 also inhibited $MPP^+$ accumulation. The $IC_{50}$ for corticosterone inhibition of $MPP^+$ accumulation reported in the present study (81 nM) is consistent with

previous studies [3, 29], and is within the physiological range reported for stress-induced corticosterone concentrations the rat brain [48, 49].

While each of the individual uptake$_2$ transporters is inhibited by corticosterone, their sensitivities to other corticosteroids have not been thoroughly characterized. Early studies in peripheral tissues demonstrated that a variety of natural corticosteroids inhibit uptake$_2$ activity or enhance the actions of epinephrine [11, 12]. However, the effects of corticosteroids other than corticosterone on individual uptake$_2$ transporters have been examined only for OCT3. Those studies demonstrated that OCT3 is inhibited by budesonide, methylprednisolone, and fluticasone [7, 28]. Our data indicate that uptake$_2$-mediated transport in the CNS is broadly sensitive to inhibition by natural and synthetic corticosteroids. While it is likely that these corticosteroids exert their effects at least in part by inhibiting OCT3, further studies are required to determine the specific contributions of each of the expressed transporters to corticosteroid-sensitive uptake.

The mineralocorticoids aldosterone and 11-DOC also inhibited $MPP^+$ accumulation by CGNs. This is consistent with previous studies, which demonstrated that these two mineralocorticoids could inhibit the clearance of epinephrine and norepinephrine, and potentiate their actions in vascular or cardiac tissue [11, 12, 50, 51]. This is the first study demonstrating mineralocorticoid-sensitive transport in CNS cells, and suggests that mineralocorticoids also exert actions in the CNS by inhibiting uptake$_2$-mediated monoamine clearance. Interestingly, OCT3 knockout mice display alterations in salt intake that are comparable to those exhibited by aldosterone-treated animals [15]. As OCT3 protein and mRNA are expressed in brain regions involved in regulation of salt intake, including circumventricular organs and medial amygdala, these data raise the possibility that this particular uptake$_2$ transporter represents a target for mineralocorticoid action on ingestive behavior [4, 15].

Comparison of the effects of corticosterone, 11-DOC and 11-dehydrocorticosterone on $MPP^+$ accumulation indicates that slight differences in corticosteroid structure significantly alter potency and efficacy of steroid-mediated inhibition of uptake$_2$. These three steroids differ in structure only at C11 of the steroid backbone. 11-

Deoxycorticosterone, which differs from corticosterone only by the lack of a hydroxyl group at C11, was a more potent and slightly more effective inhibitor of $MPP^+$ accumulation than was corticosterone, suggesting that the lack of the C11 hydroxyl group may increase the potency of 11-DOC at OCT3 and/or make it a more effective inhibitor of PMAT and/or OCT1. 11-Dehydrocorticosterone, which contains a keto- group at C11, was a much *less* effective inhibitor than either corticosterone or 11-DOC. Further studies are necessary to determine the effects of these steroids on individual $uptake_2$ transporters.

The present studies are the first to demonstrate sensitivity of $uptake_2$ activity to dexamethasone, prednisolone, and betamethasone, and to demonstrate that $uptake_2$-mediated transport in the CNS is sensitive to synthetic corticosteroids. These results are consistent with studies demonstrating that OCT3-mediated monoamine transport in bronchial smooth muscle cells is inhibited by budesonide, fluticasone and methylprednisolone [7, 28]. Again, further studies are required to determine the relative contributions of OCT3, OCT1 and PMAT to the effects of each of the tested synthetic steroids. However, these studies raise the interesting possibility that synthetic corticosteroids may acutely and potently enhance monoaminergic neurotransmission in the CNS, and that these actions may in part underlie previously described acute effects of synthetic corticosteroid treatment on behavior in humans [52-54].

Both natural and synthetic corticosteroids have powerful effects on monoamine-regulated behaviors, [52-59, 59]. Accumulating evidence suggests that $uptake_2$ transporters may play important roles in regulating monoaminergic neurotransmission in specific brain regions [1, 3, 24, 25]. The present studies demonstrate that $uptake_2$ represents an important target for corticosteroid action in the brain and that inhibition of $uptake_2$-mediated clearance of monoamines must be taken into consideration when interpreting the results of acute treatment with both natural and synthetic corticosteroid hormones.

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# Figure Captions

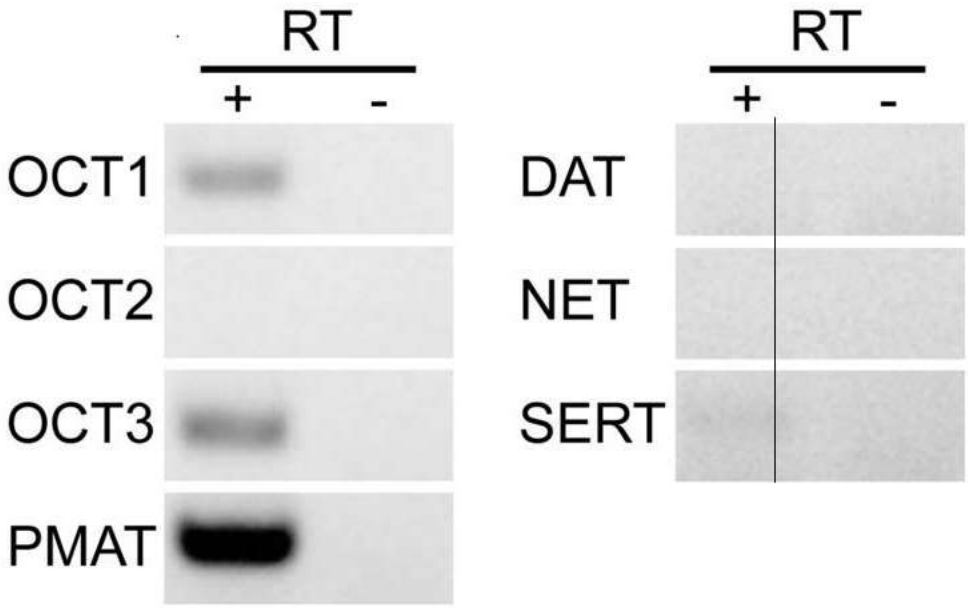


**Figure 1.** Expression of monoamine transporters in CGNs. RT-PCR was performed using total RNA extracted from CGNs and primers specific to OCTs 1, 2, and 3, plasma membrane monoamine transporter (PMAT), dopamine (DAT), norepinephrine (NET) or serotonin reuptake transporter (SERT). PCR was performed on cDNA samples generated in the presence (+) or absence (-) of reverse transcriptase. Only RT-PCR that included reverse transcriptase (+) produced distinct bands for any of the transporters. mRNAs for OCT1, OCT3 and PMAT were clearly detected, and a very small amount of mRNA for SERT was detected, in CGNs.

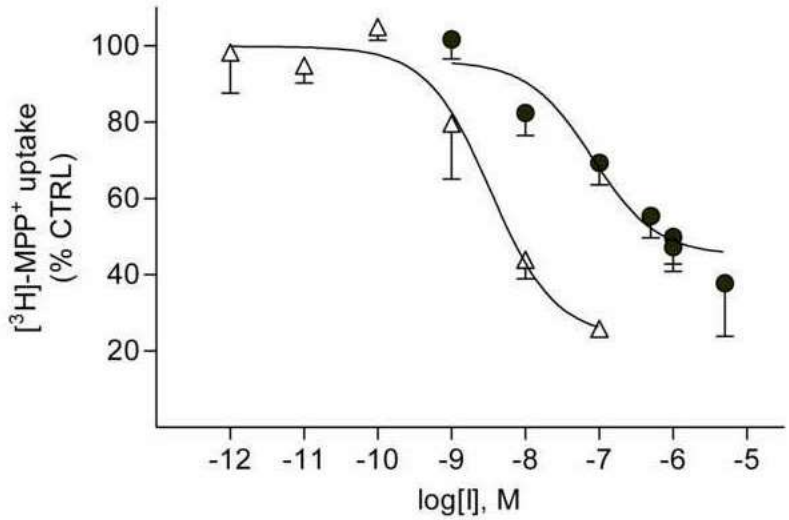


**Figure 2.** Effects of Decynium-22 and corticosterone on CGN accumulation of [$^3$H]-MPP$^+$. Cultured CGNs were incubated in uptake buffer containing the indicated

concentrations of inhibitors for 5 min, after which 20 nM [$^3$H]-MPP$^+$ was added and uptake was measured after 2 min. Decynium-22 (Δ) and corticosterone (●) inhibited [$^3$H]-MPP$^+$ accumulation in a concentration-dependent manner. Error bars represent − SE from n = 3 (decynium-22) or 8 (corticosterone) replicate wells.

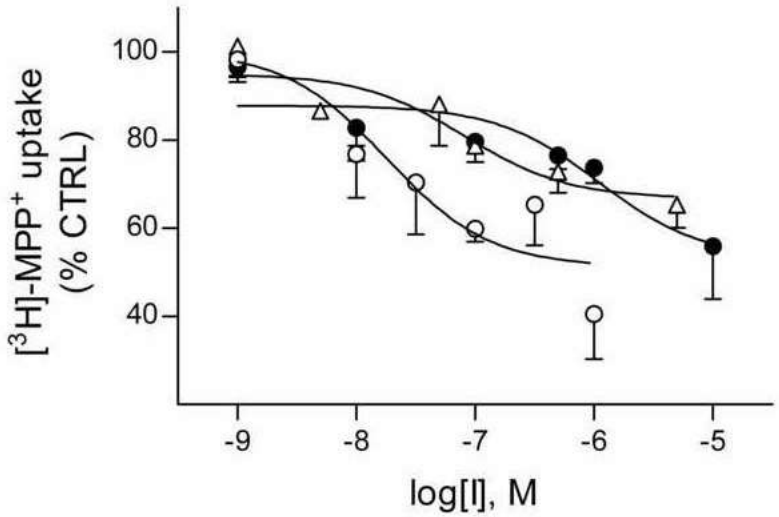


**Figure 3.** Effects of corticosteroids on CGN accumulation of [$^3$H]-MPP$^+$. Cultured CGNs were incubated in uptake buffer containing the indicated concentrations of inhibitors for 5 min, after which 20 nM [$^3$H]-MPP$^+$ was added and uptake was measured after 2 min. Aldosterone (o), cortisol (●) and cortisone (Δ) inhibited [$^3$H]-MPP$^+$ accumulation in a concentration-dependent manner. Error bars represent − SE from n = 3 (aldosterone, cortisone) or 4 (cortisol) replicate wells.

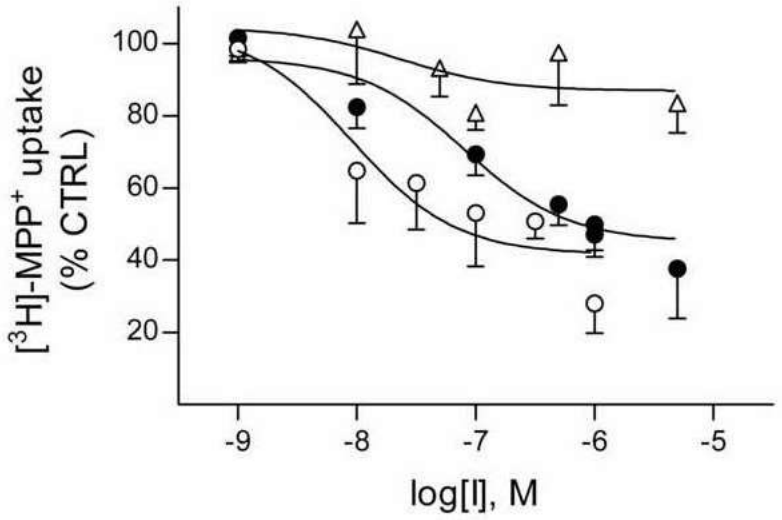


**Figure 4.** Effects of corticosterone and its metabolites on accumulation of [$^3$H]-MPP$^+$ by CGNs. Cultured CGNs were incubated in uptake buffer containing the indicated concentrations of steroids for 5 min, after which 20 nM [$^3$H]-MPP$^+$ was added

and uptake was measured after 2 min. The data for corticosterone (●) are from Figure 2, presented here for comparison to the other steroids. Both 11-deoxycorticosterone (o) and 11-dehydrocorticosterone (Δ) inhibited [$^3$H]-MPP$^+$ accumulation in a concentration-dependent manner, though with different efficacies. Error bars represent − SE from n = 3 (11-dehydrocorticosterone, 11-deoxycorticosterone) or 8 (corticosterone) independent wells.

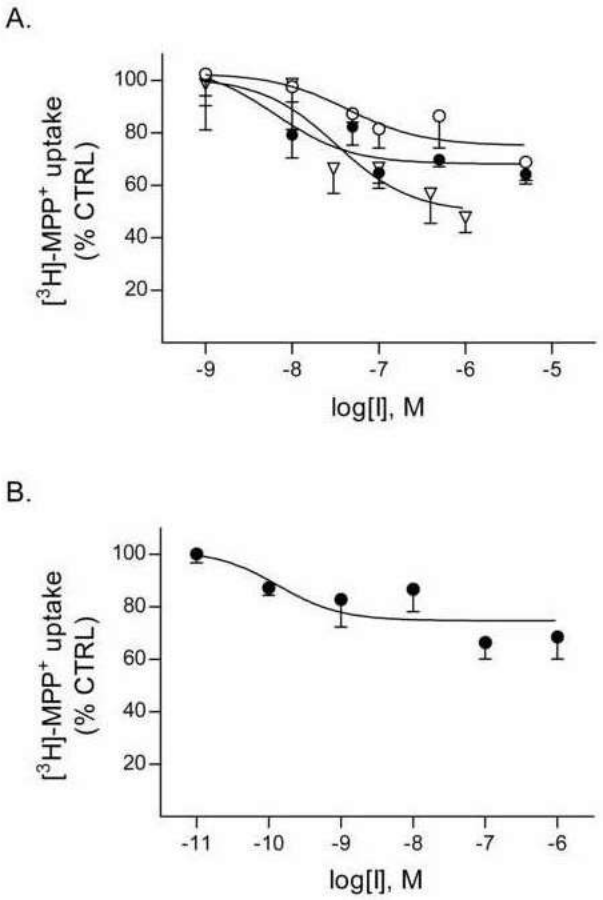


**Figure 5.** Effects of synthetic corticosteroids on accumulation of [$^3$H]-MPP$^+$ by CGNs. Cultured CGNs were incubated in uptake buffer containing the indicated concentrations of steroids for 5 min, after which 20 nM [$^3$H]-MPP$^+$ was added and uptake was measured after 2 min. **A.** Betamethasone (▽), prednisolone (●) and RU38486 (o) dose-dependently inhibited [$^3$H]-MPP$^+$ accumulation. **B.** Dexamethasone (●) dosedependently inhibited [$^3$H]-MPP$^+$ accumulation. Error bars represent − SE from n = 3 (betamethasone, prednisolone, RU38486) wells or − SD from two (dexamethasone) wells.

**Table 1.** Primers and cycling conditions for amplification of $MPP^+$ transporters in CGNs. All reactions were initiated by a 2 min incubation at 94°C, were run for 32 cycles, and ended with 10 min at 72°C.

| Gene | Primers (5'-3') | Product Size (bp) | Cycling Parameters |
|---|---|---|---|
| rOCT1a | Forward-GAT CTT TAT CCC GCA TGA GC<br>Reverse-TTC TGG GAA TCC TCC AAG TG | 477 | 45 s at 94°C, 30 s at 55°C, 45s at 72°C |
| rOCT1b | Forward-TGC AGA CAG GTT TGG CCG TAA<br>Reverse-TCG AGG CCG CTA TTG GGT AGA | 722 | 45s at 94°C, 30s at 55°C, 45s at 72°C |
| rOCT2 | Forward -CGT TGG GTA GAA TGG GCA TC<br>Reverse -GTG AGG TTG GTT TGT GTG GG | 460 | 45 s at 94°C, 30 s at 57°C, 45 s at 72°C |
| rOCT3 | Forward -TCT TCA CCC TCG GAA TCA TC<br>Reverse -TGA TAC ACC ACG GCA CTT GT | 351 | 45 s at 94°C, 30 s at 55°C, 45 s at 72°C |
| rDAT | Forward -TCC CTG ACAA GCT TCT CC<br>Reverse -GCC AGG ACA ATG CCA AGA | 305 | 1 min at 95°C, 45 s at 56°C, 45 s at 72°C |
| rNET | Forward -ACA TCG GGA AAG GTT GTC TG<br>Reverse -GTG GCG ACA TCC TCA ATC TT | 370 | 1 min at 95°C, 45 s at 60°C, 45 s at 72°C |
| rPMAT | Forward -ACC GCT ACC ATG CCA TCT AC<br>Reverse -AAG GCC AGG AGG TAA CCT G | 238 | 1 min at 95°C, 45 s at 58°C, 45 s at 72°C |
| rSERT | Forward -GTA CCA CCG AAA CGG GTG CA<br>Reverse -TGG TGG ATC TGC AGC ACA TG | 300 | 45 s at 95°C, 45 s at 60°C, 45 s at 72°C |

**Table 2.** Potencies of inhibitors of [$^3$H]-$MPP^+$ accumulation by CGNs

| Inhibitor | $IC_{50}$ nM (95% CI) | | Maximal Inhibition (%) |
|---|---|---|---|
| Decynium-22 | 3.4 | (2.4 – 4.8) | 76 ± 5 |
| 11-dehydrocorticosterone | 23 | (2.3 – 200) | 13 ± 5 |
| 11-deoxycorticosterone | 9.25 | (4.25 – 20) | 58 ± 7 |
| Aldosterone | 18 | (7.5 – 43) | 49 ± 7 |
| Betamethasone | 33 | (15 – 72) | 50 ± 8 |
| Corticosterone | 81 | (42 – 200) | 55 ± 5 |
| Cortisol | 1.1μM | (0.5 – 2.5) | 47 ± 8 |
| Cortisone | 82 | (28 – 240) | 32 ± 6 |
| Dexamethasone | 0.1 | (0.03 – 0.6) | 25 ± 4 |
| Prednisolone | 6.4 | (1.2 – 33) | 32 ± 7 |
| RU38486 | 44 | (11.5 – 200) | 25 ± 7 |

$IC_{50}$ values were determined by fitting pooled data from independent experiments to the one-site competition equation using nonlinear regression.

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