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# A sensitizing d-amphetamine dose regimen induces long-lasting spinophilin and VGLUT1 protein upregulation in the rat diencephalon

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## Abstract

Numerous studies in this lab and others have reported psychostimulant-induced alterations in both synaptic protein expression and synaptic density in striatum and prefrontal cortex. Recently we have shown that chronic d-amphetamine (d-AMPH) administration in rats increased synaptic protein expression in striatum and limbic brain regions including hippocampus, amygdala, septum, and paraventricular nucleus of the thalamus (PVT). Potential synaptic changes in thalamic nuclei are interesting since the thalamus serves as a gateway to cerebral cortex and a nodal point for basal ganglia influences. Therefore we sought to examine drug-induced differences in synaptic protein expression throughout the diencephalon. Rats received an escalating (1-8 mg/kg) dosing regimen of d-AMPH for 5 weeks and were euthanized 28 days later. Radioimmunocytochemistry (RICC) revealed significant upregulation of both spinophilin and the vesicular glutamate transporter, VGLUT1, in PVT, mediodorsal (MD), and ventromedial (VM) thalamic nuclei as well as in lateral hypothalamus (LH) and habenula. Strong positive correlations were observed between VGLUT1 and spinophilin expression in PVT, medial habenula, MD, VM and LH of d-AMPH-treated rats. No significant d-AMPH effect was seen in sensorimotor cortices for either protein. Additionally, no significant differences in the general vesicular protein synaptophysin were observed for any brain region. These findings add to evidence suggesting that long-lasting stimulant-induced synaptic alterations are widespread but not ubiquitous. Moreover, they suggest that d-AMPH-induced synaptic changes may occur preferentially in excitatory synapses.

### Keywords

synaptic plasticity; radioimmunocytochemistry; psychostimulants; behavioral sensitization

## INTRODUCTION

Long-lasting neuroadaptations associated with repeated drug administration have been proposed to underlie addiction [21, 37]. Increases in the density of synapses via morphological changes in dendritic spines has been shown to be induced by cocaine, d-amphetamine (d-AMPH), methamphetamine and nicotine and these changes can persist for up to three months after drug discontinuation [20, 23, 38, 39]. Because of their persistence,

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these dendritic alterations provide a potential neural mechanism for mediating long-lasting aspects of drug addiction.

Although numerous studies have reported drug-induced increases in spine density in striatum and prefrontal cortex [PFC; 27, 38, 39], research into psychostimulant effects on dendrites in other brain regions has been limited. Part of the reason for this is that the approach most commonly used to measure dendritic plasticity, the analysis of Golgi-stained brain sections, is labor-intensive and therefore ill-suited for evaluating neuroplastic changes in multiple brain regions. This laboratory and others have instead examined synaptic plasticity by quantifying synaptic protein expression, demonstrating that repeated psychostimulant dosing produces stable, long-lasting upregulation of both pre- and postsynaptic proteins of striatum and other limbic brain regions [5, 35, 36, 42]. Recently we reported that, after one month of d-AMPH withdrawal, spinophilin, an F-actin and protein phosphatase-1 binding protein, was upregulated in the striatum as well as in limbic brain regions including the hippocampus, septum, amygdala, cingulate cortex, and paraventricular nucleus of the thalamus [PVT; 5].

Since the thalamus serves as an entryway for incoming sensorimotor information to the cerebral cortex and a locus of efferent influences from both basal ganglia and neocortex on behavioral outputs [15, 41], synaptic changes in thalamic nuclei represent a potentially important component in psychomotor sensitization and addiction. The capacity for neuroplasticity in thalamic neurons has been previously demonstrated in studies of sensory deprivation [26], motor task learning [11], and environmental enrichment [32]; however drug-induced synaptic changes in thalamus have not yet been examined.

The present study used radioimmunocytochemistry (RICC) to examine several diencephalic regions for alterations in spinophilin expression one month after a chronic sensitizing d-AMPH regimen. Also, to examine whether excitatory afferent projections participate in these synaptic changes, we investigated VGLUT1 (a vesicular glutamate transporter found primarily in cortical projection neurons) by RICC. Additionally, we examined a general marker of synapses, synaptophysin. We hypothesized that neurons in the diencephalon would undergo synaptic alteration as a consequence of repeated d-AMPH administration, which would be effected in synaptic protein expression of specific diencephalic structures.

## MATERIALS AND METHODS

### Animals

Twenty-four male Sprague-Dawley rats (Charles River, Hollister, CA) weighing 250-300 g at the start, were used in this experiment. Animals were housed individually, allowed one week of habituation to the colony room, and handled for three days prior the start of the dosing regimen. Food and water were available *ad libitum* in their home cages. Rats were maintained in a climate-controlled facility with a 12-hour light/dark cycle (lights on at 6 AM). This experiment was approved by the Institutional Animal Care and Use Committee of the University of California, Irvine following National Institute of Health guidelines. d-AMPH sulfate was dissolved in sterile 0.9% saline. Injections were administered at a volume of 1 ml/kg i.p. (drug base).

### Sensitizing Dose Regimen

Animals were administered either saline (SAL) or d-AMPH two times a day seven hours apart for five consecutive days each week, followed by a two day washout period in order to mimic the withdrawal phase associated with human d-AMPH use [24]. Dosing was carried out for five weeks. The dosing schedule, as described previously [5, 38], followed an

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escalating pattern starting with 1 mg/kg and concluding with 8 mg/kg. Rats were dosed with d-AMPH and placed in a cage distinct from the home cage for 90 minutes.

### Sensitization testing

Procedures for evaluating behavioral sensitization to d-AMPH were previously described [5]. After the final dose of d-AMPH, animals were left undisturbed in their home cage for four weeks.

### Radioimmunocytochemistry

Animals were deeply anesthetized with sodium pentobarbital (250 mg/kg, i.p.) and decapitated 28 days after sensitization testing. Their brains were quickly removed, frozen in isopentane (-20°C), and stored at -20°C. For each rat, 8 coronal 20 μm sections were serially cut with a cryostat microtome at the level of the dorsal hippocampus (Bregma -2.25 to -2.75 mm), collected on Vectabond-coated slides (Vector Laboratories, Burlingame, CA), and stored at -20°C.

For RICC localization of spinophilin, VGLUT1 and synaptophysin, slides were thawed and air dried at room temperature (RT). Brain sections were fixed with 4% paraformaldehyde for 20 minutes then washed with 0.1 M PBS and incubated for one hour in blocking solution (0.5% Triton-X 100 in 0.1 M PBS with 5% milk for spinophilin and VGLUT1, and 5% BSA for synaptophysin) to reduce nonspecific binding. Next, sections were incubated with antibody to spinophilin (1:1000; Chemicon #AB5669, Temecula, CA), VGLUT1 (1:1000; MAb Technologies #VGT1-3, Stone Mountain, GA), or synaptophysin (1:8000; Sigma # S5768, St. Louis, MO) mixed in blocking solution for two hours. Sections were washed and incubated with $^{35}$S-labeled anti-rabbit for spinophilin and VGLUT1 and $^{35}$S-labeled anti-mouse antisera for synaptophysin at a dilution of 0.5 μCi/ml in 5% milk or 5% BSA in 0.1 M PBS for two hours. Sections were rinsed, dehydrated, air dried and exposed to Hyperfilm MP (Amersham, Piscataway, NJ). Film was analyzed for signal density at several regions using MCID analysis software (Interfocus Imaging Ltd., Linton, Cambridge, UK). Brain structures were identified from the tissue sections stained with cresyl violet, according to the rat brain atlas of Paxinos and Watson (2007). Values obtained represent the average of measurements taken from both hemispheres (except for the PVT) of all 8 brain sections per animal. Background was corrected for by subtracting the density value of corpus callosum (which should contain low levels of spinophilin, VGLUT1, and synaptophysin) from each density reading within that section. For one region (LH), some VGLUT1 densities in some sections were below callosal values, in which case background-corrected readings were converted to zero.

### Statistical Analyses

The RICC data was analyzed using two-way ANOVAs, in which treatment regimen (d-AMPH or SAL) was a grouping factor and region was a repeated-measures factor. *Post hoc* analysis was carried out using one-way ANOVAs for regions of interest (Figure 2D; (Regions of interest are listed in the legend for Figure 2). Correlations were evaluated using Pearson's *r*-test.

## RESULTS

The group of animals used in the current study was previously reported to show a sensitized locomotor response to a 1 mg/kg challenge dose of d-AMPH three days after conclusion of the dosing regimen [5].

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Spinophilin labeling in the present study was most dense in hippocampus and striatum, consistent with previous descriptions [Fig. 1C; 2, 33]. VGLUT1 labeling was similar to spinophilin, showing intense to moderate labeling throughout all layers of the cerebral cortex and hippocampus, and moderate signal throughout much of the diencephalon, confirming previous observations [Fig. 1C; 12]. In contrast, synaptophysin labeling was relatively homogenous except for the laminar pattern of labeling in hippocampus [35, 36]. To determine specific binding of [$^{35}$S]-labeled secondary antibody, some sections were subjected to RICC procedures, except that primary antibody was omitted. In absence of primary antibody, light relatively uniform, background levels of signal were obtained (Fig. 1B).

The RICC technique was used to measure differences in spinophilin, VGLUT1, and synaptophysin expression between d-AMPH-sensitized and saline-treated rats throughout several diencephalic regions, including specific thalamic, habenular, and hypothalamic regions, as well as sensorimotor cortical areas. The group of animals used in the current study was previously reported to show enhanced chronic d-AMPH-enhanced spinophilin expression in many limbic forebrain regions including the ventral striatum, cingulate cortex, septum, hippocampus, and amygdala [5].

Our chronic sensitizing regimen of d-AMPH increased spinophilin expression throughout the diencephalon (Figure 2A). A repeated-measures two-way ANOVA revealed an overall effect of treatment [$F(1,19) = 24.198$, $p < 0.001$] and a treatment-by-region interaction [$F(1,19) = 2.942$, $p < 0.01$]. *Post hoc* analysis further demonstrated significantly greater spinophilin expression in the LHb ($p < 0.01$), MHb ($p < 0.001$), PVT ($p < 0.05$), MD ($p < 0.001$), VM ($p < 0.001$), LH ($p < 0.01$), VPL ($p < 0.01$) and LD ($p < 0.01$) of d-AMPH-treated rats. No effect of d-AMPH pretreatment was observed in motor ($p = 0.690$) or somatosensory ($p = .237$) cortices.

VGLUT1 expression of the d-AMPH group was increased in many of the same brain regions as spinophilin (Figure 2B). A repeated-measures two-way ANOVA revealed an overall effect of treatment [$F(1,19) = 10.328$, $p < 0.01$]. *Post hoc* analysis demonstrated significantly greater VGLUT1 expression in MHb ($p < 0.001$), PVT ($p < 0.05$), MD ($p < 0.05$), VM ($p < 0.01$), and LH ($p < 0.01$) of d-AMPH-treated rats. No effect was observed in any other diencephalic or cortical region (p's > 0.270).

In contrast, synaptophysin expression did not differ significantly between the d-AMPH- and SAL groups (Figure 2C). A repeated-measures two-way ANOVA revealed no overall effect of treatment [$F(1,19) = 1.369$, $p = 0.256$] or treatment by region interaction [$F(1,19) = 0.002$, $p = 0.966$].

We also investigated whether the expression levels of the postsynaptic protein spinophilin were related to the expression levels of the presynaptic protein VGLUT1. For this analysis, we looked for correlations between these two proteins separately in both the d-AMPH-treated and the SAL-treated animals. In d-AMPH-treated rats, spinophilin and VGLUT1 expression showed strong significant positive correlations in several regions of the diencephalon, including the MHb ($r = 0.81$; $p <0.01$), PVT ($r = 0.91$; $p < 0.001$), MD ($r = 0.78$; $p < 0.01$), LH ($r = 0.87$; $p < 0.001$), and LD ($r = 0.87$; $p < 0.001$). No significant correlations were observed in SAL-treated rats.

## DISCUSSION

This study reports persistent increases in expression of the synaptic proteins spinophilin and VGLUT1 in several diencephalic brain regions of rats treated with a prolonged, sensitizing regimen of d-AMPH. Even four weeks after cessation of d-AMPH dosing, significant

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increases in thalamic, hypothalamic, and habenular spinophilin were revealed using RICC. In addition, significantly greater VGLUT1 expression was observed in MHb, PVT, MD, VM, and LH of d-AMPH-pretreated rats. However, synaptic protein expression was not increased in all brain regions, as d-AMPH did not increase expression of either protein in motor or somatosensory cortex. Specificity of the effect is also suggested by the finding that the same regions showed no significant synaptophysin change as a result of d-AMPH dosing. Because spinophilin [33] and VGLUT1 [4, 22] are preferentially expressed in excitatory synapses, the absence of change in synaptophysin may indicate that only a small subset of synapses in each of the measured brain regions is affected.

To date, much focus of long-term effects of drugs of abuse has been on drug-induced synaptic plasticity in nucleus accumbens (Acb), PFC, and ventral tegmental area (VTA). It has been suggested that, through repeated psychostimulant exposure, the VTA-PFC-Acb circuitry is strengthened persistently during the development of sensitization [19]. However, there is growing evidence suggesting that synaptic strengthening occurs in other brain regions in response to psychostimulant treatment [35, 36]. A separate analysis of telencephalic regions from the animals dosed in the present experiment revealed that spinophilin expression was upregulated in several limbic brain areas (e.g. septum, amygdala, hippocampus) as well as in striatum following chronic d-AMPH treatment [5]. The current results further illustrate that thalamic nuclei, hypothalamus, and habenula are loci for drug-induced alterations relevant to addiction.

Considering the prominent role of the thalamus in the processing of information both entering and exiting cerebral cortex it may not be surprising that a sensitizing regimen of d-AMPH enduringly alters synaptic proteins in some thalamic nuclei. For example, MD and PVT both receive extensive input from amygdala, frontal cortex, and ventral pallidum [14, 43], through which these limbic structures can influence cortical processing. These midline thalamic nuclei are responsive to psychostimulant administration, since acute administration of d-AMPH or cocaine has been found to dose-dependently increase Fos protein levels in the PVT and MD of rats [10, unpublished observations]. Furthermore, studies have shown that lesions of MD attenuate acquisition of cocaine self-administration in rats [44], while PVT lesions block development of cocaine-induced psychomotor sensitization [45]. Here we show that d-AMPH sensitization produces coordinated changes in both spinophilin and VGLUT1 in both MD and PVT. Similarly, the projections and physiology of neurons in VPL, VM, and LD point to their roles in somatosensory and motor functions [e.g., 18, 28] and we found that d-AMPH sensitization causes spinophilin increases in all of these structures.

The hypothalamus, specifically the orexin-containing neurons in the lateral region, has been shown to play a role in reward processing and addiction via enhancing glutamatergic PFC inputs to VTA dopamine neurons [3, 13, 17]. Previous studies have reported that repeated doses of d-AMPH increase the activation of LH neurons in response to a challenge dose of d-AMPH [30]. Our finding that d-AMPH sensitization increases both spinophilin and VGLUT1 in LH is consistent with these earlier suggestions of d-AMPH-induced remodeling of inputs to LH [1].

Finally, the LHb has been suggested to directly control reward-related dopamine release via inhibitory projections to midbrain dopaminergic neurons [7]. Electrolytic lesions of the habenula have been shown to attenuate brain stimulation reward [29]. Additionally, repeated doses of psychostimulants increase the activation of LHb neurons in response to a challenge dose [9, 16]. Our finding that a chronic sensitizing dosing regimen of d-AMPH increases spinophilin labeling in LHb is consistent with the view that the habenula is involved in a neural network underlying the expression of behavioral sensitization.

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The d-AMPH-induced coordinated alterations in diencephalic levels of spinophilin and VGLUT1 expression observed in the current study are particularly interesting because of the regions in which they occur. Considering the connectivity of the MD and PVT, long-lasting alterations in synaptic number or function within these nuclei would be expected to persistently influence a wide range of cortical and limbic structures. Additionally, d-AMPH-induced alterations in synapses within these thalamic nuclei may be amplified further as a consequence of the non-reciprocal relationship between thalamocortical and related corticothalamic projections [15]. The precise role of the PVT in response to d-AMPH is unknown, but it has been suggested that the PVT may be important in associating the rewarding aspects of psychostimulants with contextual cues [10].

Elevated LH and LHb synaptic protein expression may signify remodeling of cortical inputs in these regions. The strong positive correlation observed between VGLUT1 and spinophilin is consistent with previous reports of psychostimulant-induced LH synaptogenesis [1]. Because both the LH and LHb are known to directly control reward-related dopamine release from the VTA, long-lasting synaptic alterations in these regions could play a major role in sensitization.

The finding that synaptophysin expression was unaltered in repeated d-AMPH treated rats, while vGLUT1 and spinophilin were, suggests the interesting possibility that the synaptic change occurs only in a subset of synapses in these regions, e.g., in excitatory synapses arising from cortex.

Although the changes in pre- and post-synaptic proteins measured in the present study suggest d-AMPH-induced synaptic remodeling, there are several caveats in extrapolating these findings to earlier reports of stimulant-induced changes in dendritic spines. For example, previous studies have shown that dopaminergic denervation decreases the density of dendritic spines on striatal cells while spinophilin [6] and VGLUT1 [8] levels do not change. In addition, while spinophilin is predominantly associated with dendritic spines in both hippocampus and striatum [2, 33], excitatory afferent fibers to the thalamus more commonly synapse onto dendritic shafts [25]. Thus, the coordinated upregulation of spinophilin and VGLUT1 observed in thalamus may not necessarily signify an alteration in spine density. Alternatively, d-AMPH-induced increases in spinophilin may indicate alterations to the postsynaptic density and not the spine *per se*. In addition, it is possible that increases in synaptic protein expression could be due an enlarging of post synaptic density without an alteration to dendritic spine or synapse number. In this event it would be expected that the presynaptic terminal would also enlarge, accompanied by an increase in VGLUT1 expression. Despite these important caveats, it has been reported that even less aggressive d-AMPH regimens than that used in the present study increased asymmetrical axospinous synapse number in the medial PFC [31] and basolateral amygdala [40].

In summary, the current report demonstrates that psychostimulant-induced synaptic alterations occur throughout the diencephalon. Further study is needed to determine if these changes reflect the addition or enlargement of new synapses, but strong positive correlations between pre and post-synaptic proteins in d-AMPH-treated rats suggest these possibilities. We are hopeful that these findings will stimulate more research to be directed towards drug-induced changes in the thalamus and habenula. In particular, studies of psychostimulant-induced changes to diencephalic dendritic morphology employing either Golgi-Cox or DiI staining, would be very valuable in further understanding long-lasting synaptic changes in the diencephalon.

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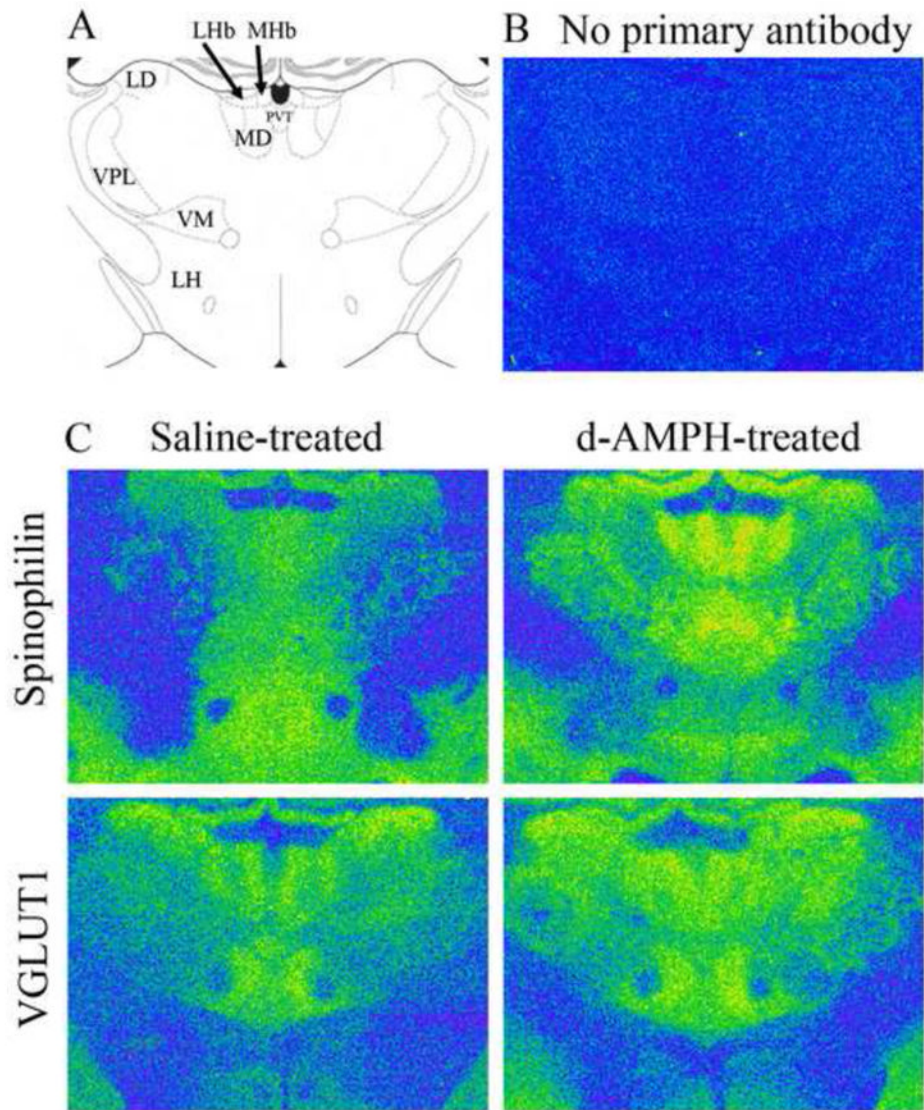


**Figure 1.**

(A) Schematic diagram of regions sampled (B) Autoradiograms with 'No primary antibody' represented by binding of [$^{35}$S]-labeled antirabbit secondary antibody in the absence of primary antibody. (C) Autoradiograms of spinophilin and VGLUT1 in saline-treated and d-AMPH-treated rat brains at the level of the diencephalon detected using RICC. Warmer colors indicate greater labeling density and cooler colors indicate lesser labeling density.

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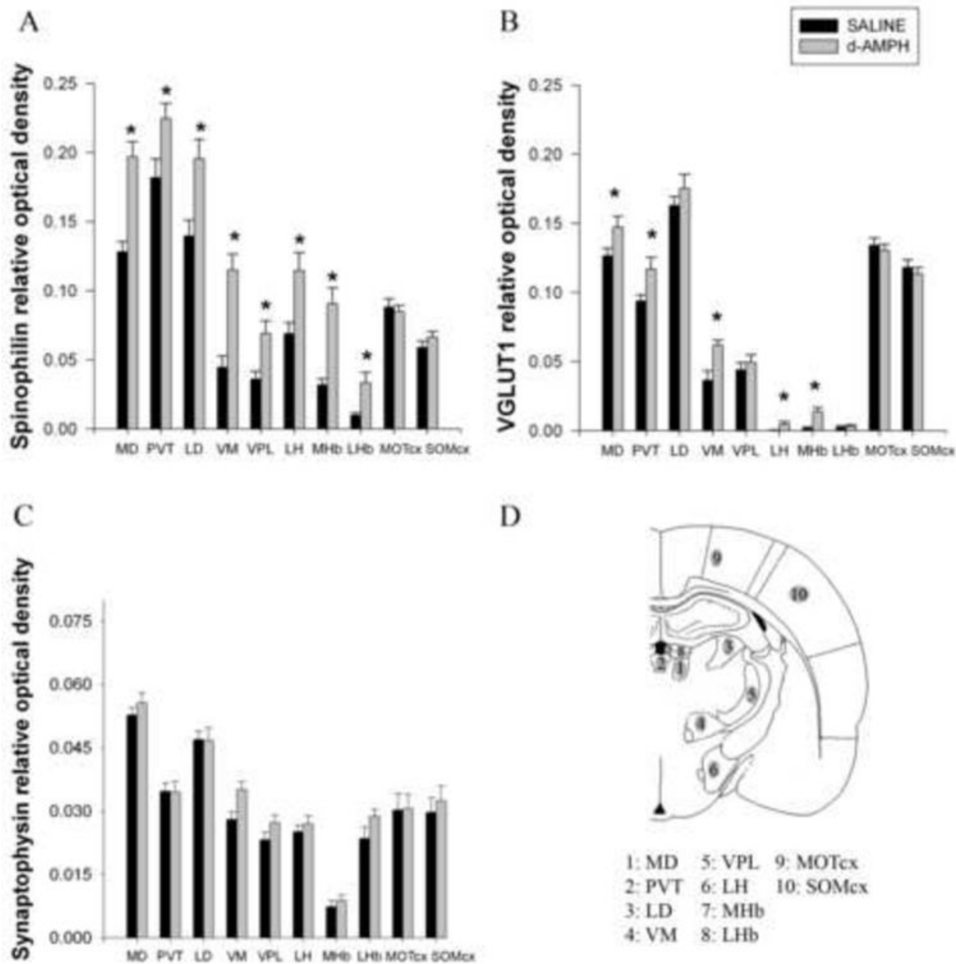


**Figure 2.**

(A, B, C) Effect of chronic d-AMPH treatment on spinophilin (A) VGLUT1 (B) and synaptphysin (C) expression using RICC. Rats treated chronically with d-AMPH demonstrated significant upregulations in spinophilin in all diencepahlic, but not cortical brain regions. VGLUT1 expression was increased in some regions and not others. Synaptophysin expression was unchanged by d-AMPH treatment. Data are expressed as mean values of corrected ROD. *$P < 0.05$ vs. saline-treated controls. (D) Schematic diagram of regions sampled. Specific brain areas were sampled for spinophilin, VGLUT1, and synaptophysin RICC, including: mediodorsal thalamic nucleus (MD), paraventricular thalamic nucleus (PVT), laterodorsal thalamic nucleus (LD), ventromedial thalamic nucleus (VM), ventroposterolateral thalamic nucleus (VPL), lateral hypothalamus (LH), medial habenula (MHb), lateral habenula (LHb), motor cortex (MOTcx), and somatosensory cortex (SOMcx). Images modified from Paxinos & Watson, 2007.