

Experimental Investigations on Dopamine Transmission Can Provide Clues on the Mechanism of the Therapeutic Effect of Amphetamine and Methylphenidate in ADHD

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SUMMARY

The aim of this review is to compare the experimental evidence obtained from in vitro studies on the effect of amphetamine and methylphenidate on dopamine transmission with the results obtained in animal models of attention deficit hyperactivity disorder (ADHD). This comparison can extend the knowledge on the mechanism of action of the drugs used in the therapy of ADHD and provide insight into

the etiology of ADHD. In particular, we considered the results obtained from in vitro methods, such as synaptosomes, cells in culture, and slices and from in vivo animal models of ADHD, such as spontaneous hypertensive rats (SHR) and the Naples high-excitability (NHE) rat lines. The different experimental approaches produce consonant results and suggest that in SHR rats, in contrast to Wistar Kyoto rats (WKY), amphetamine and depolarization by high K^+ might release different pools of dopamine-containing vesicles. The pool depleted by amphetamine might represent dopamine that is stored in large dense core vesicles, whereas dopamine released by high K^+ might be contained in small synaptic vesicles (SSV). The sustained dopamine transmission observed in the nucleus accumbens of SHR but not WKY rats can be supported by an elevated synthesis and release, which also might explain the stronger effect of methylphenidate on dopamine release in SHR but not in WKY rats. This hypothesis might enlighten the common therapeutic effect of these drugs, although their action takes place at different levels in catecholaminergic transmission.

KEYWORDS

dopamine, noradrenaline, nucleus accumbens, prefrontal cortex, SHR, VMAT2

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INTRODUCTION

Attention-deficit hyperactivity disorder (ADHD) is a clinically heterogeneous disorder with an onset in infancy characterized by the presence of three principal symptoms: hyperactivity, inattention, and impulsivity (Oades, 1998). Results from neuropsychological and neuroimaging studies suggest that abnormalities of the prefrontal cortex coexist with a dysfunction of subcortical areas that project to the prefrontal cortex, leading to the definition of 'frontosubcortical alteration', an expression that reflects the complexity of this disorder and answers questions on the site of the defect that causes ADHD (see Faraone & Biederman, 2002, for review). The treatment of ADHD is represented by drugs that raise the synaptic concentration of dopamine and norepinephrine through different mechanisms. Although the first line of treatment is represented by psychostimulants like methylphenidate and dextroamphetamine (Whalen et al., 1989; Solanto, 1998), tricyclic antidepressants can be used while weighing the risks and benefits because of their moderate to strong side effects. Antidepressants that can be used in ADHD therapy are drugs like imipramine, desipramine, clomipramine, nortriptyline, and amitriptyline that block the reuptake of norepinephrine and serotonin (Wilens et al., 1995; Spencer et al., 2002; Pliszka, 2003), but not the selective serotonin reuptake inhibitor (SSRI) (Biederman et al., 1997).

The increase of catecholamine transmission, either at the level of the basal ganglia or at the level of the prefrontal cortex, might be the key to the medication potential of psychostimulants and antidepressants. The study of synaptosomes, slices, vesicles, and cells in culture has contributed to elucidating the mechanism of action of amphetamine and its involvement in vesicle trafficking at the catecholaminergic terminal level. This issue was recently reviewed by Schmitz et al. (2003). The aim of the present review is to provide a further contribution to explaining the effect of

amphetamine, methylphenidate, and depolarization by high K^+ on dopamine release, as estimated by in vivo microdialysis in animal models of ADHD and to compare the evidence obtained with that from in vitro methods. This comparison can lead to the recognition of the advantages and the limitations of each method used to study the vesicle trafficking involved in dopamine release and provide insight into the etiology of ADHD and the mechanism of the medication effect of psychostimulants.

ANIMAL MODELS OF ADHD

In vivo experimentation on ADHD medications can be performed in different animal models of ADHD (Davids et al., 2003). Among them, spontaneously hypertensive rats (SHR) (Yamory et al., 1984) have been widely investigated because relative to their Wistar-Kyoto (WKY) normotensive controls, SHR rats show behavioral abnormalities (hyperactivity and hyper-reactivity to stress) (Knardahl & Sagvolden, 1979) that resemble those of human ADHD. Moreover, when compared with WKY controls, SHR rats exhibit increased activity when exposed to different contexts (Sagvolden et al., 1992a,b), a heightened response to stress with marked increase of plasma catecholamines (Chiueh, 1981), altered reinforcement mechanisms (Berger & Sagvolden, 1998), deficient sustained attention (Sagvolden, 2000), and impaired acquisition of operant tasks (Wyss, 1992; Mook et al., 1993; Sagvolden, 2000). Spontaneously hypertensive rats also have a reduced number of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) elements and a lower expression of the peptide product of the FOS family and ZIF-268 in the shell of the nucleus accumbens (NAc) (Papa et al., 1998). Moreover, in SHR rats, CaMKII levels can be normalized by chronic methylphenidate treatment.

In view of the evaluation of the effects of psychostimulants and norepinephrine reuptake

blockers on SHR rats, we must point out that SHR rats have a greater rate of norepinephrine uptake, mediated by the norepi-nephri-ne transporter (NET), in the frontal cortex during early development, and that such changes are accounted for by an increased V_{max} of the norepinephrine uptake mechanism (Myers et al., 1981). Moreover, the authors detected a significant decrease in dopamine uptake in the frontal cortex and striatum of the SHR at 6 weeks of age, thus suggesting an important role for dopamine in the development of both hypertension and behavioral hyperactivity exhibited by these animals. An interesting study on the expression pattern of the specific genes involved in dopamine neuron differentiation, survival, and functioning during postnatal development of the ventral midbrain in SHR males has shown that tyrosine hydroxylase (TH) and dopamine transporter (DAT) gene expression are significantly reduced in the SHR midbrain during the first month of postnatal development (Leo et al., 2003).

A promising new animal model of ADHD is represented by WKHA rats (Hendley & Fan, 1992; Hendley, 2000). The WKHA rats, selected by cross-breeding SHR and WKY, are normotensive but maintain certain behavioral features of SHR, such as hyperactivity in a novel environment and hyper-reactivity to stress; WKHA rats are less aggressive than SHR and habituate more readily to a novel environment. Neurochemical studies have suggested that the WKHA strain shows the lowest rate of norepinephrine uptake in the frontal cortex and the highest uptake rate of dopamine when compared with SHR, WKY, and WKHT (a WKY-derived strain of hypertensive rats that are not hyperactive).

Another animal model of ADHD is represented by dopamine transporter knockout mice (DAT-KO) (Giros et al., 1996) that have an elevated synaptic dopamine concentration in the striatum and the nucleus accumbens (NAc) (Carboni et al., 2001) and show hyperactivity and learning impairment

(Gainetdinov et al., 1998, 2001). Recently the Naples high-excitability (NHE) rat line was also proposed as an animal model of ADHD for its increased behavioral activity and impaired attention, which might be due to a hyperfunctioning of the meso-corticolimbic dopamine system (Sadile et al., 1993; Papa et al., 1995; 2002; Gonzales-Lima & Sadile, 2000). In summary, although several animal strains may be hyperactive and show attention deficit, the SHR strain shows the highest number of behavioral symptoms resembling those observed in ADHD (Sagvolden, 2000).

TRANSMITTERS, TRASPORTERS AND BRAIN AREAS INVOLVED IN ADHD

Investigations on dopamine transmission in the prefrontal cortex (PFCX) and in subcortical areas, i.e. caudate putamen and NAc, has provided clues on the specific neuronal mechanism that could be responsible for the behavioral features of animal models of ADHD (Linthorst et al., 1991; Sagvolden & Sergeant, 1998; Robbins et al., 2002; Spencer et al., 2002, Solanto, 2002). The PFCX and the ventral tegmental area (VTA) are reciprocally connected by dopamine afferents and glutamatergic efferents. Ventral tegmental area dopamine neurons are involved in working memory and in executive functions, such as motor planning, inhibitory-response control, and sustained attention (Fibiger & Phillips, 1986; Granon et al., 2000; Robbins, 2002). The caudate putamen and the NAc receive a dense innervation from the substantia nigra and the VTA, respectively, and play a crucial role in motor behavior and motivation (Di Chiara et al., 1992; Almaric & Koob 1993; Woodward et al., 1999). The NAc, in turn, can be subdivided into two regions, the shell and the core (Deutch & Cameron, 1992; Zham & Heimer, 1993). Dopamine transmission in the shell is involved in emotional and motivational processes related to reinforcement

(Di Chiara, 2002). In particular, dopamine transmission in the NAc might be of particular relevance in ADHD because impulsiveness and premature responding may be related to the grade of efficiency or rewarding/reinforcing mechanism (Sagvolden & Sergeant, 1998).

Children with ADHD tested in attentional tasks were shown to prefer an immediate reinforcement instead of waiting for a delayed one (Sonuga-Barke et al., 1992). Additionally, in tests addressed to show changes in rates of responding throughout fixed-interval schedules of reinforcement of bar-presses for water, SHR behavior was more sensitive to immediate reinforcement but was proportionately less sensitive to delayed reinforcement when compared with the behavior of WKY rats (Sagvolden et al., 1992a, b). In similar tests, the SHR strain can maintain high rates of responding only if reinforcers are infrequent (Sagvolden et al., 1993). Moreover, psychomotor stimulants acted by strengthening the control by delayed reinforcers, an action consistent with clinical observations that ADHD children are less willing than others to accept 'delayed gratification' and that methyl-phenidate increases the control of delayed reward over their behavior (Sagvolden et al., 1992a, b).

Although DAT-KO mice show hyperactivity and an elevated dopamine synaptic concentration, studies on the function of the DAT in ADHD suggest a higher expression of DAT sites in the basal ganglia of adults that suffered from ADHD during childhood (Dougherty et al., 1999) and in children affected by ADHD (Cheon et al., 2003). Therefore, the role of the DAT in the behavioral features of ADHD subjects or in animal models of ADHD remains to be elucidated. Moreover, a correlation between the expression of DAT and the severity of symptoms was not found in these studies. A higher expression of DAT sites was also found in the caudate-putamen of SHR compared with WKY rats, both at pre- and post-hypertensive stages, (Watanabe et al., 1997). Kujirai et al.

(1990), however, did not find any difference between SHR and WKY in ^3H -mazindol-labeled dopamine uptake sites.

Understanding the consequences of an alteration of DAT expression in ADHD is further complicated by the brain area considered. If indeed, in areas like the caudate putamen and the NAc, the DAT is the primary mechanism of clearance of dopamine released, then a different situation may be encountered in the PFCX, where the role of DAT and eventually its alteration is less clear because the clearance of transmitters is accomplished mainly by diffusion rather than by reuptake in dopamine terminals. In the PFCX, indeed a lower number of DAT sites are present as compared with the NAc and striatum (Garris & Wightman 1994; Clements 1996; Sesak et al., 1998).

A contribution to the removal of dopamine from the extracellular space in the PFCX can also be provided by norepinephrine terminals (Carboni et al., 1990, Yamamoto & Novotney, 1988). The expression of DAT in the NAc of SHR rats is unknown; how a higher expression of DAT might explain the higher basal dopamine concentration in the NAc of SHR rats as compared with WKY rats (Carboni et al., 2003) is unclear. It is indeed more likely that a higher synaptic dopamine concentration is associated with reduced DAT activity. An increased number of DAT sites and the consequent reduced dopamine synaptic concentration hardly fit with the hyperactivity typical of the ADHD syndrome. In fact, in DAT-KO mice, hypermotility and an elevated extracellular dopamine concentration in the caudate putamen and in the NAc (Carboni et al., 2001) is associated with KO (Giros et al., 1996) and knock down (KD) (Zhuang et al., 2001) of the DAT gene.

Moreover, as amphetamine reduces hypermotility in DAT-KO mice, it has been suggested that DAT-KO and DAT-KD support the hyperdopaminergic hypothesis of ADHD (Solanto, 2002; Russell, 2002). This suggestion is supported

by the finding that the dopamine agonist apomorphine, although producing biphasic effects (hypomotility at low doses and hypermotility at high doses) in wild mice and in WKY rats, induces only hypomotility in KD mice and SHR rats at low and high doses (Fuller et al., 1983; Zhuang et al., 2001).

MECHANISM OF ACTION OF DRUGS USED IN ADHD THERAPY

Most studies aiming to clarify the mechanism of action of amphetamine have been addressed to vesicle trafficking in the dopamine terminal. Amphetamine can enter the terminal, either through the DAT (Liang & Rutledge, 1982) or by membrane diffusion, due to its lipophilic properties (Mack & Bonisch, 1979). In the neuron terminal, amphetamine can label the vesicular monoamine transporter VMAT2 (Sulzer & Rayport, 1990) and, dissipating the pH that drives vesicular monoamine uptake (Sulzer et al., 1995), generates the dopamine efflux (Heikkila, 1975). Dopamine, in turn, can diffuse into the terminal cytoplasm and leave it through the inversion of the transport direction of the DAT (Sulzer et al., 1995; Jones et al., 1998). Vesicular monoamine stores, therefore, can play a significant role in locomotor activity, attention, and reinforcing processes as confirmed in heterozygotic VMAT-2 KO mice, in which amphetamine produces enhanced locomotor activity but diminished behavior reward (Takahashi et al., 1997).

A complementary hypothesis tested in chromaffin cells (Mundorf, 1999) suggests that amphetamine can disrupt vesicular stores to a sufficient degree that Ca^{++} , stored together with dopamine in the terminal vesicles, can escape and trigger the exocytosis of vesicles that are close to the plasmalemmal membrane. A similar mechanism has been proposed for neuronal systems, such as the cholinergic terminals, where acetylcholine

release can occur during large inward calcium currents involving many simultaneously opening channels but also through very small calcium transients' entry through only one channel opening at a time (Stanley, 1993).

A more intriguing action of amphetamine was observed in the rat amygdala, where it induces long-term depression of synaptic transmission by CB_1 cannabinoid receptors. Amphetamine-induced endocannabinoid release depends indeed on a rise in intracellular calcium by an undisclosed, mechanism (Huang et al., 2003). The possible effect of amphetamine on cannabinoid transmission in ADHD subjects or in animal models can be investigated further in view of the studies by Ehrenreich et al., (1999), who tested the hypothesis that chronic interference by cannabis with endogenous cannabinoid systems during peripubertal development can cause specific and persistent alterations of attentional functions. The authors indeed reported that beginning cannabis use during early adolescence could lead to enduring effects on specific attentional functions in adulthood. Inherent with this evidence, Viggiano et al. (2003) studied the influence of endocannabinoid as a neurotrophic factor on developing mesencephalic dopamine neurons in NHE rats using a prenatal elevation of anandamide. The authors reported that the offspring of rats treated with AM-404 showed a reduction in behavioral activity and attention tests.

Methylphenidate is the drug of choice for ADHD treatment (Swanson & Volkow, 2002). The drug shows a high affinity for the DAT and NET and much less for the serotonin transporter (SERT) (Gatley et al., 1996). The *in vivo* potency of methylphenidate on the DAT in the human brain is similar to that of cocaine (Volkow et al., 1999). Additionally, its psychopharmacological properties, evaluated in behavioral, assays are similar to those of cocaine (Gatley et al., 1999). The therapeutic mechanism of action of methylphenidate in ADHD is very complex and is related to chronic treatment

with oral doses. Methylphenidate, given orally to humans, blocks up to 60% of DAT sites (Volkow et al., 2002) and increases extracellular dopamine concentrations in the striatum by $16 \pm 8\%$. If we assume that DAT function is higher in ADHD patients than in unaffected subjects, then this effect could compensate the reduced dopamine function resulting from DAT overexpression. Moreover, the overexpression of DAT sites could be the primary alteration in the dopamine transmission machinery. This alteration, in the absence of an augmented firing, would therefore generate a reduction in the synaptic dopamine concentration, determining a reduced dopamine function. This possibility would be in agreement with the 'dopamine hypofunction hypothesis' (Sagvolden & Sergeant, 1998). This hypothesis is mainly based on the mechanism of action of methylphenidate, a first drug of choice in ADHD therapy, and is supported by the higher number of DAT sites found in SHR rats as compared with WKY (Watanabe et al., 1997) but not by the gene-expression studies that have shown a lower level of DAT in SHR as compared with WKY rats (Leo et al., 2003).

On the other hand, methylphenidate, at oral doses in the range of those that are used in ADHD, reportedly increases norepinephrine in the hippocampus and in the PFCX, but not in subcortical striatal areas (Kuczenski & Segal, 2002). This effect might be the result of a methylphenidate-induced blockade of the NET (Gatley et al., 1996). This effect, in turn, might be shared by antidepressants that are currently used clinically (Pliszka, 2003). Tricyclic antidepressants have indeed an efficacy comparable to that of stimulants, although several side effects may hamper their use (Spencer et al., 2002). In particular, the specific NET blocker atomoxetine increases the concentration of norepinephrine and dopamine in the PFCX, without affecting serotonin, whereas it does not change dopamine concentration in the striatum (Bymaster et al.,

2002). Atomoxetine is well tolerated and has been proposed as a valuable new treatment option for adults with ADHD (Simpson & Plosker, 2004). In agreement with a major role of norepinephrine in the amphetamine therapeutic effect is the observation that amphetamine releases norepinephrine more potently than dopamine and serotonin do, and that the oral dose of these stimulants, which produce amphetamine-type subjective effects in humans, correlates with their potency in releasing norepinephrine but not dopamine (Rotman et al., 2001).

Elevated dopamine transmission, on the other hand, has been considered a possible cause of the ADHD disorder (Papa et al., 2002; Seeman & Madras, 2002). According to this hypothesis, methylphenidate, by increasing the dopamine concentration on the D2/D3 autoreceptor, would reduce tonic dopamine release and postsynaptic dopamine function (Grace 1991, 2001).

Synaptosomes and vesicle studies

Synaptosomes are synaptic terminals in suspension, obtained by a process that includes gently grinding brain tissue in an isotonic solution and various centrifugations (Whittaker et al., 1964). Synaptosomes can be used not only to study uptake and neurotransmitter release but also to prepare neurotransmitter vesicles upon osmotic shock. The evaluation of dopamine release in synaptosomes relies on the estimation of the tracer ^3H -dopamine released together with endogenous dopamine by an artificial depolarization, usually obtained by altering the concentration of K^+ and Na^+ in the medium or by changing the electrical field stimulation (Middlemiss & Hutson, 1990; Sandoval et al., 2001; Bowyer et al., 1987).

Because ^3H -dopamine must be previously loaded in the synaptosomal vesicles to be released, the study of dopamine release from synaptosomes relies on the assumption that the dopamine captured is loaded in vesicles that are routinely

loaded with newly synthesized dopamine in the terminal. This assumption is sustained by the observation of Cerrito et al. (1980), who reported that newly synthesized and recaptured dopamine have similar compartmentation in nerve endings. Alternatively, the endogenous transmitter released from synaptosomes can be assayed by HPLC (Middlemiss & Hutson, 1990).

The effect of amphetamine on neurotransmitter release in synaptosomes and vesicles has been studied widely. Amphetamine can enter into the synaptosomal preparation through a saturable active transport, as studied by the uptake of ^3H -amphetamine (Zaczek et al., 1991). Amphetamine can determine the release of previously loaded ^3H -dopamine from striatal synaptosomes vesicles that share the feature of being the target of reserpine (Masuoka, 1982). As mentioned in the previous paragraph, besides dopamine, amphetamine and related psychostimulants like 3,4-methylenedioxymethamphetamine (MDMA), (+)-methamphetamine, and ephedrine release norepinephrine more potently than they release dopamine and serotonin (Rothman et al., 2001). Furthermore, the authors showed that whereas the psychostimulants rank order of potency for dopamine release was similar to their rank order of potency in published self-administration studies; the oral dose of these stimulants, which produce amphetamine-type subjective effects in humans, correlate with their potency in releasing norepinephrine not dopamine. This observation can influence future research on the mechanism of action of amphetamine in relation to the therapeutic effect on ADHD.

An interesting effect of amphetamine, which might also have implications in ADHD therapy, is its action on dopamine synthesis. The stimulation of dopamine synthesis in rat brain striatal synaptosomes produced by the depolarizing agent veratridine is markedly reduced by prior *in vivo* amphetamine administration (Patrick et al., 1981). On the other hand, it has been suggested that

amphetamine can increase dopamine synthesis, apparently through a mechanism that depends on a functioning uptake carrier as it is prevented by uptake inhibitors like nomifensine and benztropin (Connor & Kuczenski, 1986).

Amphetamine increases particulate protein kinase C (PKC) activity in striatal synaptoneuroosomes. The increased PKC activity is linked to the outward transport of dopamine and when the release is diminished, the inward transport of amphetamine inhibits PKC instead (Giambalvo, 2003). It would be interesting to test this evidence in synaptoneuroosomes from SHR and WKY rats to ascertain any differences that could be related to the different sensitivity of dopamine release due to amphetamine *in vivo* and in slices (Russell et al., 1998; Carboni et al. 2003).

Among other effects of amphetamine that have been shown in synaptosomal preparations and might have implications in its therapeutic effect is the ability of amphetamine to alter phosphorylation-related second messenger activities. In particular, amphetamine can enhance the phosphorylation of the neural-specific calmodulin-binding protein, neuromodulin, which, in turn, could contribute to neurochemical events leading to the enhanced release of dopamine and/or a behavioral sensitization (Gnegy et al., 1993). Amphetamine can also increase the phosphorylation of synapsin I in rat striatal synaptosomes, which in turn can play a role in enhanced dopamine release (Iwata et al., 1997). A last interesting observation was obtained in synaptosomes from SHR rats and from WKHA rats, a strain characterized by developing hyperactivity but not hypertension. In SHR rats, a significant increase in norepinephrine uptake, primarily through an increase in V_{max} in cerebral cortical areas and the cerebellum, is associated with the hypertensive trait, whereas a significant increase in dopamine uptake V_{max} in the frontal cortex is associated with the inheritance of hyperactivity among these strains (Hendley & Fan, 1992).

Slice studies

Another method that has been used to study dopamine release and the effects of drugs used in ADHD therapy is based on the use of slices obtained from a specific brain area. Slices can be a valuable tool for investigating physiological and pharmacological process occurring at the neuron terminal level because drugs can be applied in a relatively quantitative manner, and the effect on the release of a neurotransmitter can be cleaner than the one observed with in vivo methods. (Dunwiddie & Basile 1983). In slices, dopamine release can be evaluated either by assaying endogenous dopamine or by preloading storage vesicles with ^3H -dopamine. In the latter case, the amount of radioactivity released into the perfusion chamber, after either an electrically or chemically triggered depolarization, obtained by changing K^+/Na^+ concentration in the medium, allows the evaluation of the effect of drugs on depolarization-dependent dopamine release (Dunwiddie & Basile 1983; Russell 2000; Schmitz et al., 2003).

An interesting study that might have implications for ADHD therapy was performed in striatal slices (Gifford., 2000). Here the author showed that amphetamine-evoked dopamine release at a concentration of 30 micromolar reduced ^3H -raclopride binding by 77% with a 50% inhibition at a concentration of 1.6 micromolar. Amphetamine-evoked dopamine release did not have a significant effect on ^3H -SCH 23390 binding, suggesting that dopamine release evoked by low doses of amphetamine would probably act on D2 rather than on D1 receptors. This view could apply to the striatum only because an interesting role of D1 transmission in the PFCX related to attentional performance has been suggested recently (Granon et al., 2000).

Moreover, in a striatal slice preparation, Park and Park (2000) showed that 74% of ^3H -raclopride binding was displaced by the dopamine released by amphetamine, and that synaptic dopamine

concentration should correspond to 1.6 nM. The development of electrochemical recording with small carbon-fibers electrodes has permitted the advancement of the knowledge on catecholamine transmission and its presynaptic regulation (Schmitz et al., 2003).

A study using fast cyclic voltammetry reported that the synaptic dopamine concentration evoked by 1 micromolar amphetamine was about 0.6 and 0.27 micromolar in the caudate putamen and the NAc, respectively (Wieczorek & Kruk, 1994). The action of amphetamine was also investigated using fast-scan cyclic voltammetry by Jones et al. (1998). The authors confirmed not only that DAT is essential for amphetamine-induced dopamine release but also that amphetamine can pass through the plasma membrane to deplete terminal vesicles, as demonstrated by the disappearance of electrically stimulated dopamine release in striatal slices of DAT-KO mice after the application of 10 micromolar amphetamine. The authors also reported that amphetamine vesicle depletion prevents dopamine electrically stimulated release, probably by affecting all the dopamine containing vesicle in wild type mice. This observation is apparently in contrast to the observation of Anderson et al. (1998), who reported that only large dense-core vesicles (LDCV) are affected by low doses of amphetamine because it has to be considered that the results were obtained in two different models, a mammal on one side and a mollusk (*Planorbis corneus*) on the other. The evaluation of the synaptic dopamine concentration evoked by amphetamine can therefore provide useful insight into the indirect receptor activity of amphetamine, although the dopamine concentration measured differs from study to study.

The response of slices obtained from brain areas involved in ADHD, such as the caudate putamen, NAc, and PFCX, to methylphenidate and amphetamine has been widely investigated by Russell and coworkers (Russell et al., 1995, 1998; Russell, 2000, 2002). The authors reported that in

NAc slices of SHR rats, methylphenidate (10 micromolar) released dopamine to a lesser extent than in WKY rats. By contrast, amphetamine stimulated dopamine release to a greater extent in NAc slices from SHR rats than in those from WKY rats. These effects, however, were obtained only at one of the drug concentrations tested. Electrical depolarization, in the presence or in the absence of methylphenidate, on the other hand, stimulated the release of ^3H -dopamine in the NAc of SHR to a similar extent as in the NAc of WKY rats. On the other hand, the depolarization (25 mM K^+)-induced release of dopamine from NAc slices of SHR rats was significantly lower than in those from WKY rats (Russell, 2000).

The possibility that amphetamine interacts with dopamine release triggered by high potassium was investigated in striatal synaptosomes and slices by Bowyer et al., (1987). The authors reported that amphetamine evokes ^3H -dopamine release and inhibits the subsequent K^+ -evoked ^3H -dopamine release at a flow perfusion that prevents reuptake, whereas at a flow rate that allows the reuptake, the effect of amphetamine is of lesser extent. On the basis of these results, Bowyer et al. suggested that amphetamine can open voltage-dependent Ca^{++} channels that are sensitive to cobalt but not to nifedipine, and that amphetamine can desensitize these voltage-dependent Ca^{++} channels and inhibit their activation by K^+ depolarization. This observation parallels the amphetamine effect of preventing the electrical stimulation of dopamine release in striatal slices (Jones et al., 1998).

Whole cell studies

Some interesting insight into the mechanism of action of amphetamine was recently provided by Khoshbouei et al. (2003). The authors, using the patch clamp technique in the whole-cell configuration combined with amperometry on human embryonic kidney HEK-293 cells stably transfected with the

human DAT, observed that the dopamine efflux mediated by DAT is voltage dependent, electrogenic, and dependent on the intracellular Na^+ concentration in the recording electrode. On this basis, the authors suggested that the transport of amphetamine after binding to the DAT causes an inward current, which in turn increases the intracellular concentration of Na^+ , thereby enhancing the DAT-mediated reverse transport of dopamine toward the synaptic space.

Studies on cells have also suggested a different action of amphetamine on terminal vesicles. Indeed, it has been reported that using carbon fiber microelectrodes, currents transients corresponding to individual exocytotic events can be recorded from the cell body of the dopamine-containing neuron of the giant dopamine cell of *Planorbis corneus* (Anderson et al., 1998). In these preparations, amphetamine at low doses selectively caused the depletion of LDCV, leaving intact the small synaptic vesicles (SSV), although SSV vesicles should be located densely packed in the plasma membrane vicinity. At high doses, amphetamine depletes small vesicles more strongly than large vesicles but continues to deplete large vesicles in a dose dependent manner. On the other hand, in rat NAc catecholamine terminals, identified by immunocytochemical localization of TH, both LDCV and SSV were detected (Bouyer et al., 1984). The authors also reported that a certain proportion of TH-containing terminals within the NAc are morphologically distinct from the catecholaminergic terminals within the dorsal striatum.

If we now assume that in the rat neuron, specifically in NAc terminals, some of the above described mechanisms occur, then it becomes challenging to speculate that the higher amount of dopamine released by amphetamine in SHR rats than in WKY rats (Carboni et al., 2003) could be due to the capability of low doses of amphetamine to deplete a pool of LDCVs that might be located in greater proportion in SHR terminals. On the other

hand, the larger dopamine release observed in WKY rats resulting from depolarization by high K^+ might be explained, supposing that high K^+ acts at least preferentially on the SSV-containing dopamine pool, which is larger in WKY rats than in SHR rats. Therefore, it cannot be excluded that a different distribution of LDCV and SSV in the catecholamine terminals of basal ganglia and eventually in other brain areas, might be a morphological and functional feature that plays a role in the etiology of ADHD.

In vivo studies

Among the methods used to elucidate the mechanisms involved in the release of a neurotransmitter, microdialysis offers the advantage of evaluating the concentration of a transmitter in the dialysate, which directly reflects the extracellular concentration at the synaptic clefts. The concentration at the synaptic clefts, in turn, depends on the amount released by a firing- and calcium-dependent process and on the efficiency of the reuptake system in removing the transmitter from the synaptic cleft.

Dopamine release from both dendrites and terminals can be assayed *in vivo* in unanesthetized rats using the microdialysis method (Di Chiara, 1990; Di Chiara et al., 1996; Justice, 1993; Westerkamp, 2000; Carboni, 2003). The microdialysis technique, therefore, allows the evaluation of endogenous dopamine that, after being released by a depolarization/ calcium-dependent process, escapes from the synaptic cleft, and reaches the microdialysis fiber to be collected in the artificial cerebrospinal fluid. Moreover, the synaptic dopamine concentration is dependent on the capturing capability of the DAT and therefore depends on the firing activity of the terminal and on the density of DAT sites in the area studied. Even in microdialysis, it is possible to study dopamine release triggered by an artificial depolarization, which can be achieved in the area surrounding the microdialysis fiber by perfusing

the fiber with an artificial cerebrospinal fluid containing an elevated K^+ concentration.

The acute and chronic effects of amphetamine and/or methylphenidate have been widely studied by microdialysis (Carboni et al., 1989; Cadoni et al., 1995; Kuczenski et al., 1995; Carboni et al., 2003). Cadoni et al. (1995), investigating the role of vesicular and newly synthesized dopamine in the action of amphetamine, reported that the increase of extracellular dopamine evoked by amphetamine (0.2 and 0.5 mg/kg s.c.) in the dorsal striatum, was only partially prevented by the blockade of dopamine synthesis by alpha-methyl-p-tyrosine pretreatment or by the inactivation of vesicular amine uptake by reserpine pretreatment. The combined treatment instead produced a dramatic reduction of the amphetamine-evoked dopamine output.

Kuczenski et al. (1995) reported that both amphetamine and methylphenidate (2 mg/kg s.c.) promote the equivalent increase of dopamine output in the caudate, whereas amphetamine is more potent in raising the synaptic concentration of norepinephrine in the hippocampus. We recently reported (Carboni et al., 2003) that the systemic administration of amphetamine (0.25 and 0.5 mg/kg s.c.) and methylphenidate (1 or 2 mg/kg i.p.) produces a higher increase in dialysate dopamine in the shell of SHR than in the shell of WKY rats. In contrast, when the microdialysis fiber was perfused with 30 or 60 mM K^+ through reverse dialysis, the increase of the dialysate dopamine was lower in SHR rats than in WKY rats. The results, compared and critically discussed with the results of other studies investigating the effect of amphetamine, methylphenidate, and high potassium depolarization, using *in vitro* preparations, suggest the existence of different dopamine-containing vesicle pools in the NAc shell dopamine terminals of SHR and WKY rats.

Moreover, we reported that the basal extracellular dopamine concentration in the NAc shell is higher in SHR rats than in WKY rats. On the

other hand, no difference between SHR and WKY rats, in both basal and amphetamine-induced striatal dopamine release and metabolites, was found by Ferguson et al. (2003). This result is in contrast with what we recently reported (Carboni et al., 2003), but an explanation can be found in the different age of the rats used (6-week-old rats in our study versus 19-week-old rats in the Ferguson study) and in the brain area investigated (NAc in our study, striatum in Ferguson study).

Yu et al. (1990) found no difference between dopamine levels in the striata and NAc from 9-week-old WKY and SHR rats. In groups treated acutely with cocaine, the dopamine levels in these two brain regions were surprisingly unaffected, whereas 2 h after the administration of cocaine, both strains showed a significant increase in striatal HVA. Subacute cocaine administration in WKY and SHR, however, affected dopamine levels in the striata and NAc differently. One more study reported a positive, linear correlation between the extracellular levels of dopamine and cocaine for the 60-min period following acute cocaine administration in both SHR and WKY rats. On the other hand, the slope of the linear regression plots obtained from the data of each 15-min sample was slightly, but significantly, higher in conscious SHR than in conscious WKY (Inada et al., 1992).

In summary, *in vivo* microdialysis studies have been revealed as a very useful tool for investigating the effect of amphetamine and methylphenidate in either normotensive or hypertensive rats, although the differences in the brain area investigated and in the age of the rats hamper a direct comparison of the results.

DISCUSSION

Synaptic release and consequently the extracellular concentration of dopamine measured depend on a sequence of events that begins with

terminal depolarization and ends with the reuptake or the diffusion the transmitter. The higher extracellular concentration of dopamine observed in the NAc of SHR rats than in the NAc of WKY rats (Carboni et al., 2003) suggests that in SHR rats, synaptic transmission in the NAc occurs by means of a higher dopamine concentration in the synaptic cleft. The elevated synaptic dopamine concentration cannot be recognized as being due to either elevated tonic or elevated phasic activity because phasic activity can be measured on a subseconds to seconds timescale (Venton et al., 2003; Grace, 1991, 1995), whereas the amount of transmitter assayed by the microdialysis technique represents an estimate of the average dopamine concentration present at the synaptic cleft for the collecting time (20 min). Therefore, the higher synaptic dopamine concentration observed in the NAc of SHR rats can be attributed to a dysfunction at different levels, such as dopamine synthesis, dopamine storage, dopamine metabolism, dopamine reuptake, autoreceptor function, postsynaptic receptor activity, and transduction mechanisms. Unfortunately, basal release cannot be estimated in synaptosomes and might be hampered in slices because of an interruption in the cell body-terminal connections; therefore, no comparison with the results obtained *in vitro* can be made.

Although the differences observed in NAc-basal dopamine release is of limited entity, this dissimilarity could represent a crucial transmission alteration that might be involved in the expression of the typical behavioral observed in SHR rats (Knardahl & Sagvolden, 1979), and it is possible to speculate that children affected by ADHD might have in common with SHR rats an elevated dopamine transmission in the NAc. This view is supported by the increase in TH activity found in another animal model of ADHD, the Naples high-excitability (NHE) rat (Viggiano et al., 2000, 2002). If one assumes that the SHR phenotype is a model of ADHD, then the higher dopamine basal concentration found in the NAc of SHR as

compared with WKY rats (Carboni et al., 2003) favors a hyperdopaminergic hypothesis of this condition. Moreover, SHR rats showed a more pronounced increase of extracellular dopamine in the same area after challenge with amphetamine and methylphenidate. The clear-cut difference between SHR and WKY rats in the responsiveness of NAc-shell dopamine to methylphenidate is particularly relevant, given that this drug is to date the treatment of choice for ADHD. An increased dopamine synthesis in SHR rats seems also supported by the higher expression of TH mRNA levels (Kumai et al., 1996) and TH gene expression (Reja et al., 2002) in the medulla oblongata of SHR rats. On this basis, it has been speculated that an increased dopamine output in the NAc would be compensating for a reduced transduction mechanism (Papa et al., 1998). Consistent with this proposal might be the reduced number of CaMKII-positive elements identified in the anterior portion of the shell together with a lower expression of *c-FOS*.

The larger increase of dialysate dopamine caused by a low doses of amphetamine in SHR, as compared with WKY rats (Carboni et al., 2003), is difficult to attribute to a specific mechanism, considering the existence of multiple evidence and a hypothesis on the mechanism of action of amphetamine. Although many differences are due to the methodology used, the comparison of our data with those from a few in vitro studies in slices conducted by Russell et al. (1998) allows us to point out some common evidence. The authors indeed showed that in slices obtained from the accumbens of SHR and WKY rats, the effect of amphetamine on dopamine release, at the lower dose tested, was higher in SHR than in WKY rats. Moreover, amphetamine at higher doses produced a bigger increase in dopamine release in PFCX slices from SHR than in those from WKY rats. The data are in agreement with a recent observation in our laboratory. We indeed found that amphetamine releases more dopamine in the

PFCX of SHR rats than in WKY rats (Carboni et al., unpublished observations).

When dopamine release is evoked by high K^+ concentrations, SHR and WKY rats respond in a strikingly different manner (Carboni et al., 2003). Therefore, if amphetamine releases more dopamine from the NAc shell of SHR rats than from the shell of WKY rats, then the depolarization obtained by perfusion with high- K^+ Ringer's solution releases more dopamine in WKY than in SHR rats. The data suggest that (a) two pools of releasable dopamine might be available in accumbal dopamine terminals; (b) the pools are differently affected by amphetamine and high K^+ , and (c) they represent a different source of releasable dopamine in the rat strains SHR and WKY. Our result is in agreement with that reported by Russell (2000). The authors reported indeed that the 25 mM K^+ -evoked dopamine release in NAc slices from SHR rats is much lower than that observed in slices from WKY rats. It is interesting to note that such a difference was not observed when depolarization was triggered by electrical stimulation (Russell et al., 1998). A higher effect of high K^+ in SHR rats was reported recently (Yousfi-Alaoui et al., 2001). The authors showed that 15 mM K^+ -evoked 3H -dopamine overflow is lower in the striatum and PFCX synaptosomes from SHR as compared with WKY rats. This evidence allows us to suggest that 3H -dopamine used as a tracer in both synaptosomes and slice preparations is evenly distributed among vesicles, no matter if two pools of vesicles eventually are differently mobilized by high K^+ or by amphetamine.

We recently reported that methylphenidate enhances the extracellular dopamine concentration in the NAc of SHR more than in WKY rats (Carboni et al., 2003). The data from that study, when compared with the data obtained by Russell et al. (1998), are different regarding the effect of methylphenidate. Indeed Russell et al. reported that the methylphenidate effect on the electrically stimulated release of 3H -dopamine was not different

in slices obtained from the NAc of WKY or SHR rats. At the moment, we do not have an explanation for such diversity, but the different density and location of DAT sites in areas such as the NAc, the caudate, and the PFCX may be relevant. The DAT indeed can be densely packed in the synaptic cleft vicinity but also more sparsely located far from the synaptic cleft (Sesak et al., 1998), and this feature might affect the results obtained by different methods. If indeed the dialysate concentration of dopamine measured through the microdialysis technique depends strongly on the DAT function in the striatum, and in the cortex it has been considered that dopamine can be captured also by the NET (Carboni et al., 1989, 2001), therefore the evaluation of dopamine release and the influence of DAT in slices, and even more in synaptosomes, may be different because dopamine is released in much larger spaces.

Upon studies on slices, Russell et al. (2000) concluded that in SHR rats, the DAT function is not probably different from that in WKY rats. If we assume that this conclusion is correct, our data would suggest that the higher effect of methylphenidate observed in SHR rats as compared with WKY rats is merely due to a higher release of dopamine in the synaptic cleft, which is, in turn, in agreement with the higher dopamine basal concentration found in the NAc of SHR rats as compared with WKY rats. This point of view would therefore strengthen the hypothesis (Russell, 2000) that the difference between SHR and WKY rats regarding the response to amphetamine and to high K^+ lies at the vesicle level.

A further mechanism in which amphetamine is involved is the so called 'redistribution' of VMAT-2 in dopamine terminals (Sandoval et al., 2001, 2002; Fleckenstein & Hanson, 2003). A single dose of amphetamine indeed, causes a reversible decrease in the function of dopamine uptake in a purified vesicle-enriched fraction and a consequent rise in the extracellular dopamine concentration, with more chances that reactive species derived

from oxygen degradation products are formed in the dopamine terminal. An opposite effect has been reported for methylphenidate that instead, it increases dopamine uptake in the vesicle-enriched fraction whilst increasing the number of VMAT-2 sites in the same fraction and decreasing them in plasmalemma-enriched fractions (Fleckenstein & Hanson, 2003). It is hard to position VMAT-2 'redistribution' in the mechanism of action of psychostimulants in ADHD therapy because both amphetamine and methylphenidate have been used alternatively with therapeutic benefit, but considering the localization of VMAT2 in VTA neurons (Nirenberg et al., 1996) and the effect of amphetamine in KA1 VMAT2 mutant mice (Patel et al., 2003), it cannot be excluded that the defect present in SHR rats and eventually in ADHD is at the level of different LDCV and SSV density and/or VMAT2 expression in the vesicles.

In conclusion, this study supports the usefulness of investigating dopamine release in SHR and WKY rats using different methods for improving either the knowledge on vesicle trafficking at the dopamine-terminal level or the knowledge of the mechanism of action of amphetamine and methylphenidate, therefore contributing to the understanding of ADHD etiology.

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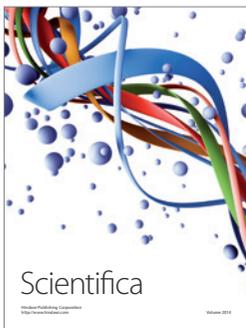
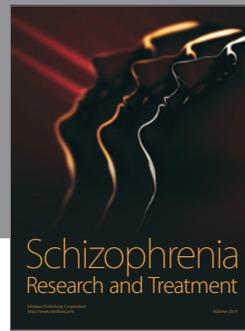
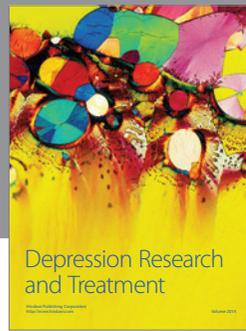
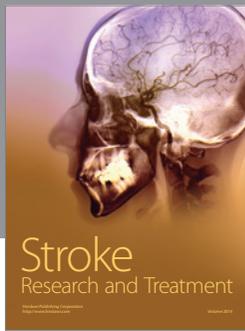
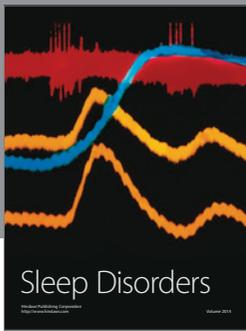
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