Dopamine reuptake transporter (DAT) "inverse agonism" — A novel hypothesis to explain the enigmatic pharmacology of cocaine

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ABSTRACT

The long held view is cocaine's pharmacological effects are mediated by monoamine reuptake inhibition. However, drugs with rapid brain penetration like sibutramine, bupropion, mazindol and tesofensine, which are equal to or more potent than cocaine as dopamine reuptake inhibitors, produce no discernable subjective effects such as drug "highs" or euphoria in drug-experienced human volunteers. Moreover they are dysphoric and aversive when given at high doses. *In vivo* experiments in animals demonstrate that cocaine's monoaminergic pharmacology is profoundly different from that of other prescribed monoamine reuptake inhibitors, with the exception of methylphenidate. These findings led us to conclude that the highly unusual stimulant profile of cocaine and related compounds, eg methylphenidate, is not mediated by monoamine reuptake inhibition alone.

We describe the experimental findings which suggest cocaine serves as a negative allosteric modulator to alter the function of the dopamine reuptake transporter (DAT) and reverse its direction of transport. This results in a firing-dependent, retro-transport of dopamine into the synaptic cleft. The proposed mechanism of cocaine is, therefore, different from other small molecule negative allostereric modulators of the monoamine reuptake transporters, eg SoRI-6238, which merely reduce the rate of inward transport. Because the physiological role of DAT is to remove dopamine from the synapse and the action of cocaine is the opposite of this, we have postulated that cocaine's effect is analogous to an inverse agonist. If this hypothesis is validated then cocaine is the prototypical compound that exemplifies a new class of monoaminergic drugs; DAT "inverse agonists".

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1. Introduction

It was our experience in developing the centrally-acting, anti-obesity drug, sibutramine (Meridia®, Reductil®), which raised serious doubts about the hypothesis that cocaine's pharmacological effects were solely mediated by monoamine reuptake inhibition. Sibutramine is an interesting molecule because it contains the β-phenylethylamine substructure that is present in many mono-amine releasing agents, eg D-amphetamine, methamphetamine and MDMA. In addition, sibutramine's active metabolites inhibited the reuptake of noradrenaline (norepinephrine), 5-hydroxytryptamine (5-HT, serotonin) and dopamine (Cheetham et al., 1993, 1996; Heal et al., 1998b), which raised the question of its pharmacological similarity to cocaine. How could sibutramine, which was in effect

10-fold more potent as a dopamine reuptake inhibitor (Cheetham et al., 1993, 1996; Heal et al., 1998b) than cocaine (Kula and Baldessarini, 1991; Hyttel, 1982; Richelson and Pfenning, 1984; Rothman et al., 2001) and very rapidly penetrated the brain (Heal et al., 1992), fail to mimic the psychostimulant actions of cocaine? As a consequence, an enormous amount of preclinical and clinical testing was performed to attempt to demonstrate that sibutramine was pharmacologically different from both D-amphetamine and cocaine. The most compelling evidence came from studies in drug-experienced human volunteers where sibutramine did not produce positive effects, eg "Drug liking", "High", "Euphoria" or "Want to take drug again" (Cole et al., 1998; Schuh et al., 2000). On the contrary, sibutramine produced no discernable subjective effects at low dose and was dysphoric and aversive at high dose (Cole et al., 1998; Schuh et al., 2000).

Subsequently, it has emerged that the clinical findings with sibutramine were not an anomaly, and have now been replicated many times with other monoamine reuptake inhibitors, eg bupropion, mazindol and tesofensine. Furthermore, *in vivo* experiments in

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animals have revealed that cocaine's monoaminergic pharmacology is profoundly different from that of clinically used monoamine reuptake inhibitor drugs with the exception of methylphenidate. Together, these observations have led us to the conclusion that the highly unusual, stimulant profile of cocaine and related compounds is not mediated by reuptake inhibition alone. In this review, we put forward the hypothesis that cocaine allosterically modulates the function of the dopamine reuptake transporter (DAT) to reverse its direction of transport, resulting in a firing-dependent retrotransport of dopamine into the synaptic cleft. The proposed action of cocaine is, therefore totally different from that of related, small molecule, negative allostereric modulators of monoamine reuptake transporters, eg SoRI-6238, which only reduce the rate of inward transport (Nandi et al., 2004). Since the physiological role of DAT is to remove dopamine from the synapse, we have postulated that cocaine's effect is analogous to that of an inverse agonist. If this hypothesis proves to be valid then cocaine is the prototypical compound that exemplifies a new class of monoaminergic drugs; the DAT "inverse agonists".

2. Exploring the myths

When a drug has been the subject of scientific research for decades, there is often a prevailing view that its mechanism of action has been fully elucidated and there is nothing new to be learnt. In this situation there is a tendency for a hypothesis that is based on sound scientific observations to become dogma. Dogma which is often accompanied by an unwillingness to concede that when the facts no longer fit the hypothesis, it is the hypothesis and not the facts that needs to be re-evaluated. Cocaine may turn out to be another classic example.

Although it was already well established that cocaine potentiated the effects of noradrenaline in peripheral tissues and this effect was pronounced in areas where there was dense sympathetic innervation (Trendelenburg, 1965, 1966, 1969), it was the seminal work of Iversen and colleagues that led to the discovery that cocaine was a competitive inhibitor of high-affinity, noradrenaline uptake transport (Uptake-1) in the heart. By this action it prevented clearance of noradrenaline from the synapse (see reviews of Iversen, 1970, 1971). Furthermore, Iversen also demonstrated that cocaine did not impede the transfer of noradrenaline into storage vesicles within the noadrenergic neurones (Iversen, 1970, 1971), thereby inferring that in contrast to the β-phenylethylamine releasing agents, cocaine's pharmacological effects took place on the external cell surface of noradrenergic neurones. The presence was discovered of similar reuptake transporters on noradrenergic neurones in the brain and related transporters on dopaminergic and serotonergic neurones (Iversen, 1970, 1971). The finding that cocaine was an inhibitor of the reuptake transporters for dopamine (DAT) and 5-HT (SERT) as well as noradrenaline (NET) (Hyttel, 1982; Richelson and Pfenning, 1984; Andersen, 1989) translated cocaine's monoamine reuptake inhibition properties into the sole pharmacological mechanism mediating its stimulant, discriminative and reinforcing actions. The replication of cocaine's uptake inhibitory properties many times in many laboratories, the robustness of the finding, and the failure to discover an alternative mechanism have all served to reinforce the prevailing view that monoamine reuptake inhibition, especially dopamine reuptake inhibition, adequately explains cocaine's pharmacology (Ritz et al., 1987; Di Chiara and Imperato, 1988; Galloway, 1988; Volkow et al., 1997, 1999; Carroll et al., 2004a,b). The DAT inhibitor hypothesis has also directed research into pharmacological approaches to tackling the problems of cocaine abuse and addiction for the last 20 years (eg Rothman, 1990; Deutsch et al., 1996; Volkow et al., 1997; Katz et al., 2000; Schenk, 2002; Schweri et al., 2002; Carroll et al., 2004a,b). In the

following sections, we will critically assess the validity of the evidence in support of the DAT inhibitor hypothesis for cocaine.

2.1. All dopamine reuptake inhibitors are stimulant euphoriants

Although observations made using animal models have excellent predictive validity with regard to the recreational abuse liability of compounds from many pharmacological classes, the monoamine reuptake inhibitors are problematic in this respect (see review by Huskinson et al., 2014). In recent years, there has been increasing pressure from regulators to explore recreational abuse liability of novel CNS-active drug-candidates in drug-experienced human volunteers. From these observations, there is a growing body of definitive information from reuptake inhibitors with a widely differing balance of reuptake inhibitory actions across the spectrum of monoamines that sheds light on the question of whether all DAT inhibitors have the capacity to be stimulant euphoriants.

As shown in Table 1, cocaine, which is a non-selective monoamine reuptake inhibitor (Hyttel, 1982; Richelson and Pfenning, 1984; Andersen, 1989), unequivocally and reproducibly produces pleasurable energisation (stimulation), and feelings of wellbeing and elation (euphoria) that are reflected in subjective reports of "drug liking".

Methylphenidate, which is a catecholamine reuptake inhibitor (Hyttel, 1982; Richelson and Pfenning, 1984; Andersen, 1989), also evokes subjective effects in drug-experienced human volunteers and normal healthy subjects that are similar to those produced by the highly abused psychostimulants, i.e. cocaine or amphetamine (Martin et al., 1971; Smith and Davis, 1977; Heishman and Henningfield, 1991; Rush et al., 2001; Rush and Baker, 2001; see also reviews by Kollins et al., 2001; Huss and Lehmkuhl, 2002; Klein Schwartz, 2002; Kollins, 2003, Table 1). Specifically, under "blinded" experimental conditions, methylphenidate has been reported to generalise to the cocaine cue in normal human volunteers and stimulant abusers who have been trained to discriminate between cocaine and placebo (Rush and Baker, 2001; Rush et al., 2002b). These subjects also reported levels of "drug liking" and "stimulation" in these sessions similar to those reported for cocaine and other abused stimulants (Rush and Baker, 2001; Rush et al., 2001, 2002b; Stoops et al., 2003, 2004).

Volkow et al. (1996) performed a positron emission tomography (PET) study with [11C]D-*threo*-methylphenidate in a group of normal, healthy subjects to explore the relationship between levels of striatal DAT occupancy and the "high" produced by intravenous administration of methylphenidate and concluded that >80% occupancy was necessary for methylphenidate's euphoriant effect. What was surprising was even though DAT sites were ~80% occupied by methylphenidate, this blockade did not prevent the subsequent "high" induced by a second intravenous injection of methylphenidate given 60 min later. This finding casts doubt on the notion that the elevated synaptic dopamine concentration resulting from prolonged DAT blockade is implicated in its psychostimulant actions. What it does indicate is that methylphenidate's "high" is effected by a mechanism that is much faster and greater than inhibition of dopamine reuptake. Consistent with the findings reported by Volkow et al. (1996), Stoops et al. (2012) observed that not only did the DAT inhibitor, bupropion, fail to block the high evoked by insufflated ("snorted") cocaine by preventing cocaine from reaching its site of action, bupropion actually potentiated the latter's "good drug" effect. By contrast, blockade of NET sites with administration of atomoxetine neither increased nor decreased the stimulant effects of snorted cocaine (Stoops et al., 2008).

Consistent with these clinical data *in vivo* microdialysis experiments have also shown that substantial blockade of DAT sites in the brains of rats produced only minor reductions in the ability of

Table 1
Subjective effects of various dopamine reuptake inhibitors in human volunteers.

Compound	Subjects	Dose(s)	Active comparator(s)	Positive effects	Stimulant effects	Negative effects	Reference
Methylphenidate	Normal volunteers	0.375 mg/kg K iv	N/A	✓	✓	✓	Volkow et al. (1996)
	Normal volunteers	60 mg po	N/A	✓	✓	Not stated	Volkow et al. (2001)
	Recreational drug users	90 mg, po	N/A†	✓	✓	✓	Jasinski et al. (2008)
	Stimulant drug abusers	15, 30, 60, 150 mg/70 kg, sc	N/A	✓	✓	✓	Martin et al. (1971)
	Normal volunteers	20 and 40 mg, po	N/A	✓	✓	✓	Kollins et al. (1998)
	ADHD subjects	20 and 40 mg, po	N/A†	✓	✓	Not stated	Kollins et al. (2009)
	Normal volunteers	20 and 40 mg, po	D-amphetamine 10 and 20 mg	✓	✓	Not stated	Rush et al. (2001)
	Stimulant drug abusers	16, 32 and 48 mg, po	D-amphetamine 8, 16 and 24 mg po	✓	✓	Not stated	Stoops et al. (2004)
	Cocaine abusers	15, 30,60 and 90 mg, po	Cocaine 50, 100, 200 and 300 mg, po	✓	✓	×	Rush and Baker (2001)
	Normal volunteers	20 and 40 mg, po	D-amphetamine 10 and 20 mg po L-amphetamine 10 and 20 mg po	✓	✓		Smith and Davis (1977)
	Recreational drug users	45 and 90 mg, po	N/A†	✓	✓	✓	Jasinski (2000)
	Recreational drug users	7.5, 15, 30 and 60 mg, po	D-amphetamine 30 mg po	✓	✓		Heishman and Henningfield (1991)
	Recreational drug users	30, 60 and 90 mg, po	Cocaine 150 mg, po	✓	✓	Not stated	Rush et al. (2002a)
	Stimulant drug abusers	10, 20 and 30 mg intranasal	N/A	✓	✓	Not stated	Stoops et al. (2003)
Cocaine	Cocaine abusers	50, 100, 200 and 300 mg, po	N/A	✓	✓	×	Rush and Baker (2001)
	Cocaine abusers	12.5, 25 and 50 mg, iv	N/A	✓	✓	×	Preston et al. (1993)
Mazindol	Normal volunteers	0.5 and 2 mg, po	Benzphetamine 25, 50 mg po Phenmetrazine 25 50 mg po	×	×	✓	Chait et al. (1987)
	Amphetamine dependant subjects	1, 2 and 4 mg, po	DL-amphetamine 50 mg po	×	×	×	Götestam and Gunne (1972)
	Normal volunteers	2 mg	N/A	×	×	×	Holmstrand and Jonsson (1975)
	Cocaine abusers	1 and 2 mg, po	Cocaine 12.5, 25 and 50 mg, iv	×	✓	×	Preston et al. (1993)
	Normal volunteers	0.5 and 2 mg, po	D-amphetamine 10 mg po	×	×	✓	Chait et al. (1986)
Bupropion	Normal volunteers	50 and 100 mg, po	D-amphetamine 5 and 10 mg po	×	×	×	Peck et al. (1979)
	Psychostimulant abusers	100, 200 and 400 mg po	D-amphetamine 15 and 30 mg po	×	×	×	Griffith et al. (1983)
	Drug abusers	100, 200 and 400 mg po	D-amphetamine 15 and 30 mg po	×	×	×	Miller and Griffith (1983)
	Normal volunteers	100 and 200 mg po	D-amphetamine 5 and 10 mg po	×	×	×	Hamilton et al. (1983)
	Recreational drug users	400 mg, po	D-amphetamine 15 and 30 mg po	×	×	×	Schoedel et al. (2010)
Nomifensine	Normal volunteers	100 mg, po	D-amphetamine 5 and 10 mg po	×	×	×	Hamilton et al. (1983)
	Normal volunteers	100 mg, po	D-amphetamine 15 mg po	×	×	×	Taeuber et al. (1979)
Tesofensine	Recreational drug users	1, 6, 9 mg, po	D-amphetamine 15 and 30 mg po	×	×	×	Schoedel et al. (2010)
Sibutramine	Polydrug abusers	25 and 75 mg, po	D-amphetamine 20 mg, po	×	×	✓	Schuh et al. (2000)
	Stimulant drug abusers	30 and 30 mg, po	D-amphetamine 20 and 30, po	×	×	✓	Cole et al. (1998)
	Recreational drug users	25, 50 and 75 mg, po	Cocaine 150 mg, po	×	×	Not stated	Rush et al. (2002a)
GBR12909	Normal volunteers	50, 100, 150, 200 and 300 mg, po	N/A	×	×	✓	Søgaard et al. (1990)

N/A† – compound was the reference comparator.

cocaine to increase dopamine efflux when administered peripherally (Baumann et al., 1994) or by reverse-dialysis (Rothman et al., 1991). Together, these findings point to a number of conclusions. First, dopamine appears to mediate the stimulant effects of cocaine and methylphenidate, second, the pharmacological effect of cocaine and methylphenidate is not attenuated by DAT blockade, and finally, it indicates that the actions of these stimulants are not mediated by blockade of dopamine reuptake.

These human studies are underpinned by a wealth of data on methylphenidate obtained from *in vitro* and *in vivo* experiments, which have demonstrated that methylphenidate's neurochemical and behavioural profile (see later sections of this review), its discriminative properties (Wood and Emmett-Oglesby, 1988; Kleven et al., 1999; Schweri et al., 2002; Rush et al., 2002b), its reinforcing potential (Aigner and Balster, 1979; Schenk and Partridge, 1999; Wee and Woolverton, 2004; Burton et al., 2010; Heal et al., 2013a,b) and its liability for tolerance and dependence (Parran and Jasinski, 1991) are similar to those of cocaine. Schenk and Partridge (1999) have also reported that the potency and efficacy of cocaine and methylphenidate were comparable in reinstating cocaine self-administration in rats. In their review of the evidence, Kollins et al. (2001) concluded that in almost every

respect methylphenidate was indistinguishable from cocaine, although this view has been toned down in more recent publications (Volkow et al., 2002a,b; Volkow and Swanson, 2003; Swanson and Volkow, 2003).

The results obtained with the conventional, competitive, monoamine reuptake inhibitors are in complete contrast to those obtained with cocaine and methylphenidate. Drugs with a broad range of monoamine reuptake inhibition profiles have been tested and none has been found to evoke behavioural stimulation or euphoria in humans (Table 1). The list of compounds tested includes high and low potency, selective dopamine reuptake inhibitors, i.e. GBR12909 (vanoxerine) and bupropion, respectively, catecholamine reuptake inhibitors nomifensine and mazindol (Hyttel, 1982; Richelson and Pfenning, 1984; Andersen, 1989), and non-selective reuptake inhibitors, i.e. sibutramine (Cheetham et al., 1993, 1996; Heal et al., 1998b; Rowley et al., 2000; Balcioglu and Wurtman, 2000) and tesofensine (Lehr et al., 2008). Many of these clinical trials included one or more recreationally abused stimulants as positive controls in the protocol design (Table 1). In every case, the reference comparator stimulants produced the expected psychological stimulation and drug liking, which eliminated the possibility that the lack of effect of the reuptake inhibitors was an artefact of the trial. Not only did the classical monoamine reuptake inhibitors fail to produce "drug liking" in humans in these trials, but in several cases, eg sibutramine, mazindol and tesofensine, their subjective effects were often experienced as dysphoric and aversive (Table 1).

Another strand of evidence that points to the difference between the conventional monoamine reuptake inhibitors and cocaine is their singular failure to demonstrate efficacy as treatments for cocaine abuse and dependence. Methadone and Suboxone® (buprenorphine + naloxone) are established and reasonably successful μ-opioid receptor partial agonist therapies for treating morphine and heroin addiction. Bupropion comes closest to cocaine in terms of its potency as a dopamine reuptake inhibitor (Table 2), but it has not proven effective in the management cocaine abuse/dependence (see meta-analysis by Castells et al., 2007). In double-blind, placebo-controlled trials, bupropion did not reduce cocaine use in methadone-maintained, opiate-dependent, cocaine abusers (Margolin et al., 1995b; Poling et al., 2006), in methadone-maintained patients with ADHD, who were also cocaine abusers (Levin et al., 2006), or when combined with cognitive behavioural therapy (CBT) in heroin-maintained, subjects who were dependent on both opiates and cocaine (Dürsteler-MacFarland et al., 2013).

Table 2
In vitro profile of cocaine as a dopamine reuptake inhibitor compared with examples from various classes of DAT ligand.

Compound	[³H]Dopamine uptake inhibition (Ki = nM)	Cocaine site [³H]WIN 35,428 (Ki = nM)	Competitive inhibitor site [³H]GBR 12935 (Ki = nM)
Cocaine binding site ligands			
Cocaine	270[1] 310[5] 478[2] 404[a, 6] 204[3] 690[a, 7] 250[4] 370[a, 15]	173[a, 6] 89[a, 14] 189[10] 23[b, 16] 47[13] 240[c, 17]	351[3] 525[a, 7] 1,450[a, 15]
DL-*threo*-Methylphenidate	160[1] 281[a, 7] 110[4] 240[a, 15] 224[a, 6]	83[a, 6] 21[c, 17] 185[10] 17[a, 11]	158[3] 855[a, 15] 500[a, 7]
D-*threo*-Methylphenidate	270[a, 18] 1,300[a, 19]	– – 88[a, d, 20]	
WIN 35,065-2	8[3]	87[10] 23[a, 14]	46[3]
WIN 35,428	83[a, 6]	22[10] 3[b, 16] 10[12] 10[c, 17] 5[13]	ND
Competitive reuptake inhibitors			
GBR 12909	4[2] 1[a, 7] 0.3[3] 4[a, 15]	12[10] 4[c, 17] 3[13]	0.1[3] 5[a, 15] 4[a, 7]
GBR 12935	4[2]	13[10]	ND
Mazindol	26[2] 18[5] 80[3] 29[a, 7] 28[4] 37[a, 15]	16[10] 4[b, 16] 7[12] 24[c, 17]	0.8[3] 7[12] 93[a, 7] 72[a, 15]
Nomifensine	118[3] 134[a, 7] 88[4] 49[a, 15] 48[5]	43[10] 16[b, 16] 42[c, 17]	49[3] 269[a, 7] 250[a, 15]
Bupropion	630[1] 600[5] 831[3] 648[a, 7] 409[4]	373[10] 55[b, 16] 950[c, 17]	2,500[a, 7]
Competitive substrates			
Dopamine	66[1] 230[a, 7] 38[2]	3,000[b, 16] 3,100[c, 17]	2,300[a, 7]
S(+)-Amphetamine	82[1] 132[8] 34[2] 400[a, 7] 225[3] 78[9]	21,000[c, 17]	9,600[a, 7]
S(+)-Methamphetamine	114[2]	ND	ND

[1] Richelson and Pfenning (1984), [2] Rothman et al. (2001), [3] Kula and Baldessarini (1991), [4] Cheetham and Heal (data on file), [5] Hyttel (1982), [6] Deutsch et al. (1996), [7] Andersen (1989), [8] Heal et al. (1998b), [9] Rowley et al. (2000), [10] Katz et al. (2000), [11] Meltzer et al. (2003), [12] Xu and Reith (1997), [13] Chen et al. (1996), [14] Carroll et al. (2004a), [15] Berger et al. (1985), [16] Aloyo et al. (1995), [17] Pristupa et al. (1994), [18] Ferris et al. (1972), [19] Patrick et al. (1987), [20] Schweri et al. (1985).
ND – No data were found.
[a] IC₅₀ not Ki.
[b] Data from rabbit caudate membranes.
[c] Data from cloned human DAT expressed in COS-7 or CHO cells.
[d] Data obtained using [³H]methylphenidate as the radioligand.

Bupropion also did not reduce cocaine abuse or dependence when combined with CBT (Shoptaw et al., 2008) or when combined with the dopamine agonist, bromocriptine (Montoya et al., 2002). The pharmacological actions of cocaine are of course not limited to dopamine; however, an identical outcome was obtained when the catecholamine reuptake inhibitor, mazindol, was evaluated for the management of cocaine abuse. Thus, in two double-blind, placebo-controlled trials, mazindol treatment did not separate from placebo in terms of positive urine analyses or subjective ratings of abuse in cocaine-dependent subjects (Stine et al., 1995) and it did not reduce the rate of relapse in methadone-maintained, opiate-dependent, cocaine abusers (Margolin et al., 1995a). More recently, the potent selective reuptake inhibitor, GBR12909, entered clinical development as a treatment for cocaine dependence, but the trials were stopped for safety reasons leaving it as a matter of conjecture whether or not it would have proven to be effective.

2.2. Cocaine is a potent dopamine reuptake inhibitor

As a general "rule of thumb", the potency of monoamine reuptake inhibitors can be classified as according to their Ki values; thus, <1.0 ηM = highly potent; 1–10 ηM = potent; 10–50 ηM = moderately potent; 100–1000 ηM = weak and Ki > 1000 ηM = inactive. Furthermore, when assessing Ki values, data obtained from measurement of [³H]monoamine uptake into synaptosomes is of greater relevance than determinations of radioligand affinity for DAT, SERT and NET in physiologically relevant tissues because the former evaluates the efficacy of the compound to inhibit the dynamic process of reuptake.

The data in Table 2 show that cocaine has Ki (or IC$_{50}$) values for the inhibition of [³H]-dopamine uptake of 270–690 ηM, which according to the criteria above, places cocaine firmly into the category of weak dopamine inhibitors. The corresponding Ki values for inhibition of noradrenaline and 5-HT reuptake are 155–367 ηM and 180–389 ηM, respectively (Hyttel, 1982; Richelson and Pfenning, 1984; Andersen, 1989). Cocaine is, therefore, a weak, non-selective inhibitor of the monoamine reuptake inhibitor with a potency rank order of noradrenaline = 5-HT > dopamine.

Methylphenidate's potency as a dopamine reuptake inhibitor is similar to that of cocaine (Table 2) and methylphenidate is also a weak inhibitor of noradrenaline reuptake (Richelson and Pfenning, 1984; Andersen, 1989); however, methylphenidate differs from cocaine by virtue of having no pharmacologically relevant effect on 5-HT reuptake (Richelson and Pfenning, 1984; Andersen, 1989). The D-enantiomer of methylphenidate is approximately 10-fold more potent as a catecholamine reuptake inhibitor than the L-enantiomer, and therefore, contributes the 90% of the pharmacological effect of racemic methylphenidate (Heal and Pierce, 2006). The results for D-*threo*-methylphenidate provided in Table 2 are consistent with the potency difference between the D- and L-enantiomers.

The tropane analogues of cocaine, WIN 35,428 [2β-carbomethoxy-3β-(4-fluorophenyl)tropane] and WIN 35,065 [troparil; (−)-2β-carbomethoxy-3β-phenyltropane], are more potent dopamine reuptake inhibitors than cocaine itself (Table 2). Numerous tropane molecules and analogues of methylphenidate have been synthesised, characterised as DAT inhibitors, and had their reinforcing and interoceptive cues shown to be similar to cocaine (Katz et al., 1999; Carroll et al., 2004a). Many of these new compounds have much higher potency as DAT inhibitors than cocaine or methylphenidate (Katz et al., 1999; Carroll et al., 2004a).

In contrast, GBR 12909 and GBR 12935 are classified as potent or highly potent dopamine uptake inhibitors. With an IC$_{50}$ value of 6.5 ηM, tesofensine also potently inhibits [³H]dopamine uptake into synaptosomes (Lehr et al., 2008). In the next tier of potency

as dopamine reuptake inhibitors comes mazindol, nomifensine (Table 2) and sibutramine's secondary and primary amine metabolites (Heal et al., 1998a,b). Bupropion is the only DAT inhibitor to have been extensively used in humans that has potency which approximately equivalent to cocaine and methylphenidate (Table 2).

In summary, therefore, the experimental data show that although cocaine and methylphenidate are powerful psychostimulants, in absolute terms, they are weak dopamine uptake inhibitors. These stimulants are also much less potent DAT inhibitors than many other compounds that have been shown to be non-stimulant in humans.

2.3. Cocaine's stimulant properties can be explained by rapid brain penetration

In the search for greater highs and the maximum effect from each gram of drug taken, abusers frequently employ self-administration routes that deliver the maximum amount of drug into the brain in the shortest possible time. Non-clinical routes used for methylphenidate include snorting and intravenous injection. Cocaine is abused by snorting, intravenous injection and by smoking as "crack". In the case of snorting and smoking, the drug is not only delivered to the CNS as a rapid bolus, but it also evades first-pass, hepatic metabolism. This link between the speed of CNS penetration and the abuse/addiction potential of substances of abuse has been coalesced into the "rate hypothesis", which states that the strength of positive psychological effects (i.e. abuse liability) is proportional to the rate of drug binding to its site of action (Balster and Schuster, 1973; Gorelick, 1998). Consistent with this hypothesis, delayed delivery of stimulants as in the cases of osmotically-released methylphenidate or the D-amphetamine prodrug, lisdexamfetamine, substantially reduces the stimulant, euphoriant experience they produce compared with immediate-release methylphenidate (Kollins et al., 1998) or D-amphetamine (Jasinski and Krishnan, 2009a,b). In our experience, the leftward shift in potency that is observed with cocaine and methylphenidate is more pronounced than for any class of substance of abuse. Heal et al. (2013a) reported that a 10 mg/kg oral dose of methylphenidate was required to generalise to D-amphetamine in a rat drug-discrimination study with a ~3-fold increase in potency when methylphenidate was given by intraperitoneal injection. Compare this with methylphenidate's intravenous reinforcing dose of 0.1 mg/kg. In the same drug-discrimination model, cocaine generalised to the D-amphetamine cue at a dose of 10 mg/kg ip (Gosden et al., 1996), but maintained self-administration in rats at a dose of 0.32 mg/kg iv (Heal et al., 2013a,b). Thus, for both stimulants there is an ~30-fold increase in stimulant potency when switching from the intraperitoneal to the intravenous route, and in the case of methylphenidate it is 100-fold more potent when administered intravenously than orally.

In summary, the animal and human data unequivocally demonstrate that rapid CNS entry markedly potentiates the stimulant effects of methylphenidate and cocaine. However, the results in Table 1 show that these drugs also produce stimulation and euphoria when taken orally. What the findings do not imply is that conventional, competitive DAT inhibitors will mimic the actions of cocaine and methylphenidate if they rapidly cross the blood–brain barrier.

Tsukada et al. (2000) compared the effects of intravenously administered cocaine or GBR12909 on DAT occupancy and dopamine synthesis measured by PET, and striatal dopamine efflux by *in vivo* microdialysis in a separate cohort of monkeys. Despite cocaine having 100-fold lower DAT affinity than GBR12909, both compounds produced similar levels of DAT occupancy with the maximum degree of DAT blockade at the first post-dose time-point.

Their effects on dopamine efflux, however, looked very different. Cocaine (5 mg/kg, iv) evoked a sharp 630% increase in dopamine efflux with a peak at 15 min, whereas the same dose of GBR12909 produced a maximum 480% increase that did not reach a peak until 45 min after intravenous dosing. Thus, increasing the rate of entry of GBR12909 into the brain did not profoundly alter the pharmacodynamics of its actions on dopamine efflux *in vivo*.

Reverse-dialysis overcomes the problems of absorption, metabolism and the rate and extent of brain penetration. Since access to the site of action in the brain is virtually instantaneous, it provides an opportunity to compare the effects of cocaine and methylphenidate versus competitive DAT inhibitors, eg GBR12909, and competitive DAT substrates, eg D-amphetamine. Nomikos et al. (1990) studied the effects of reverse-dialysis of various drugs on dopamine efflux in the striata of freely-moving rats. GBR12909 produced gradual concentration-dependent increases in dopamine efflux. Its maximum effect occurred 30—45 min after the start of reverse-dialysis and as its concentration was increased, a ceiling to its effect at concentrations ≥100 nM was clearly evident (Fig. 1). In contrast methylphenidate and D-amphetamine produced rapid increases in extracellular dopamine and the size and speed of onset of the maximum effect became larger as their concentrations were increased (Fig. 1). Since these compounds were all applied directly onto dopaminergic nerve terminals, these results demolish the argument that cocaine-like drugs produce rapid increases in dopamine efflux simply as a result of rapid brain penetration. This is clear because instantaneous application of GBR 12909 did not cause it to mimic cocaine's or methylphenidate's effect dopamine efflux; on the contrary, it produced no change or augmentation of its pharmacological effect.

2.4. Cocaine's neurochemical effects are consistent with those of classical reuptake inhibitors

As discussed in Section 2.1, evidence from experiments performed in humans has revealed that drugs that are proposed to increase synaptic dopamine concentrations by acting as DAT uptake inhibitors fall into two distinct categories. Those like cocaine, methylphenidate and related compounds, which are stimulant, euphoriant and subject to recreational abuse, and those like bupropion, sibutramine and tesofensine which are not (Table 1). Importantly, because these evaluations of their CNS effects were conducted in placebo-controlled, blinded trials, the possible contribution of conditioning cues to the subjective experience was eliminated. Thus, the subjective experience evoked by these drugs was dictated by their pharmacology and pharmacokinetics.

Results presented in Section 2.2 demonstrate that *in vitro* cocaine, methylphenidate and related compounds have low affinity for the competitive reuptake inhibitor site on DAT (labelled with [³H]GBR12935), the cocaine binding site on DAT (labelled with [³H]WIN 35,428) and are weak inhibitors of [³H]dopamine uptake, contradicting the idea that the psychostimulant and euphoriant actions of these compounds are the result of powerful inhibition of dopamine reuptake *in vivo*.

In this section, we will review the results from a range of *in vitro* and *in vivo* experiments which support the hypothesis that cocaine-like drugs are not only different from both the competitive DAT reuptake inhibitors and the DAT substrate releasing agents, but also are part of a novel class of compounds that we believe serve as DAT "inverse agonists".

Consistent with cocaine-like compounds having a unique pharmacology, we observed that high concentrations of cocaine and methylphenidate increased the overflow of [³H]dopamine from preloaded rat striatal slices (Heal et al., 1996; Table 3). This releasing action was not observed with most competitive reuptake inhibitors, and is different from that observed with the competitive DAT substrates, which evoke [³H]dopamine release at very low concentrations (Heal et al., 1996; Table 3). As shown in Table 4, this dopamine releasing effect of cocaine and methylphenidate has been reported by others, but only in experiments employing tissue slice preparations; the effect is generally not observed when synaptosomes or DAT transfected cell-lines are used. DAT substrate releasing agents show no such tissue specificity in their releasing actions (Table 4). Together, these results suggest that cocaine and some related compounds have a dopamine releasing action that occurs only at high concentrations and this effect is dependent on aspects of intact neuroanatomical architecture and/or intracellular signalling processes.

Moving to whole animal systems, we used *ex vivo* measurements of the changes in the concentration of dopamine and its major metabolites (see Fig. 3) to identify the patterns ("neurochemical fingerprints") produced by the dopaminergic drugs with different presynaptic mechanisms. Many years ago, we demonstrated that increases in the concentration of 3-methyoxytyramine (3-MT) measured *ex vivo* were an excellent marker of dopamine release evoked by the competitive DAT substrate releasing agents, eg D-amphetamine and methamphetamine (Heal et al., 1990). In mice, the neurochemical fingerprint of the DAT substrate releasing agent, D-amphetamine, in the striatum comprised an increase in the concentration of 3-MT, with accompanying decreases of DOPAC and HVA (Heal et al., 2009b; Cheetham et al., 2010). This mirrors effects occurring *in vivo* because an identical pattern of changes
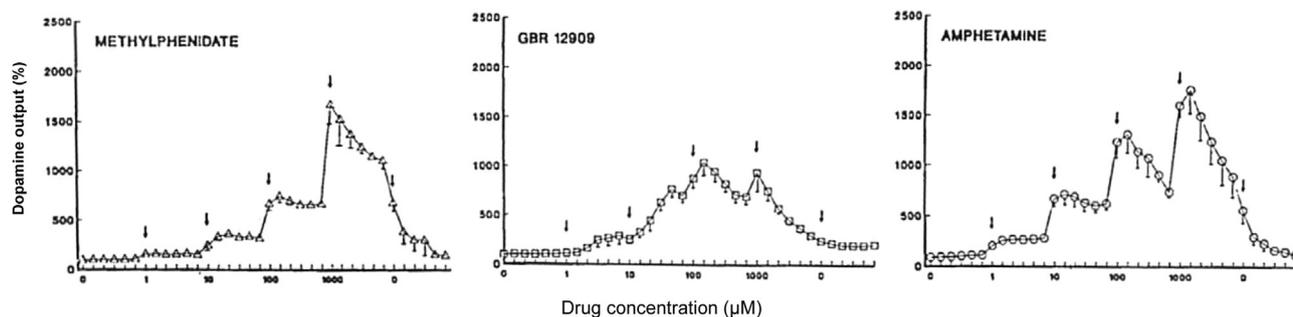
Fig. 1. Reverse-dialysis experiments demonstrating that the stimulant profile of methylphenidate cannot be explained by rate of access to the brain. The effects of reverse dialysis of increasing concentrations of the dopamine reuptake inhibitor, GBR 12909, or the DAT "inverse agonist", methylphenidate, on extracellular concentration of dopamine in striata of conscious rats. Arrow indicates the first sample taken after commencing reverse-dialysis at each concentration tested. Methylphenidate induced rapid, large increases in extracellular dopamine with no dose-effect ceiling to its action. The greater the concentration of methylphenidate, the faster the time to reach peak dopamine efflux. In contrast, reverse dialysis of GBR 12909 produced a gradual increase in extracellular dopamine. Increasing the concentration of GBR 12909 did not accelerate the onset of peak dopamine efflux and there is a clear dose-effect ceiling. Figures reproduced from Nomikos et al. (1990) with permission.

Table 3
Effects of various DAT ligands on [³H]dopamine release from rat striatal slices.

Compound	Pharmacological classification	Percentage increase in [³H]dopamine release	
		10^{-7} M	10^{-5} M
Sibutramine	Competitive reuptake inhibitor	NS	NS
BTS 54 354	Competitive reuptake inhibitor	NS	NS
BTS 54 505	Competitive reuptake inhibitor	NS	NS
Bupropion	Competitive reuptake inhibitor	NS	NS
Nomifensine	Competitive reuptake inhibitor	NS	$20 \pm 6^*$
D-Amphetamine	Competitive substrate releasing agent	$56 \pm 9^{**}$	$138 \pm 15^{***}$
Methamphetamine	Competitive substrate releasing agent	$37 \pm 10^*$	$140 \pm 10^{***}$
Cocaine	DAT "inverse agonist"	NS	$54 \pm 19^*$
Methylphenidate	DAT "inverse agonist"	NS	$29 \pm 7^{***}$

Mean $\pm$ SEM ($n \geq 4$) $^*P < 0.056$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Williams test).
NS = Not significantly different from basal [³H]dopamine overflow.
Data taken from Heal et al. (1996).

was observed after D-amphetamine administration in microdialysis experiments in freely-moving mice (Heal et al., 2009b).

Competitive DAT inhibitors, eg GBR 12909, produce falls in DOPAC, but no change in the concentrations of 3-MT or HVA (Heal et al., 2009b). The fall in DOPAC has long been accepted to reflect a homeostatic reduction in the synthesis and release of dopamine in response to elevated synaptic concentrations following DAT blockade (Cheng and Wooten, 1982; Hallman and Jonsson, 1984; Heal et al., 2009a,b). Administration of methylphenidate to mice produced a small decrease in the concentration of DOPAC, no change in 3-MT, and a dose-dependent increase in HVA (Heal et al., 2009b; Cheetham et al., 2010). These results suggest that rather than reducing the firing-rate of dopaminergic neurones and decreasing the exocytotic release of dopamine, cf the actions of the competitive DAT inhibitors, methylphenidate produces the opposite effect, i.e. it increases the release and metabolism of dopamine. The hypothesis that D-amphetamine and methylphenidate both increased the rate of dopamine release, but by different mechanisms, was supported by the finding that the degree of stereotypy was linearly correlated with the striatal concentration of 3-MT

(Cheetham et al., 2010) for D-amphetamine and HVA for methylphenidate (Fig. 3). Together, the results from these *in vitro* and *ex vivo* experiments indicate that cocaine-like drugs can be differentiated not only from the DAT substrate releasing agents, which is well accepted, but also from the competitive DAT reuptake blockers.

In vivo microdialysis experiments can be used to differentiate between monoamine transporter substrate releasing agents and competitive reuptake inhibitors. Results from investigations conducted by Gundlah et al. (1997) and Tao et al. (2002) identified criteria to define the pharmacological characteristics of both types of compound (Table 5). Although these criteria were developed by Gundlah et al. (1997) and Tao et al. (2002) for competitive SERT reuptake inhibitors and substrates, they apply equally to the catecholamine neurotransmitter reuptake transporter ligands (Wortley et al., 1999; Géranton et al., 2003; Heal et al., 2009a, 2012).

The results shown in Fig. 4 compare the effects of various doses of methylphenidate on dopamine efflux versus D-amphetamine and sibutramine. Sibutramine and D-amphetamine respectively show the pharmacodynamic profiles of a competitive reuptake inhibitor and transporter substrate releasing agents, respectively. The pharmacodynamics of D-*threo*-methylphenidate do not resemble those of the reuptake inhibitor, sibutramine, but show a striking similarity to those of the dopamine releaser, D-amphetamine. Thus, there is a rapid onset of peak effect (30 min post-dose in both cases), a rapid decline in dopamine overflow after its zenith, a large effect size (>1000% of baseline efflux) and the lack of dose-effect ceiling. On the contrary, for both stimulants the increments in peak dopamine efflux became larger as the doses of the stimulants were increased. Thus, in almost every regard the actions of methylphenidate differ from those of the competitive reuptake inhibitors, and moreover, better fit the criteria for the releasing agents. There is good indirect evidence to indicate that the actions of methylphenidate and cocaine are mediated through binding to monoamine reuptake transporters because methylphenidate, which has affinity for DAT and NET, but not SERT (Richelson and Pfenning, 1984; Andersen, 1989) increases the efflux of dopamine and noradrenaline, but not 5-HT, in the CNS, (Kuczenski and Segal, 1997; Rowley et al., 2014). In contrast, cocaine which binds to DAT, NET and SERT (Richelson and Pfenning, 1984; Hyttel, 1982;

Table 4
Contrasting effects of cocaine binding site ligands versus DAT transporter substrates on monoamine release from various *in vitro* preparations.

Drug	Reference	Superfused tissue slices	Dopamine	Noradrenaline	5-HT
Cocaine binding site ligands					
DL-*threo*-Methylphenidate	1	Yes	✓	–	–
	2	Yes	✓	–	–
	3	Yes	✓	–	–
	4	Yes	✓	✓	–
	5	No	✓/✗	✓/✗	✓/✗
	6	No	✗	✗	–
	7	No	✗	–	–
D-*threo*-Methylphenidate	6	No	✗	✗	–
	8	No	✗	✗	–
L-*threo*-Methylphenidate	6	No	✗	✗	–
Cocaine	2	Yes	✓	–	–
	7	No	✗	–	–
WIN 35,428	7	No	✗	–	–
DAT transporter substrates					
D-Amphetamine	9	Yes	✓	✓	✓
	5	No	✓	✓	✓
	7	No	✓	–	–
Methamphetamine	10	Yes	✓	–	–
	7	No	✓	–	–

✓ = Causes release; ✗ = Inactive; ✓/✗ = Equivocal result; – = Not tested.
[1] Vickroy and Johnson (1982), [2] Heal et al. (1996), [3] Russell et al. (1998), [4] Easton et al. (2007), [5] Wall et al. (1995), [6] Patrick et al. (1987), [7] Eshleman et al. (1994), [8] Ferris et al. (1972), [9] Heal et al. (1998a), [10] Heal et a.l (1992).
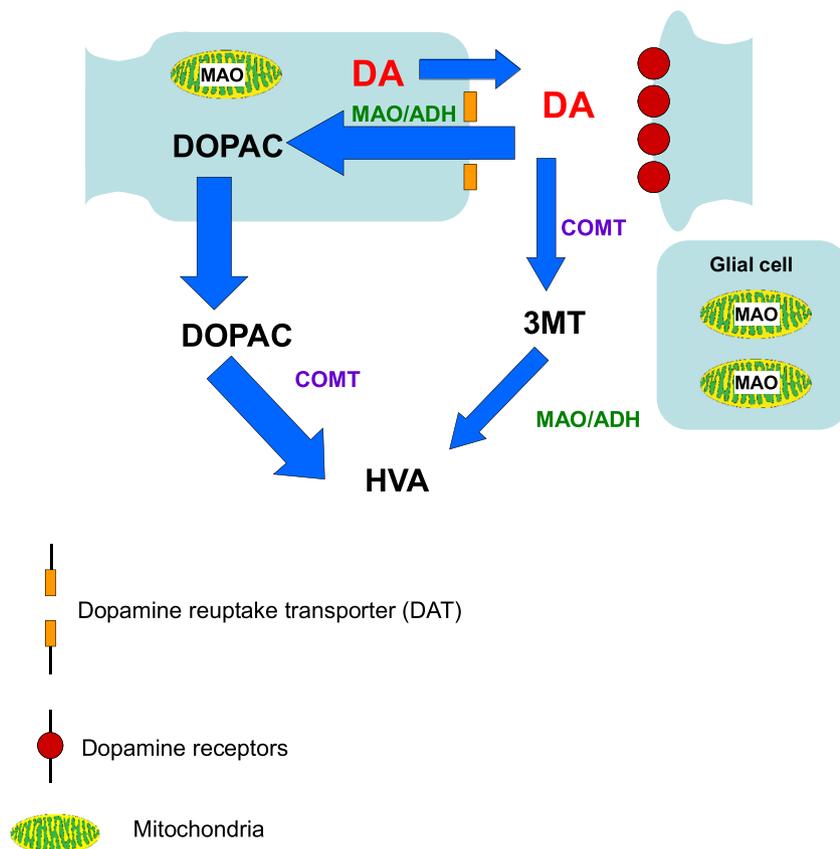
Fig. 2. Regulation of nigrostriatal or mesolimbic dopaminergic neurotransmission. The major routes for the regulation of dopaminergic signalling in the nigrostriatal and meso-limbic dopaminergic neurones are illustrated above. Most of the dopamine that is neuronally released in the striatum or nucleus accumbens is taken back up into dopaminergic neurones via DAT. Dopamine is metabolised intraneuronally to DOPAC by MAO/ADH. DOPAC passes across the cell membrane where it is converted to HVA by the extraneuronal enzyme, COMT. A minor extraneuronal pathway is the conversion of dopamine to 3-MT by COMT and then to HVA by MAO/ADH. HVA is the common final end product of both metabolic pathways. The "neurochemical fingerprints" of the different classes of compound with presynaptic dopaminergic mechanisms are determined by their effects on do-pamine's metabolite concentrations. DA = dopamine; DOPAC = dihydroxyphenylacetic acid; 3MT = 3-methoxytyramine; HVA = homovanillic acid; MAO/ADH = monoamine oxidase/aldehyde dehydrogenase; COMT = catechol-O-methyltransferase.
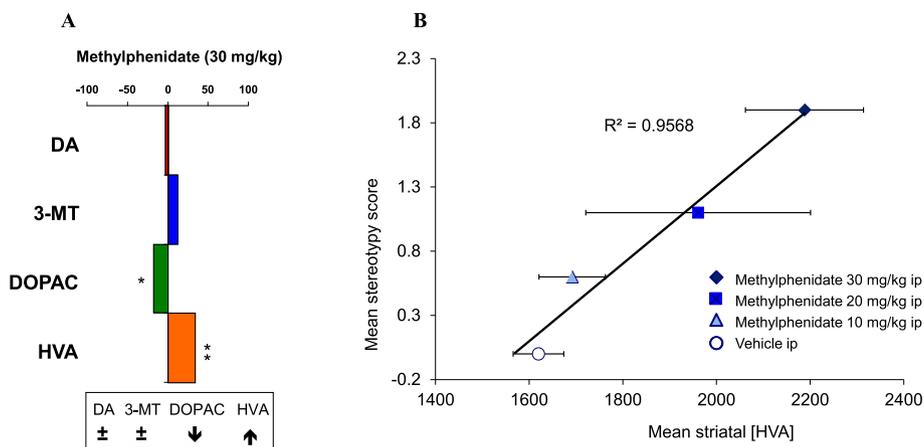
Fig. 3. Effect of methylphenidate on (A) the striatal concentrations of dopamine and its metabolites measured *ex vivo* in mice and (B) correlation between stereotypy scores and striatal homovanillic acid (HVA) concentrations. The effects of methylphenidate on (A) *ex vivo* concentrations of dopamine and its metabolites ("Neurochemical fingerprint") in mouse striatum and (B) the correlation between HVA concentrations and stereotypy scores in mice. C57/Bl/6 mice were injected with methylphenidate (10, 20 or 30 mg/kg, ip) and 60 min later their stereotypy was scored (0–2) in a blinded fashion. Mice were then immediately killed and the striatal concentrations of dopamine, DOPAC, and HVA were measured as described by Cheetham et al. (1996) and 3-MT by Heal et al. (1990). Increased release and utilisation of dopamine is demonstrated by the high degree of correlation between the increases in stereotypy and HVA concentrations. Results taken from Cheetham et al. (2010). DA = dopamine; DOPAC = dihydroxyphenylacetic acid; 3-MT = 3-methoxytyramine.

Table 5
Differences in the pharmacological characteristics of monoamine reuptake transporter competitive inhibitors and competitive substrate releasing agents.

Competitive reuptake inhibitors	Competitive substrate releasing agents
Gradual increase in extracellular monoamine concentration	Rapid increase to peak efflux
Long duration of effect	Rapid decline after peak efflux
Moderate effect size (few hundred percent of baseline)	Large effect size (several thousand percent of baseline)
Dose-effect ceiling	No dose-effect ceiling
Drug effect subject to physiological auto-inhibitory modulation	Drug effect not subject to physiological auto-inhibitory modulation
Not applicable	Drug action prevented by monoamine reuptake transporter blockade

Andersen, 1989) enhances the overflow of all three monoamines *in vivo* (Bradberry et al., 1993; Chen and Reith, 1994; Reith et al., 1997; Pum et al., 2007).

One of the important, recent, advances in high performance liquid chromatography with electrochemical detection (hplc-ecd) has been the ability to measure multiple monoamines and metabolites in a single microdialysate sample. A comparison of the effects on the extracellular concentrations of dopamine, DOPAC and HVA produced by methylphenidate and the competitive DAT inhibitor, nomifensine, is shown in Fig. 5. The mechanisms responsible for the exocytosis, reuptake and catabolism of dopamine are illustrated in Fig. 2 which shows that the major route of catabolism of dopamine is reuptake followed by intraneuronal catabolism to DOPAC. Thus, the observation that the increase in extracellular dopamine produced by nomifensine is accompanied by a concomitant sharp fall in extracellular DOPAC indicates that the blockade of DAT sites is preventing the reuptake of dopamine and metabolism by MAO in presynaptic dopaminergic nerve terminals, and simultaneously reducing the rate of dopamine release. The impact of this action is also reflected by a transient decrease in extracellular HVA. This finding is supported by identical results that we have obtained using sibutramine to inhibit dopamine reuptake (data not shown). It is also consistent with previously reported findings using other competitive DAT inhibitors, i.e. bupropion, nomifensine and GBR 12909 (Nomikos et al., 1989,

1990; Kaakkola and Wurtman, 1992; Adachi et al., 2001). In contrast, methylphenidate produced a larger increase of dopamine efflux, but there was no concomitant fall in the extracellular concentration of DOPAC, and extracellular HVA was substantially increased. For the sake of clarity, we have only shown the effect of a single dose of methylphenidate, but Rowley et al. (2014) reported an identical pattern of effects when methylphenidate was tested at lower doses of 3 and 10 mg/kg, indicating that not only was this a robust phenomenon, but also one that appeared across a wide range of pharmacological doses. In Fig. 5, it is evident that the baseline concentration of DOPAC and HVA was decreasing over time. In the publication by Rowley et al. (2014), the D-amphetamine prodrug, lisdexamfetamine, and the enigmatic stimulant, modafinil, were also studied over a pharmacologically equivalent dose range. Both drugs increase dopamine efflux, but lisdexamfetamine decreased extracellular HVA, and at the highest dose, also DOPAC, whereas modafinil had no effect on either metabolite (Rowley et al., 2014). Since methylphenidate, modafinil and lisdexamfetamine consistently produced increases, no change and decreases in HVA, we can be confident that the effect of methylphenidate shown in Fig. 5 was not an artefact created by the falling HVA baseline. Returning to Fig. 2, the absence of a fall in extracellular DOPAC after methylphenidate administration indicates that the drug is not inhibiting the reuptake and intraneuronal metabolism of dopamine. Furthermore, as HVA is the final common product of dopamine catabolism, the increase in this metabolite reveals that the rate of dopamine release and metabolism (turnover) is not being reduced, cf DAT inhibitors, but increased. Segal and Kuczenski (1999) reported that dopamine efflux produced by high dose methylphenidate was similarly accompanied by increases in extracellular HVA in nucleus accumbens and striatum. A dose-dependent enhancing effect of methylphenidate on dopamine efflux is also shown by the *ex vivo* results shown in Fig. 3.

One key difference between the competitive DAT substrate releasing agents and the cocaine-like stimulants is the latter require sodium channel-dependent neuronal firing for their actions. Local application of tetrodotoxin (TTX) did not impair the releasing effect of D-amphetamine (Westerink et al., 1987, 1989; Nomikos et al., 1990; Benwell et al., 1993), but it abolished the increases evoked by cocaine or methylphenidate (Nomikos et al., 1990; Butcher et al., 1991; Benwell et al., 1993). The susceptibility of the actions of cocaine and methylphenidate to TTX blockade applies also to the effects of the competitive DAT inhibitors on dopamine efflux (Westerink et al., 1987, 1989; Nomikos et al., 1990; Butcher et al., 1991).
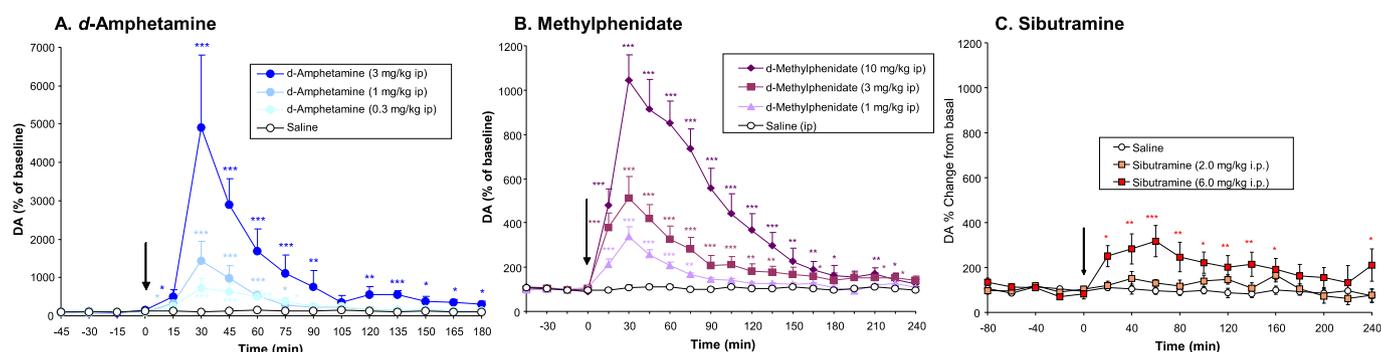
Fig. 4. Pharmacodynamic effects of D-amphetamine and methylphenidate on dopamine efflux in the striatum and sibutramine on dopamine efflux in the nucleus accumbens of freely-moving rats. Similarity between the effects of the DAT releasing agent, D-amphetamine, and the DAT "inverse agonist", D-*threo*-methylphenidate, on striatal dopamine efflux measured in freely-moving rats. Both stimulants rapidly induce large increases in extracellular dopamine that reach a maximum in <1.0 h. There is no dose-effect ceiling. The efflux curves show the pattern of a rapid increase followed by a relatively sharp decline in extracellular dopamine that is characteristic of stimulant drugs of abuse. Significantly different from vehicle control $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. D-Amphetamine data (A) from Heal et al. (2009a). Methylphenidate data (B) from Heal et al. (2008). Sibutramine data taken from Rowley et al. (2000).
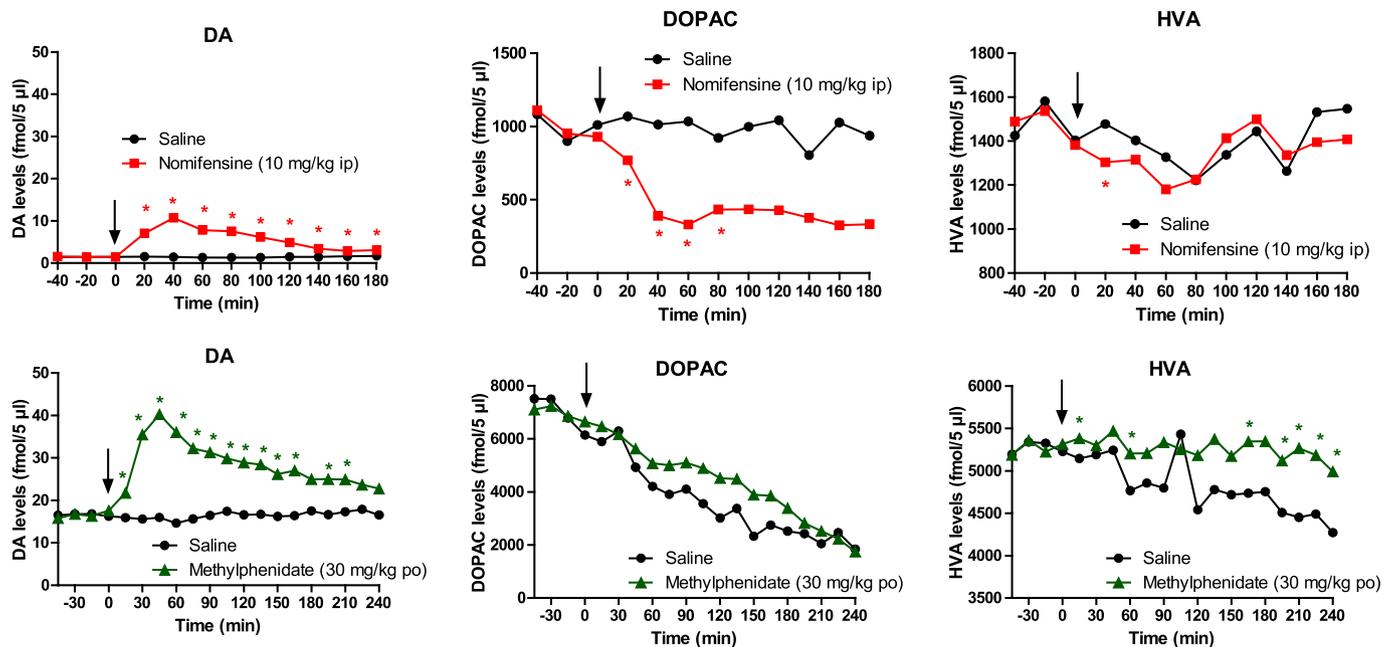
Fig. 5. A comparison of the effects of methylphenidate and nomifensine on the striatal efflux of dopamine and its major metabolites. The dopamine reuptake inhibitor, nomifensine (10 mg/kg, ip), increased striatal dopamine efflux with a concomitant decrease in the extracellular concentration of DOPAC and a transient decrease in HVA. Experiments performed in freely-moving mice ($n = 6$/group) (Cheetham and Heal, data on file). The DAT "inverse agonist", methylphenidate (30 mg/kg, po), increased striatal dopamine efflux, but in contrast to nomifensine, it did not decrease extracellular DOPAC, and also produced a robust increase in HVA. Experiments performed in freely-moving rats ($n = 6$/group). Significantly different from vehicle control *$p < 0.05$. Results from Rowley et al (2014). DA = dopamine; DOPAC = dihydroxyphenylacetic acid; HVA = homovanillic acid.

What occurs to the firing-rate of dopaminergic neurones and efflux of dopamine after administration of cocaine and methylphenidate is a complex subject. We could find no published studies on electrophysiology combined with microdialysis in conscious, freely-moving rats, although there two reports from experiments in anaesthetised rats (Lodge and Grace, 2005; Panin et al., 2012). Panin et al. (2012) reported that cocaine produced a gradual dose-dependent decline in the rates of dopaminergic neuronal firing and burst firing; however this effect was much less profound than the abolition of firing that was produced by the D2 receptor agonist, quinpirole. Microdialysis was only performed using highest dose of cocaine which induced a large increase in dopamine efflux in the nucleus accumbens with a minor decrease in DOPAC (<20%) indicating very little evidence of DAT inhibition. Lodge and Grace (2005) tested a single, 10 mg/kg dose of cocaine and observed a moderate (30%) decrease in dopaminergic firing in one experiment and no significant reduction in the second, a ~50% reduction in burst-firing, and a rather slow and moderate increase of dopamine efflux in the nucleus accumbens. Thus, both studies reveal that after cocaine administration dopaminergic firing is maintained at a reasonable level that is consistent with this stimulant's action on dopamine efflux being dependent on intact dopaminergic neuronal firing (Nomikos et al., 1990; Butcher et al., 1991; Benwell et al., 1993). Shi et al. (2000, 2004) demonstrated that the stimulants D-amphetamine, cocaine and methylphenidate have an excitatory as well as an inhibitory effect on dopaminergic neuronal firing; the former being mediated by increased noradrenaline efflux activating α1-adrenceptors. This may explain why a functional level of dopaminergic neuronal firing is maintained after cocaine and methylphenidate administration. Although these data are supportive, any correlation between dopaminergic neuronal firing-rates and dopamine efflux they should be regarded with a considerable degree of caution because anaesthesia, irrespective of whether it is gaseous or chloral hydrate, has a profoundly confounding effect on microdialysis measurements of dopamine efflux

and on the actions of reuptake inhibitors and releasing agents (Hamilton et al., 1992; el-Maghrabi and Eckenhoff, 1993; Fink-Jensen et al., 1994; Adachi et al., 2001).

It is also important to note that although the firing-rate of dopaminergic neurones is an important component in determining the magnitude of the extracellular concentrations of dopamine and its metabolites, many other factors contribute to these outcomes, eg terminal autoreceptor modulation of exocytotic release, rate and efficiency of reuptake and synthesis of dopamine and noradrenaline by the rate-limiting enzyme, tyrosine hydroxylase. Microdialysis measures the composite effect of all of these contributory factors.

Blockade of DAT prevents the effects of transporter substrate releasing drugs, eg D-amphetamine or methamphetamine, on dopamine efflux (Butcher et al., 1988; Baumann et al., 1994, 2002), demonstrating that entry into the presynaptic nerve terminal is a prerequisite for their dopamine releasing action. This is another key difference between the DAT substrate releasing agents and cocaine or methylphenidate, which are physically too large to serve as DAT substrates. In contrast, attempts at blocking cocaine's effect on dopamine efflux using the high affinity competitive DAT inhibitor, GBR 12909, produced some minor reduction, but no abolition, of cocaine's effect (Rothman et al., 1991; Baumann et al., 1994), despite the 200-fold greater affinity of the former (see Table 2) and an abundance of GBR 12909 in the cytosol (shown by shifts in the Kd of [3H]GBR 12935 binding) (Rothman et al., 1989, 1991). Baumann et al. (1994), who used the intravenous route for both compounds, reported similar modest reductions in cocaine's ability to increase dopamine overflow in the nucleus accumbens. The inability of competitive DAT inhibitors to block increased dopamine efflux evoked by cocaine is very reminiscent of the failure of extensive DAT blockade to prevent a second injection of methylphenidate from producing "highs" in human subjects reported by Volkow et al. (1996).

Wu et al. (2001) combined paced electrical stimulation of the forebrain bundle with voltammetry to compare the effects of

cocaine and the non-competitive DAT inhibitor, RTI-76, on dopamine overflow in the nucleus accumbens *in vivo*. Some of the data are reproduced in Fig. 6, which shows that RTI-76 augments the electrically-induced increase of extracellular dopamine. RTI-76 does not alter the time of the peak effect but it delays the clearance of transmitter from the cytosol (reuptake inhibition). On the other hand, cocaine increases the rate of electrically-evoked dopamine release, noticeably advancing the onset of the peak of 60 Hz (stimulated release), but does not alter the rate of dopamine clearance from the cytosol (no reuptake inhibition). The results, therefore, provide evidence for impulse-dependent dopamine release by cocaine, but none for delayed clearance of the monoamine as a result of DAT blockade.

When viewed overall, the findings from a wide range of experimental techniques do not question the hypothesis that cocaine binds to DAT and can under certain conditions serve as a DAT inhibitor, nor do they contradict the view that dopamine is an important mediator of the psychostimulant and euphoriant actions of cocaine, methylphenidate and related compounds. The scientific observations provide strong evidence to support the view that cocaine-like compounds do not function like competitive DAT reuptake inhibitors *in vivo* and this conclusion is merely consistent with the results from numerous trials in human volunteers (Table 1). The evidence from the preclinical studies reveals that cocaine and cocaine-like compounds share more pharmacological characteristics with the competitive DAT transporter substrate releasing agents than the competitive DAT inhibitors, but the cocaine-like compounds are different from both of these classes of DAT ligand. These similarities and differences are summarised in Table 4. On the basis of the scientific evidence provided, we postulate that cocaine, methylphenidate and related compounds produce the majority of their pharmacological effects through firing-dependent release of dopamine and other monoamines, rather than via inhibition of their reuptake.

3. Evidence to support the DAT inverse agonist hypothesis

3.1. Structure and function of the dopamine reuptake transporter

In this section, we have focused on those aspects of DAT structure and function which are especially relevant to the transport of dopamine and to the different mechanisms of action pertaining to cocaine and related cocaine binding site ligands, competitive DAT inhibitors and competitive DAT substrate releasing agents. For a more comprehensive overview of the structure and function of DAT, readers are directed to the excellent reviews of Eriksen et al. (2010a) and Manepalli et al. (2012).

DAT is a member of the neurotransmitter/sodium symporter family that includes the transporters for noradrenaline (NET), 5-HT (SERT) and also glycine and γ-aminobutyric acid (GABA). The importance of DAT function in regulating dopaminergic neurotransmission and stimulant drug action was amply demonstrated by the DAT knock-out mouse which displayed hyperlocomotion, an inability to maintain intra-neuronal stores of dopamine, and resistance to the pharmacological effects of D-amphetamine and cocaine (Giros et al., 1996).

DAT is a single gene product comprising 619 amino acids with an approximate mass of 80 kDa (Giros et al., 1992). Hydrophobicity analysis of DAT predicts 12 transmembrane (TM) regions, each consisting of about 25 hydrophobic amino acids. There is a large extracellular loop between TM3 and TM4 with potential sites for N-glycosylation, and on the intracellular side of the transporter,
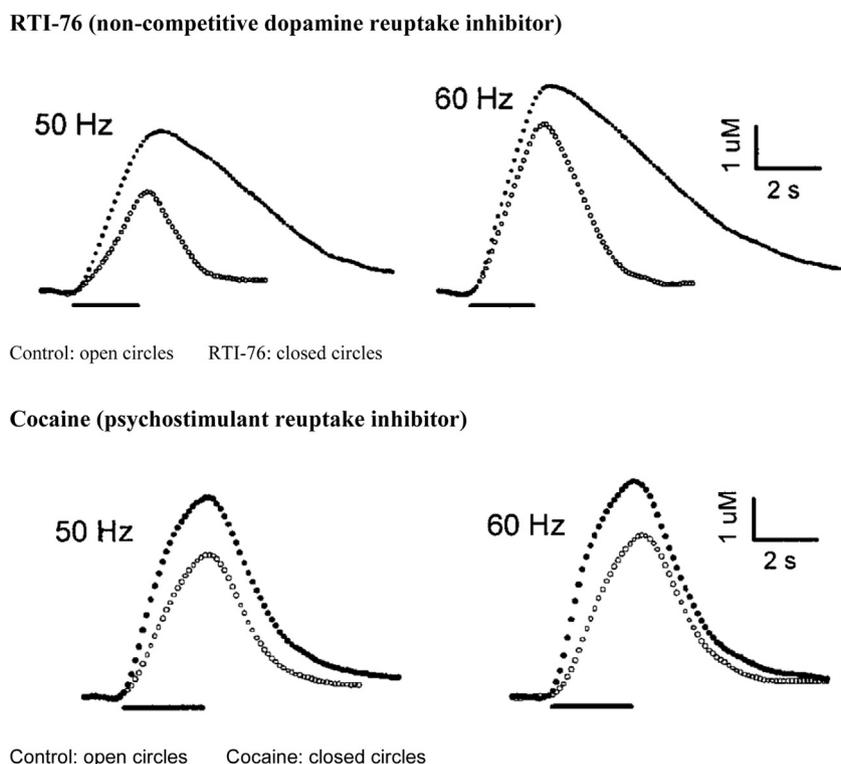
Fig. 6. Frequency-stimulated release, but lack of reuptake inhibition of dopamine in the nucleus accumbens by cocaine demonstrated by *in vivo* voltammetry in anaesthetised rats. Results obtained from anaesthetised rats with stimulating electrodes placed in the ipsilateral forebrain bundle (constant-current, biphasic, square-wave, 2 s, 300–400 μA pulses of 10–60 Hz). Extracellular dopamine in the nucleus accumbens was measured by fast cyclic voltammetry. Each point represents a 100 msec interval. The non-competitive DAT inhibitor, RTI-76 (100 ηMol, icv) substantially delayed the clearance of dopamine after electrical stimulation. In contrast, cocaine (40 mg/kg, ip) increased electrically stimulated release of dopamine, but did not delay its clearance from the extracellular space. Figures reproduced from Wu et al. (2001) with permission.

large N- and C-terminal domains containing potential phosphorylation sites (Olivier et al., 2000; Kilty et al., 1991; Shimada et al., 1991; Giros et al., 1992). Experimental evidence indicates that the transportor lies in the cell membrane as an oligomeric structure (Hastrup et al., 2001; Kilic and Rudnick, 2000; Schmid et al., 2001; Sitte et al., 2004; Farhan et al., 2006). The first 5 TMs are believed to be involved in functions common to all members of Na^+ and Cl^- dependent transporters, which use the concentration gradients of these ions as an energy source. TM6 through to TM8 are the likely target sites for inhibitors, whilst regions TM9 through to the C-terminus contain the determinants for substrate affinity and stereo-selectivity (see review by Schenk, 2002).

The ATP-driven Na^+/K^+ ion exchanger maintains a Na^+ ion gradient across the neuronal plasma membrane, i.e. high $[Na^+]$ outside and low $[Na^+]$ inside, and DAT, like other members of the neurotransmitter/sodium symporter family, uses the power of this ionic gradient to transport dopamine from the synaptic space into the presynaptic terminal. In this process a protonated (positively charged) dopamine molecule, two Na^+ ions and one Cl^- ion are co-transported into the presynaptic terminal. Because the concentration of dopamine inside the terminal is much higher than outside in the synaptic space, DAT requires the motive force of the inward-directed Na^+ ion gradient to transport dopamine into the neurone against its own outward-directed concentration gradient.

Site-directed mutagenesis of the transporter has contributed significantly to our increased understanding of DAT and the differences in characteristics and locations for the binding of competitive substrates, competitive inhibitors and cocaine-binding site ligands. Results from these experiments have demonstrated that the arginine (Arg-85) and the aspartic acid (Asp-76) residues in TM1 and two serine residues in TM7 are essential for dopamine binding in the model proposed by Edvardsen and Dahl (1994). The Asp-345 in the third intracellular loop that is conserved in all neurotransmitter/sodium symporters appears to be critical for the binding of dopamine and various DAT inhibitors including cocaine and GBR12935 (Chen et al., 2003). These researchers also showed that inward and outward (reverse) transport of dopamine was also profoundly decreased when they made substitutions to this amino acid residue (Chen et al., 2003). The function of Asp-345 is likely to be mediated both by its volume and its negative charge and Chen et al. (2003) proposed that mutations of this residue fixed DAT in its inward-facing conformation. The rat homologue of DAT has 21 arginine residues in it and although their role in dopamine transport has not been investigated by site-directed mutagenesis they have been investigated using phenylglyoxal, which covalently binds to the arginine residues. Covalent binding of phenylglyoxal markedly reduced the transporter's velocity (Volz et al., 2004) indicating that arginine residues in DAT are also important for the binding and transport of dopamine.

Although the crystal structure of DAT has not been elucidated, a wealth of insights about its structure and function has been obtained by studying the leucine transporter (LeuT) which is a bacterial homologue of DAT.

DAT function is a complex process comprising (i) the binding of the substrate, dopamine, and the co-transported Na^+ and Cl^- ions while the transporter is an outward-facing conformation (ii) a change in the conformation of DAT whereby the transporter shifts from an outward-facing to an inward-facing state that results in translocation of dopamine across the plasma membrane, (iii) release of dopamine and the co-transported ions into the intracellular cytosol and (iv) 3-dimensional reorientation of DAT to an outward-facing conformation for the process to be repeated. C Zhao, Y Zhao and their respective co-workers have performed seminal work to elucidate these mechanisms (Shi et al., 2008; Zhao et al., 2010a,b, 2011, 2012; Zhao and Noskov, 2013).

In the model adopted by Edvardsen and Dahl (1994), DAT is visualised as a channel with a single binding site that transports dopamine. The single site model has also been used in the theoretical simulations by Zhao and Noskov (2013). In this construct, binding of Na^+ ions rather than the substrate (dopamine) stabilises the transporter in the outward-facing conformation. The binding of substrate leads to closure of the transporter, transition to the inward-facing conformation, and ultimately, dissociation of the substrate and co-transported ions into the intracellular cytosol.

More recent evidence has indicated a two-site model for the transport of dopamine (Beuming et al., 2008: Bisgaard et al., 2011; Shan et al., 2011) DAT. This two-site model is not only consistent with observations on the transport of dopamine, but it also fits more closely with the different binding and pharmacological characteristics of the cocaine binding-site ligands and the competitive DAT inhibitors. In this model, DAT possesses two binding sites for dopamine designated S1 and S2. The S1 site is proposed to be located in the central region of the transporter and is postulated to bind both dopamine and Na^+ ions. The S2 site is proposed to be 10A° above it on the outward-facing part of the transporter. In this two-site model, dopamine binding can occur simultaneously at both the S1 and S2 sites. However, it is binding at the S2 site, which is responsible for the conformational change that leads (i) to the translocation of the dopamine and co-transported ions across the membrane and (ii) the simultaneous movement of the dopamine molecule docked at S2 into S1 ready for translocation. Dissociation of dopamine and Na^+ ions into the neuronal cytoplasm leads to reconfiguration into the outward-facing conformation that allows binding of another dopamine molecule at the S2 site.

The dynamics of DAT function are regulated by a multiplicity of factors including ions, regulatory proteins, GPCRs (eg D_2 receptors), phosphorylation (eg Ca^{2+}/calmodulin-dependent protein kinase II) and transporter internalisation (Eriksen et al., 2010b). Another complication is that the function of DAT is not identical in all brain regions. In the medial prefrontal cortex, DAT appears to behave quite differently from DAT sites in the striatum and nucleus accumbens because transport is not totally Na^+-dependent as in the other two areas, and it is not as sensitive to the actions of DAT inhibitors, eg cocaine and GBR 12909 (see Schenk, 2002).

The role of Na^+, Cl^- and Zn^{2+} ions is especially relevant because they probably have a role not only in the rate of dopamine transport, but also the direction of substrate flow through the dopamine transporter. These mechanisms are pivotal to the pharmacological actions of the DAT substrate releasing agents, and we believe, also to the mode of action of cocaine and related compounds. Uptake experiments performed *in vitro* indicate that the rate of dopamine transport by DAT is both Na^+ and Cl^- dependent with a maximum velocity achieved at concentrations of 100 μM for both Na^+ and Cl^- ions. By using cell-lines transfected with *h*DAT, the contribution of other intracellular mechanisms to dopamine efflux can be avoided. When the extracellular concentration of Na^+ ions was reduced to 0 or 5 mM, it produced reverse-transport of preloaded dopamine from the cells via DAT sites. Moreover, at zero Na^+, reverse-transport of dopamine was resistant to inhibition by a high concentration of cocaine (Pifl et al., 1997). This effect was unique to Na^+ ions and not replicated by Cl^- ions even though reverse-transport of dopamine could be observed at low Cl^- concentrations. Extrapolating these findings to the *in vivo* situation suggests that under conditions where the Na^+ ionic gradient is markedly reduced cocaine would not serve as a DAT inhibitor, and furthermore, binding of cocaine to DAT sites would not impede the release of dopamine by reverse-transport.

Zn^{2+} ions are widely distributed in the CNS including in areas that are densely innervated with dopaminergic neurones (Pérez-Clausell and Danscher, 1985). Zn^{2+} may serve as chelated counterions for neurotransmitters stored in presynaptic terminals and Zn^{2+} ions are co-released by exocytosis into the synapse along with neurotransmitters (Berneis et al., 1969; Howell et al., 1984). A more recent development has been the discovery that there is a Zn^{2+} binding site present on *h*DAT with its binding co-ordinated by His-193 on extracellular loop between TM3 and TM4 and His-373 and Glu-396 on the extracellular loop between TM7 and TM8 (Norregard et al., 1998, 2003). Zn^{2+} ions appear to play a complex role in DAT function. Physiologically relevant concentrations of Zn^{2+} ions non-competitively inhibit dopamine uptake by *h*DAT (Norregard et al., 1998; Scholze et al., 2002) and it has been suggested this effect occurs as a result of preventing the conformational change of DAT that leads to dopamine translocation (Norregard et al., 1998). The presence of Zn^{2+} ions in the incubation medium also increased the number of sites available for binding dopamine and the cocaine analogue, WIN35,428 (Norregard et al., 1998). Subsequent site-directed mutagenesis experiments revealed that Ala-399 of TM7-TM8 was protected against cysteine modification by cocaine, but not dopamine (Norregard et al., 2003). Together, the results indicate that Zn^{2+} ions bind and stabilise *h*DAT in the outward-facing conformation, dopamine and cocaine bind at different locations on *h*DAT, and furthermore, they may stabilise *h*DAT in different outward-facing conformation states.

Scholze et al. (2002) showed that the presence of physiological concentrations of Zn^{2+} ions facilitated amphetamine-induced reverse-transport of dopamine by DAT. Intriguingly, the results also suggested that the only interaction that was required for dopamine reverse-transport was the binding of amphetamine to *h*DAT and not its transport into the cell (Scholze et al., 2002). This finding raises the question if the extracellular binding of amphetamine to DAT sites in the presence of Zn^{2+} ions can evoke reverse-transport of dopamine, can this effect be reproduced by other DAT ligands, eg cocaine which show Zn^{2+} sensitivity in their binding to *h*DAT.

The evidence above reveals that DAT is not simply a revolving door for dopamine transport driven by the Na$^+$ gradient. DAT function is complex and we still do not have validated mechanisms for either dopamine reuptake or reverse-transport. Substrates, inhibitors and cocaine probably bind at different locations on the transporter and may stabilise DAT in different conformational states. The ionic environment is an additional complexity that modifies both DAT function and drug action. The evidence also suggests that in the presence of Zn^{2+} ions it is feasible for stimulants to evoke reverse-transport of dopamine by binding to the extracellular domain of DAT.

3.2. The cocaine binding and substrate recognition sites on DAT

The majority of research performed to define cocaine's mechanism of action has focused on DAT, and in particular, on cocaine's interaction with two sites on the complex, i.e. the *substrate recognition site* and the *cocaine binding site*.

The Ki values of various classes of DAT ligand for the competitive inhibitor site on DAT (labelled by [^{3}H]GBR12935), the cocaine binding site (labelled by [^{3}H]WIN35,428) together with their Ki's for [^{3}H]dopamine uptake inhibition are reported in Table 2. It is evident that in many instances, there is substantial variation between the results reported from different laboratories. Taking that factor into account, the results reveal that cocaine and methylphenidate have 6–7-fold greater affinity for the cocaine binding site than for the competitive inhibitor site. The Ki values of cocaine-like ligands for the cocaine binding site are also higher than their respective Ki values for [^{3}H]dopamine uptake inhibition. In contrast, the competitive DAT inhibitors and substrates have similar Ki's for displacement of [^{3}H]WIN35,428 and [^{3}H]GBR12935. In the case of the competitive DAT inhibitors, there is little difference between their Ki values for the cocaine binding site, the competitive inhibitor site and [^{3}H]dopamine uptake inhibition. In the case of the competitive DAT substrates, their potency to inhibit [^{3}H]dopamine uptake inhibition is several hundred-fold lower than their Ki values for either the cocaine binding or competitive inhibitor sites reflecting the fact that they are merely molecules in the queue for the "revolving door" of the dopamine transporter.

Although it was originally thought that dopamine and cocaine competed for the same site on the dopamine transporter complex, ligand-receptor binding (Calligaro and Eldefrawi, 1988; Maurice et al., 1991) and thermodynamic (Bonnet et al., 1990) experiments have suggested there may be separate or overlapping, but not identical, substrate and stimulant binding sites on this transporter. Thus, there is a *substrate recognition site* on DAT, which binds dopamine, the classical, monoamine reuptake inhibitors, eg GBR 12909, mazindol and bupropion, and also the β-phenylethylamine releasing agents, eg the enantiomers of amphetamine or methamphetamine, and a *cocaine binding site*, which preferentially binds cocaine and related psychostimulants, eg WIN 35,428 and methylphenidate. In the model of Edvardsen and Dahl (1994), these authors proposed that the *substrate recognition site* for dopamine on DAT is located on TMs 1, 7 and 10–12 and the *cocaine binding site* is located on TMs 1, 7 and 9–11.

The ability of phenylglyoxal to block arginine residues on DAT was concentration-dependently inhibited by pre-incubation with cocaine, WIN35,428 or WIN35,065-2, indicating that binding of these ligands prevented the reagent from accessing the critical arginine residues (possibly Arg-282). In contrast, GBR12909, GBR12935 and benztropine were ineffective in preventing the phenylglyoxal reaction showing that these competitive inhibitors bind at a different locus on DAT than cocaine and related compounds.

Site-directed mutagenesis experiments have provided a wealth of evidence to support the hypothesis that cocaine and related stimulants bind not only to sites distinct from those which bind substrates and competitive inhibitors, but also to different conformational states of the transporter. Loland et al. (2002) identified Tyr-335, which is located on the third intracellular loop between TM6 and TM7, as a Zn^{2+} activated inhibitor of transporter function. In the resting state, DAT adopts an outward-facing conformation that binds both dopamine and cocaine. The binding of Zn^{2+} ions to this site inhibits dopamine transport by shifting DAT to the inward-facing, translocation conformation. Substituting Tyr-335 for alanine in the Y335A mutant stabilizes DAT in the inward-facing conformation, and binding of Zn^{2+} has the opposite effect of activating dopamine transport by this *h*DAT mutant. The affinity of dopamine and other substrates for the Y355A mutant, *h*DAT, was increased 5–20 fold, compared with the wild-type transporter, whereas the affinity of cocaine and WIN35,428 was decreased by >100-fold. However, the affinity of the competitive DAT inhibitor, GBR12909, was only marginally altered by this mutation, i.e. a 14-fold shift in Ki. The authors concluded that substrates, cocaine and GBR12909 bind at separate (possibly overlapping) loci and in different conformational states of the transporter. Thus, the active, inward-facing conformation favoured substrate binding, the inactive, outward-facing state favoured cocaine binding. GBR12909 bound with approximately equal affinity to both the inward- and outward-facing conformations of the transporter. Loland et al. (2008) extended support for this hypothesis by investigating the abilities of representatives from the various classes of DAT ligand to protect against covalent chemical reaction with Cys-159 in TM3.

They conducted further studies using the Y335A hDAT mutant, which revealed cocaine and related stimulants showed >100-fold decrease in affinity for the Y335A mutant compared with the wild-type hDAT, all generalised fully to the cocaine cue in rodent drug-discrimination testing. On the other hand, benztropine and rimcazole analogues, which were competitive DAT inhibitors, exhibited much smaller shifts in affinity did not generalise to cocaine. Loland et al. (2008) concluded that the reuptake inhibitors and cocaine-like stimulants bind to different conformational states of DAT, i.e. active, inward-facing and inactive, outward-facing respectively.

There are 26 tryptophan and acidic residues on the 12TM spanning units and on the extra- and intracellular cellular loops of hDAT, which are highly conserved across the Na^+/Cl^- family of transporters and these residues are essential for the binding of dopamine and its transport into the presynaptic terminal. Chen et al. (2001) demonstrated that mutagenesis of aspartate-313 on the extracellular loop linking TM5 and TM6 abolished dopamine transport and markedly reduced the binding of WIN35,428. All of the mutations studied exhibited reduced WIN35,428 binding, but not all of the DAT mutations had a reduced Vmax for dopamine uptake indicating the presence of some dissociation between these two parameters thus supporting earlier work suggesting that the binding domains for dopamine and cocaine are overlapping, but not identical. This point was emphasised by mutation of aspartate-345 on the intracellular loop between TM6 and TM7 on DAT which maintained the ability to transport dopamine, but did not bind WIN35,428 (Chen et al., 2001).

Chen, Reith and colleagues had previously shown that site directed mutagenesis of Trp-84 in TM1, i.e. leucine for tryptophan (W84L), and Asp-313 on the extracellular face of TM6, i.e. asparagine for aspartate (D313N), resulted in mutations of hDAT where the transporter was fixed in the outward-facing conformation. The observation that cocaine analogues had higher affinity for the W84L mutant in which hDAT was predominantly fixed in the outward-facing state, but had lower affinity for W-L mutants in which hDAT was more readily fixed in the inward-facing (Chen et al., 2004) is consistent with their previous conclusion that the outward-facing conformation is preferred for cocaine binding (Chen and Justice, 1998).

However, this group was similarly intrigued why cocaine and some related compounds were powerful positive reinforcers, but many other DAT inhibitors were not and so they performed additional DAT mutagenesis experiments to explore the reasons for this conundrum.

In a pivotal study, Schmitt et al. (2008) used the W84L and D313N hDAT mutations to study the pharmacological characteristics of a wide range of DAT ligands, including the stimulants, cocaine, WIN 35,428 and methylphenidate, and bupropion, GBR 12909 and benztropine which are not psychostimulant in humans. A summary of their findings is presented in Table 6. Schmitt et al. (2008) observed that in the presence of a physiological, 130 mM concentration of Na^+ ions, the affinity of cocaine, WIN35,428 and methylphenidate for the W84L mutated DAT was increased in comparison to their affinities for the wild-type hDAT. In contrast, the affinities of GBR12909, bupropion and benztropine were all decreased. A similar picture emerged for the D313N mutation where at 130 mM Na^+ ions, the psychostimulants had increased affinity, whereas the affinity of benztropine, bupropion and GBR12909 decreased or was unchanged. The affinities of both groups of DAT ligands for the W84L and D313N were markedly reduced in the absence of Na^+ ions (Schmitt et al., 2008), showing that the binding of all of the compounds to these mutated DAT transporters was Na^+-sensitive which is analogous to their binding to the wild-type hDAT. At zero Na^+ ions, the affinities of cocaine, WIN35,428 and methylphenidate for W84L were increased compared

Table 6
A summary of the effects of the W84L and D313N mutations on the binding affinity of cocaine and related compounds compared with DAT inhibitors.

Compound	Affinity relative to wild type hDAT kapp hDAT/kapp W84L or Kapp hDAT/kapp D313N			
	W84L		D313N	
	130 mM Na$^+$	0 mM Na$^+$	130 mM Na$^+$	0 mM Na$^+$
Psychostimulants				
Cocaine	↑	↑	↑	↑
WIN 35,428	↑	↑	↑	↑
Methylphenidate	↑	↑	↑	↑
DAT inhibitors				
Bupropion	↓	↓	↓	±
GBR 12909	↓	↓	±	↑
Benztropine	↓	↓	↓	±

Data taken from Schmitt et al. (2008).
Kapp = Apparent equilibrium dissociation constant.

with wild-type hDAT, whereas the affinities of GBR12909, bupropion and benztropine were decreased. The psychostimulants also had greater affinity for D313N compared with the wild-type hDAT, whereas the non-psychostimulants had unchanged affinity with the exception of GBR 12909. Since the W84L mutation disrupts the transition between the outward- and inward-facing conformations of DAT, the authors concluded that increased affinity of cocaine, WIN35,428 and methylphenidate for W84L compared with the wild-type hDAT was consistent with them preferentially binding to the outward-facing conformation of DAT (Schmitt et al., 2008). In contrast, the reduction of affinity for W84L that was observed with GBR12909, bupropion and benztropine suggested that they preferentially bound to DAT in its inward-facing conformation (Schmitt et al., 2008). Similarly, the D313N mutation also has the ability to stabilise DAT in the outward-facing conformation and a similar increase of affinity of the psychostimulants for D313N compared with wild-type hDAT further supports this conclusion.

Finally, Chen et al. (2006) created a knock-in mouse that expressed a DAT mutation, which transported dopamine, but did not bind cocaine. In mice with this DAT mutation, the ability of cocaine to inhibit dopamine uptake was reduced by ~1000-fold, cocaine administration did not reduce the rate of dopaminergic neuronal firing, it did not increase dopamine efflux in the nucleus accumbens, and it did not induce locomotor activation or conditioned place-preference (Chen et al., 2006). These experiments therefore unequivocally established that binding of cocaine to the transporter was essential dopaminergic pharmacology and for its reinforcing effect.

Together, the ligand-receptor binding and site-directed mutagenesis experiments demonstrate that the binding of cocaine to DAT is essential to its actions on dopaminergic neuronal function and to its pyschostimulant and reinforcing properties. Cocaine binds at an overlapping but different domain on DAT than those which bind conventional competitive inhibitors or substrates. The experimental findings indicate that dopamine binds at the S2 to initiate the transition of DAT from the outward-facing to the inward-facing conformation, cocaine and related psychostimulants bind to S2 of DAT in its outward-facing conformation, and conventional competitive DAT inhibitors like bupropion and GBR12909, bind to S2 of DAT in its inward-facing conformation. The proposed interactions of the various types of DAT ligand with the transporter are illustrated in Fig. 7.

3.3. The hypothesis that cocaine, methylphenidate and related cocaine binding site ligands are DAT "inverse agonists"

The discovery of inverse agonists came from observations of β-carboline, benzodiazepine receptor ligands and their highly

A   Neuronal reuptake of dopamine by DAT

DAT in the outward-facing conformation

Binding of a second molecule of dopamine at S2 causes transition of DAT to the inward-facing conformation

Reversion to outward-facing conformation

Translocation of dopamine into the neurone

Dopamine transport driven by outward/inward Na$^+$ concentration gradient
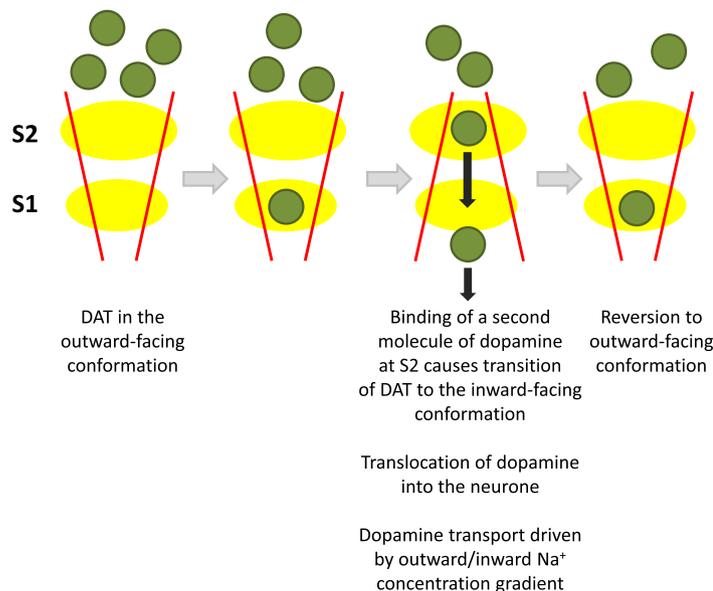
B   Proposed mechanisms for DAT inhibitors and cocaine

DAT inhibitor binds at S2 site stabilising the inward-facing conformation of DAT preventing translocation of dopamine molecules

Cocaine binds at S2 site on DAT stabilising the outward-facing conformation of DAT

Reverse transport of dopamine driven by the inward/outward concentration gradient

Dopamine reuptake transporter | S1 and S2 binding domains | Dopamine molecule | Reuptake inhibitor | Cocaine

Fig. 7. Proposed function of DAT showing the interactions with DAT inhibitors and cocaine as a DAT "inverse agonist". Although cocaine is shown bound to DAT during the reverse transport of dopamine, it is also feasible that rapid dissociation and association by cocaine occurs during the process.

unusual pharmacological interactions with the GABA$_A$ receptor. Unlike the conventional benzodiazepines that are positive allosteric modulators that prolong the GABA-mediated opening of the GABA$_A$ receptor Cl$^-$ ion channel, the novel ligand, β-CCE (β-carboline-3-carboxylate), is a negative allosteric modulators that produces the opposite effect of reducing the duration of GABA-mediated opening of the Cl$^-$ channel (Cowen et al., 1981; Polc et al., 1982). Unlike the benzodiazepine GABA$_A$ receptor agonists which are anxiolytic and anticonvulsant, the benzodiazepine inverse agonists are proconvulsant and anxiogenic leading to them as being called "inverse agonists" (Polc et al., 1982).

SoRI-6238 is a novel, synthetic, small molecule that serves as a negative allosteric modulator of the serotonin transporter by increasing the binding affinity of [^{125}I]RT1-55 to SERT, slowing its rate of association and dissociation and by inhibiting the Vmax of 5-HT transport (Nandi et al., 2004). It is important to note that although SoRI-6238 is a negative allosteric modulator of SERT, it does not reverse the direction of 5-HT transport via the transporter. Although cocaine is a negative allosteric modulator of DAT, the pharmacological action that we are proposing for cocaine and related psychostimulants is not to reduce the inward transport of dopamine via DAT, it is to modify allosterically the conformation of the transporter which results in DAT serving as a channel that allows reverse-transport of dopamine out of the neurone and into the synapse. This mechanism is totally different from that of a compound like SoRI-6238, and to avoid confusion, we have avoided using the term negative allosteric modulator to describe cocaine. We have, therefore, borrowed the term DAT "inverse agonist" for cocaine and related psychostimulants because binding of dopamine to S2 triggers a conformational change that translocates dopamine into the neurone (a type of allosteric "agonism"), whereas binding of cocaine produces the opposite conformational shift that leads to dopamine efflux via DAT (allosteric "inverse agonism").

Much of the scientific evidence to support the hypothesis that cocaine, methylphenidate and related compounds serve as DAT "inverse agonists" is contained in the granted UK patent (Heal, 2008).

A schematic representation of the reuptake mechanism of DAT is shown in the left-hand panel of Fig. 8. At resting potential the fast-gated Na^+ channels are closed and the ATP-driven Na^+/K^+ ion exchanger maintains the membrane potential of the neurone by pumping out two Na+ ions and pumping in one K^+ ion. This leads to high $[Na^+]$ outside the plasma membrane and low $[Na^+]$ inside. As a member of the neurotransmitter/sodium symporter family DAT uses the power of this ionic gradient to transport dopamine from the synaptic space into the presynaptic terminal. In this process a protonated (positively charged) dopamine molecule, two Na^+ ions and one Cl^- ion are co-transported into the presynaptic terminal. Because the concentration of dopamine inside the terminal is much higher than outside in the synaptic space, DAT requires the motive force of the inward-directed Na^+ ion gradient to transport dopamine into the neurone against its own outward-directed concentration gradient.

Although various hypotheses have been put forward to explain how the competitive DAT transporter substrates release dopamine (eg see Pifl et al., 1997; Fleckenstein et al., 2007; Sitte and Freissmuth, 2010; Eriksen et al., 2010a), there is no unequivocally proven mechanism at the current time. What is undisputed is DAT is not a one-way inward channel. Competitive DAT substrate releasing agents can reverse the direction of transport of dopamine via DAT by displacing dopamine from the newly-synthesised cytosolic pool. These drugs also increase the amount of neurotransmitter that is available for release from the cytosol by entering the dopamine storage granules via VMAT-2 (vesicular monoamine transporter-2) and displacing the neurotransmitter from here also. Since the rate of uptake by DAT is not regulated by either autoreceptor inhibition or neuronal-firing rate, the only factor that controls the rate of entry of a DAT substrate releasing agent into the presynaptic terminal is the concentration which is present in the synaptic environment adjacent to the transporter sites. By the same logic, the entry of the releasing agent into the dopamine storage granules is dependent only on its concentration in the presynaptic terminal that is available for transport by VMAT-2. These factors explain the pharmacodynamics of the DAT substrate releasing agents on dopamine efflux, i.e. the bigger the dose administered and the more rapidly it reaches DAT sites in the brain, the greater and more rapid the resulting release of dopamine. Furthermore, since every molecule of a DAT substrate releasing agent transported into the presynaptic terminal is accompanied by two Na^+ and one Cl^- ions, the influx of Na^+ ions with a DAT substrate releasing agent
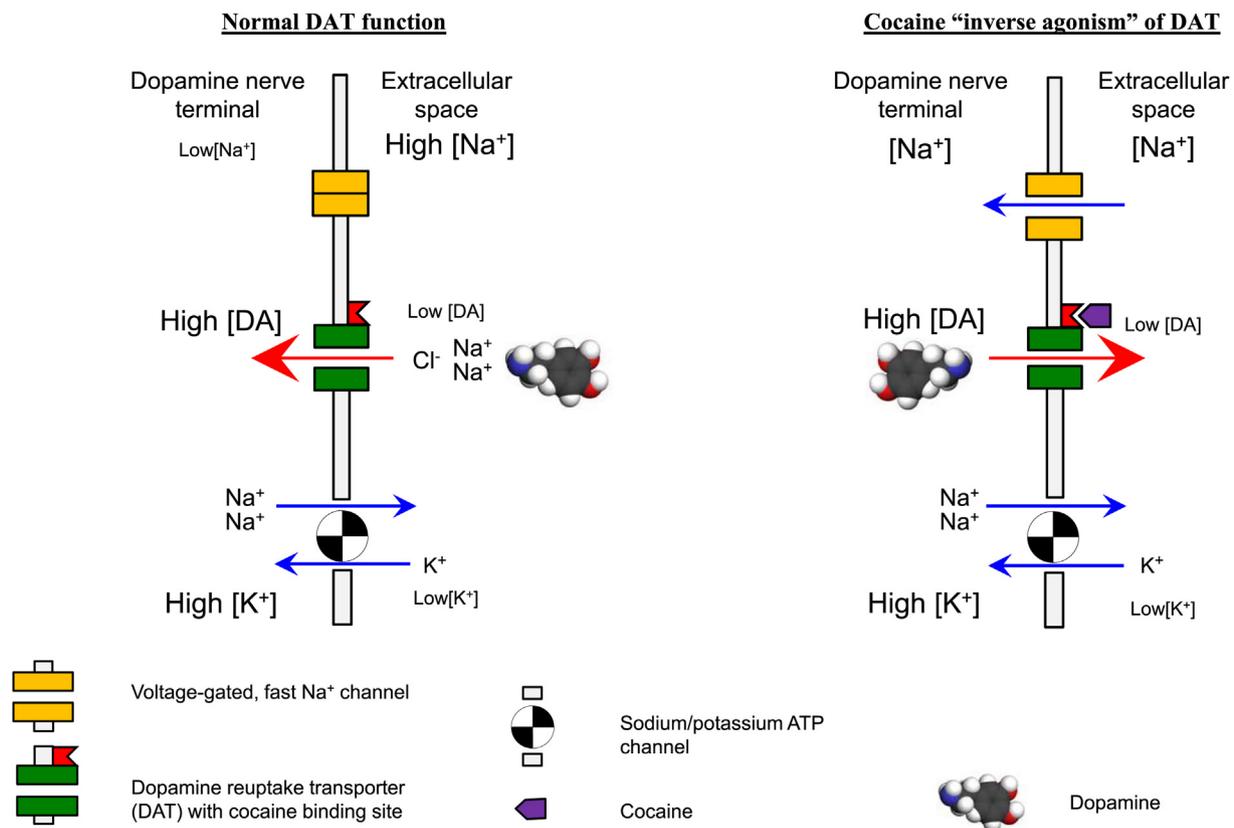
Fig. 8. Proposed "inverse agonist" mechanism of cocaine and related cocaine binding site ligands. **Normal DAT function**: Dopaminergic signalling is terminated by switching-off cell firing and the active removal of dopamine from the synaptic region by DAT. In this situation, voltage-gated fast Na^+ ion channels are closed. One molecule of dopamine, one Cl^- and two Na^+ ions are translocated into the cytosol of the presynaptic terminal via DAT and driven by the power of the Na^+ ionic gradient across the neuronal plasma membrane. The Na + ionic gradient across the cell membrane is maintained by the ATP-driven Na^+/K^+ exchanger. **Cocaine "inverse agonism" of DAT**: Cocaine has an extraneuronal site of action on DAT and its release mechanism is dependent on voltage-gated, fast Na^+ ion channel opening. The action of cocaine is also greatest when high concentrations of the drug are rapidly presented to DAT sites. Dopaminergic neuronal firing is associated with opening of the voltage-gated Na^+ ion channels which temporarily reduces the magnitude of the Na^+ ionic gradient across the neuronal cell membrane. Cocaine binding to DAT in its outward-facing conformation leads to temporary opening of the transporter channel that in turn facilitates the reverse-transport of dopamine from the neuronal cytosol into the synapse. This process is driven by the dopamine concentration gradient that exists between the cytosol of the presynaptic nerve terminal and the extracellular space. The pharmacodynamics of the effect of cocaine on dopamine efflux from the nerve terminal would be similar to reverse transport of dopamine caused by competitive DAT substrate releasing agents. Because unlike the DAT substrates, cocaine has an extra-neuronal site of action, it is not able to evoke release of dopamine from the vesicular storage pool. This may explain why the maximum effect of competitive DAT substrate releasing agents on dopamine efflux is greater than that of DAT "inverse agonists".

is one plausible mechanism for DAT reversing its direction of transport. The massive influx of Na^+ ions disrupts the inward Na^+ ionic gradient to the point it no longer has sufficient motive force to pump dopamine into presynaptic terminal against its own concentration gradient. The direction of flow in the DAT channel reverses because of disruption of the Na^+ gradient and the outward dopamine gradient causes the monoamine to diffuse out of the terminal and into the synaptic cleft.

The reverse-transport of dopamine evoked by the DAT substrate releasing agents demonstrates that this ion channel can operate in both directions. Heal (2008) postulated that the cocaine binding site on DAT allosterically controls the opening of the channel. Binding of cocaine, methylphenidate and similar cocaine-like compounds leads to it opening to allow dopamine to reverse-transport from the presynaptic terminal into the extracellular space.

As shown in Fig. 7, conventional competitive DAT inhibitors bind to the S2 site of DAT which also binds Na^+ and Cl^- ions and is the translocation site for the entry of dopamine into the cytosol of the presynaptic terminal. This is the binding site labelled by [^{3}H]GBR12909 and docking of the reuptake inhibitor causes DAT to adopt the inward-facing conformation that prevents dopamine molecules from docking at the S1 site. The result is blockade of dopamine transport until the DAT inhibitor is displaced from S2 by competition from synaptic dopamine. Because in most instances competitive DAT inhibitors have high or very high affinity for DAT, only a relatively low concentration of the compound is required to occupy and block a significant proportion of the available DAT sites.

A representation of the binding of cocaine to the S2 binding site of the outward-facing conformation of DAT leading to the reverse-transport of dopamine is illustrated in Fig. 7. A schematic representation of the DAT "inverse agonist" mechanism of action of cocaine is illustrated in the right-hand panel of Fig. 8.

Cocaine and related compounds bind to a unique domain on the S2 site of DAT, which is labelled by [^{3}H]WIN 35,428, and binding of the proposed "inverse agonists" causes DAT to adopt the outward-facing conformation. We postulate that the opening of voltage-gated Na^+ ion channels which mediates neuronal firing leads to the rapid influx of Na^+ ions and a transient reduction of the inward Na^+ ionic gradient across the presynaptic plasma membrane. When this occurs, cocaine and other DAT "inverse agonists" allosterically effect the opening of the transporter channel. In this situation, the motive force of the Na^+ ion gradient is insufficient to drive the transport of dopamine into the presynaptic terminal via DAT. The direction of transport is reversed and dopamine flows down its outward concentration gradient into the synaptic cleft. Since cocaine binding site ligands have only weak affinity for both the

cocaine binding site (unique domain of S2) and the substrate binding site (overlapping, but non-identical, domain of S2) on DAT, they are easily displaced by dopamine that is exiting in large quantities via DAT. The sum effect is to produce rapid efflux of dopamine into the synapse which is analogous to the actions of the DAT substrate releasing agents; the larger the concentration and more rapid the delivery of a DAT inverse agonist to its site of action, the greater the resulting efflux of dopamine. This outcome is assisted by the fact that administration of cocaine and related compounds produce only a moderate reduction of dopaminergic neuronal firing and these psychostimulants also have excitatory properties (Shi et al., 2000, 2004). This mechanism would rationalise the similarity between the effects of cocaine and methylphenidate on dopamine efflux and those produced by the DAT substrate releasing agents like D-amphetamine and methamphetamine, i.e. (i) rapid onset of peak efflux, (ii) short duration of effect and (iii) lack of a dose-effect ceiling. It also explains why all of these drugs are powerful stimulants and highly reinforcing (Table 7).

The hypothesis also highlights important differences that exist between the mechanism of the DAT "inverse agonists" and the DAT substrate releasing agents (Table 7). Since DAT "inverse agonists" rely on Na^+ entry into the neurone via voltage-gated Na^+ channels to evoke dopamine release, the rapid presentation of high concentrations of cocaine and related compounds is critical to their ability to effect large amounts of dopamine efflux. Gradual delivery of DAT "inverse agonists" allows the brain's auto-inhibitory mechanisms to blunt their dopamine releasing action. This is consistent with the 100-fold increase in potency that is observed when cocaine and methylphenidate are switched from the oral to the intravenous route of administration. Cocaine and related DAT "inverse agonists" are too large to serve as DAT substrates, and consequently, they cannot mobilise dopamine which is contained within the vesicular storage pool. This factor may explain why the DAT "inverse agonists" can compete with the DAT substrates in terms of speed of dopamine release, but the maximum size of their effect will always be smaller than that of the competitive DAT substrate releasing agents because of their inability to augment cytosolic dopamine with neurotransmitter displaced from the vesicular storage pool.

Although the inverse agonist hypothesis has been exemplified using DAT, *in vivo* microdialysis experiments have unequivocally shown that the highly unusual pharmacodynamics of cocaine and related compounds extends to noradrenaline in the case of methylphenidate (Kuczenski and Segal, 1997; Heal et al., 2009a, 2012; Rowley et al., 2014) and noradrenaline and 5-HT in the case of

Table 7
Proposed classification of dopamine reuptake transporter ligands.

Proposed class	Examples	Pharmacological characteristics in microdialysis experiments				Firing-dependent effect	Site of action	Euphoriant and/or stimulant
		Time of peak effect	Magnitude of effect	Effect ceiling	Duration of action			
DAT "inverse agonists"	Cocaine[1, 2, 3] Methylphenidate[4, 5] D-Methylphenidate[4, 6]	Rapid (<1 h)	Large (500-1500%)	No	Relatively short (≤3 h)	Yes	Extra-neuronal	Yes
Competitive DAT inhibitors	Bupropion[7, 8] GBR12909[1, 3, 8, 9, 10] Sibutramine[11, 12]	Slow (>1 h)	Moderate (≤500%)	Yes	Long duration (≥3 h)	Yes	Extra-neuronal	No
Competitive DAT substrate releasing agents	D-Amphetamine[1, 4, 5, 8, 11, 13, 14] L-Amphetamine[13, 15] Methamphetamine[9, 10, 13, 14] Phentermine[11]	Rapid (<1 h)	Large (≥1000%)	No	Relatively short duration (≤3 h)	No	Intra-neuronal	No

[1] Tanda et al. (1997); [2] Hemby et al. (1999); [3] Baumann et al. (1994); [4] Heal et al. (2008); [5] Heal et al. (2009a); [6] Ding et al. (1997); [7] Sidhpura et al. (2007); [8] Bredeloux et al. (2007); [9] Tsukada et al. (1999); [10] Zolkowska et al. (2009); [11] Rowley et al. (2000); [12] Balcioglu and Wurtman (2000); [13] Kuczenski et al. (1995); [14] Shoblock et al. (2003); [15] Cheetham et al. (2007).

cocaine (Bradberry et al., 1993; Chen and Reith, 1994; Reith et al., 1997; Pum et al., 2007). Hence, these observations suggest that these stimulants may also serve as "inverse agonists" of NET, and in the case of cocaine, SERT.

In summary, the hypothesis that cocaine, methylphenidate and related compounds act as "inverse agonists" of DAT and possibly also of other monoamine reuptake transporters, fits more closely with their pharmacology as stimulants and also helps explain why they are anomalies as competitive DAT reuptake inhibitors. The inverse agonist hypothesis does not contradict the fact that these stimulants are competitive DAT inhibitors, it merely proposes that DAT "inverse agonism" is the major contributor to their pharmacological actions, especially when they are given at high doses and by routes that promote rapid entry to the brain.

4. Future directions

Although we have proposed DAT "inverse agonism" as a mechanism to rationalise the highly unusual pharmacology of cocaine, methylphenidate and other drugs, which are powerful stimulants, but are not DAT transporter substrates, alternative hypotheses can be put forward that would explain the pharmacodynamics of their actions on dopaminergic neurotransmission. For example, it could be hypothesised that cocaine and related stimulants may substantially augment the quantal release of dopamine by exocytosis.

It would be very satisfying if new experimental data emerged to support the hypothesis that cocaine serves as a DAT "inverse agonist", but it was not the primary objective for writing this review. The most important aim was to stimulate debate and new ideas about the pharmacological mechanism of action of cocaine and related stimulants because the more that we learn about the pharmacology of these intriguing drugs, the more obvious it becomes that they are not conventional DAT inhibitors. It is now almost 50 years since it was first reported that cocaine could potentiate sympathetic tone by inhibiting the synaptic clearance of catecholamines and the rigid adherence to reuptake inhibition as the mechanism responsible for cocaine's pharmacology has ossified research in the field and become a major impediment to progress. Pigeon-holing cocaine as a competitive DAT inhibitor steered drug treatment for cocaine dependence in the direction of high affinity DAT inhibitors. Despite all of the efforts that have been expended over more than a decade, this approach has delivered no promising new treatments for this serious unmet medical need. Moreover, the competitive DAT inhibitor theory for the mechanism of cocaine has raised the spectre of cocaine-like abuse potential over every new competitive DAT inhibitor that has been taken into clinical development and regulatory review. Although our knowledge is limited to information that is in the public domain, the available results reveal that none of these novel DAT inhibitors exhibits a pharmacological profile which is similar to that of cocaine or methylphenidate.

The evidence still points to dopamine being the most important mediator of the psychostimulant and euphoriant actions of cocaine and methylphenidate; it is only the mechanism by which these drugs achieve this effect that is debated. Twenty years ago when Prof Sid Auerbach (Rutgers University, New Jersey) and ourselves were attempting to elucidate the very different neurochemical profiles of monoamine reuptake transporter inhibitors and β-phenylethylamine releasing agents (Gundlah et al., 1997; Tao et al., 2002), the relative importance of monoamine release as a contributor to the pharmacology of drugs like ᴅ-amphetamine and d-fenfluramine was barely appreciated. Now, a determination of DAT substrate properties is an essential component of characterising novel stimulant substances of abuse. We believe it is an appropriate time to put aside the competitive DAT inhibitor hypothesis as an explanation of cocaine's psychostimulant actions and to start the search for novel pharmacological mechanisms that better fit with cocaine's intriguing pharmacology.

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