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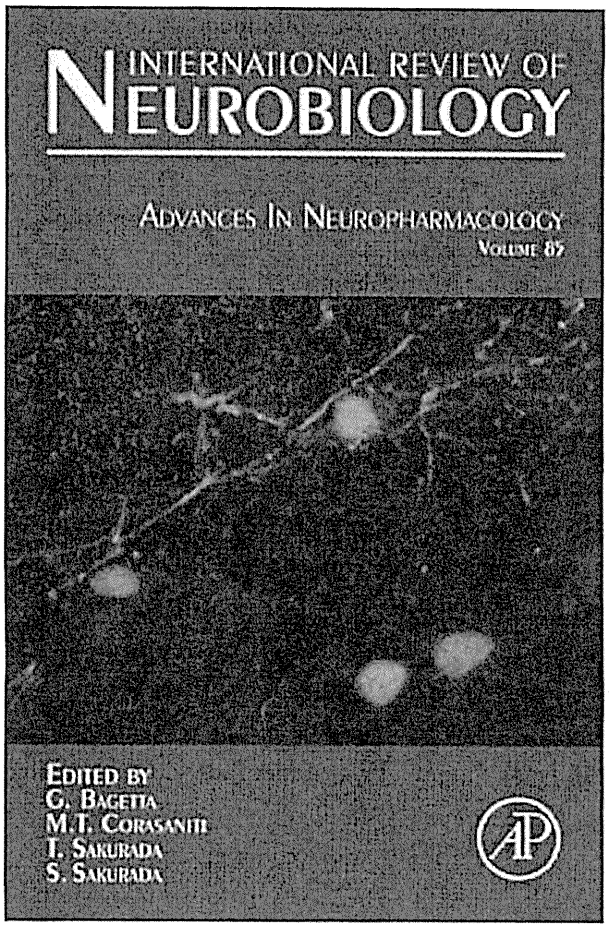

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MONOAMINE TRANSPORTER AS A TARGET MOLECULE FOR PSYCHOSTIMULANTS

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Methamphetamine (MAP), a drug of abuse known worldwide for its addictive effects and neurotoxicity, causes somatic and psychiatric disorders. MAP enters terminals/neurons via monoamine transporters, displaces both vesicular and intracellular monoamines, and facilitates the release of monoamines into the extraneuronal space through synaptic transport via the monoamine transporters. Chronic psychostimulant abusers exhibit psychotic features, including delusions and auditory hallucinations. The dopamine transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2) play pivotal roles in the action of MAP, including locomotor effects. The deletion of DAT attenuates the locomotor effects of MAP and may play larger role in behavioral responses to MAP compared to the deletion of VMAT2. MAP produces hyperthermia and/or neuronal toxicity in most species. The effects of MAP in DAT or serotonin transporter (SERT) single knockout (KO) mice and DAT/SERT double KO mice suggested that DAT and SERT are key molecules for hyperthermia and neuronal toxicity of MAP.

I. Introduction

Methamphetamine (MAP) is a psychostimulant that induces enhanced arousal and euphoria acutely, and psychosis and addiction chronically. MAP enters the terminals/neuron via the monoamine transporters (dopamine transporter: DAT, serotonin transporter: SERT, or norepinephrine transporter: NET), displaces

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both vesicular and intracellular monoamines, and facilitates release of monoamines into the extraneuronal space by synaptic transport in the monoamine transporters (Seiden *et al.*, 1993). The large release of monoamine produced by psychostimulant is thought to contribute to the drug's effects in the brain.

II. MAP-Induced Behavioral Sensitization

The acute and chronic pharmacological consequences of MAP in human users have been observed in behavioral experiments in animals, including both hyperactivity and sensitization of locomotor responses (Segal and Schuckit, 1983). Behavioral sensitization is a phenomenon whereby repeated intermittent exposure to MAP-like psychostimulant elicits a progressive enhancement of those responses, which persists for extended time periods following withdrawal from the drug and are easily reinstated by exposure to the drug or psychosocial stress (Robinson and Becker, 1986). This process closely resembles the course of the relapse in MAP-induced psychosis or schizophrenia, thus sensitization in animals has been suggested to model these psychoses (Sato *et al.*, 1983). Behavioral sensitization is thought to be an early and enduring manifestation of neuronal plasticity associated with changes in mesolimbic dopamine neurotransmission (Kalivas *et al.*, 1993). MAP induces dopamine release through exchange diffusion of plasma membrane DAT (Seiden *et al.*, 1993), and release of vesicular dopamine into the cytosol by acting on the vesicular monoamine transporter 2 (VMAT2) (Sulzer *et al.*, 2005). The dopamine releasing effect of MAP has been postulated to mediate its locomotor stimulant and rewarding effects (White and Kalivas, 1998). Therefore, DAT and VMAT2 should play pivotal roles in the mechanisms underlying the actions of MAP.

DAT knockout (KO) mice and VMAT2 KO mice have been used to investigate the roles of DAT and VMAT2 in dopamine neurotransmission and pharmacological mechanisms underlying the actions of psychostimulants. Homozygous deletion of the DAT gene has been reported to produce a 10-fold increase (Shen *et al.*, 2004) or fivefold elevation (Jones *et al.*, 1998) of extracellular dopamine concentrations in the striatum measured by *in vivo* microdialysis, while heterozygous deletion of DAT was not found to significantly increase extracellular dopamine (Shen *et al.*, 2004) or to produce a smaller twofold elevation (Jones *et al.*, 1998) of dopamine in the striatum. Homozygous DAT KO mice show growth retardation and hyperactivity, whereas heterozygous DAT KO mice did not show gross abnormalities in either development or baseline behavioral parameters (Sora *et al.*, 1998). Habituated homozygous DAT KO mice do not show any significant cocaine-induced increase in locomotion (Sora *et al.*, 1998, 2001; Uhl *et al.*, 2002).

We examined locomotor activity and sensitization in heterozygous DAT KO ($DAT^{+/-}$), heterozygous VMAT2 KO ($VMAT2^{+/-}$), double heterozygous DAT/VMAT2 KO ($DAT^{+/-}$ $VMAT2^{+/-}$), and wild-type (WT) mice to evaluate the roles of DAT and VMAT2 in MAP-induced locomotor behavior (Fukushima *et al.*, 2007). In $DAT^{+/-}$ $VMAT2^{+/-}$ mice, all of MAP-induced behavioral responses were similar to those in $DAT^{+/-}$, but not $VMAT2^{+/-}$ mice. The behavioral effects of both acute and chronic MAP administration were suppressed in heterozygous DAT KO mice, whether or not it was combined with heterozygous VMAT2 KO. Contrary to the effect observed in heterozygous DAT KO mice, acute MAP administration produced greater locomotor responses in heterozygous VMAT2 KO mice. These findings indicate that the half deletion of DAT plays a major role in both acute and chronic behavioral responses to MAP, while the effect of the half deletion of VMAT2 is less prominent.

III. MAP-Induced Hyperthermia and Neuronal Toxicity

MAP abuse causes serious health hazards including irreversible neuronal degeneration, seizures, hyperthermia, and death in human and experimental animals (Davidson *et al.*, 2001). Among these side effects, MAP produces hyperthermia and/or dopaminergic neurotoxicity in most species. Clinical reports and animal studies indicate that lethality by MAP closely correlates with hyperthermia, which may be the primary cause of death. Animal studies suggest that dopamine receptor activation is crucial for MAP-induced hyperthermia (Broening *et al.*, 2005) and lethality (Bronstein and Hong, 1995). There has also been an assumption that the hyperthermia that follows MAP administration is serotonin receptor-mediated (Green *et al.*, 2003).

We examined hyperthermic and lethal toxic effects of MAP in DAT, SERT, and DAT/SERT double KO mice to elucidate the role of these two transporters in MAP-induced hyperthermia and lethality (Numachi *et al.*, 2007). MAP caused significant hyperthermia even in the mice with a single DAT gene copy and no SERT copies ($DAT^{+/-}$ $SERT^{-/-}$ mice). Mice with no DAT copies and a single SERT gene copy ($DAT^{-/-}$ $SERT^{+/-}$ mice) showed significant but reduced hyperthermia when compared to WT mice after MAP. These results demonstrate that MAP exerts a hyperthermic effect via DAT, or via SERT, in the absence of DAT. DAT gene deletion in mice strikingly increased LD_{50} of MAP by 1.7–1.8 times that of WT mice, suggesting that the lethal toxic effect of MAP is mainly dependent on DAT. Although DAT and SERT were shown here to be involved in both the effects of MAP on temperature as well as MAP lethal toxicity, the mechanisms are nonetheless different; DAT single KO mice exhibited hyperthermia but greatly reduced MAP lethality, and the lethality was no different from

DAT/SERT double KO mice that had hypothermic responses to MAP. Thus, although the lethal toxic effect of MAP is mainly dependent on DAT, with some contribution from SERT, hyperthermia is not prerequisite for MAP-induced lethality.

In conclusion, these findings lead us to hypothesize that DAT variants may have more profound effects than VMAT2 or SERT variants on the clinically important consequences of acute and chronic MAP abuse in humans.

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COCAINE-CONDITIONED LOCOMOTION IN DOPAMINE TRANSPORTER, NOREPINEPHRINE TRANSPORTER AND SEROTONIN TRANSPORTER KNOCKOUT MICE

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Abstract—The behavioral effects of cocaine are affected by gene knockout (KO) of the dopamine transporter (DAT), the serotonin transporter (SERT) and the norepinephrine transporter (NET). The relative involvement of each of these transporters varies depending on the particular behavioral response to cocaine considered, as well as on other factors such as genetic background of the subjects. Interestingly, the effects of these gene knockouts on cocaine-induced locomotion are quite different from those on reward assessed in the conditioned place preference paradigm. To further explore the role of these genes in the rewarding effects of cocaine, the ability of five daily injections of cocaine to induce conditioned locomotion was assessed in DAT, SERT and NET KO mice. Cocaine increased locomotor activity acutely during the initial conditioning session in SERT KO and NET KO, but not DAT KO, mice. Surprisingly, locomotor responses in the cocaine-paired subjects diminished over the five conditioning sessions in SERT KO mice, while locomotor responses increased in DAT KO mice, despite the fact that they did not demonstrate any initial locomotor responses to cocaine. Cocaine-induced locomotion was unchanged over the course of conditioning in NET KO mice. In the post-conditioning assessment, conditioned locomotion was not observed in DAT KO mice, and was reduced in SERT KO and NET KO mice. These data reaffirm the central role of dopamine and DAT in the behavioral effects of cocaine. Furthermore, they emphasize the polygenic basis of cocaine-mediated behavior and the non-unitary nature of drug reward mechanisms, particularly in the context of previous studies that have shown normal cocaine-conditioned place preference in DAT KO mice. © 2009 Published by Elsevier Ltd on behalf of IBRO.

Key words: transgenic mice, dopamine transporter, serotonin transporter, norepinephrine transporter, cocaine, conditioned locomotion.

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Abbreviations: ANOVA, analysis of variance; CPP, conditioned place preference; DAT, dopamine transporter; GFP, green fluorescent protein; KO, knockout; NEO, neomycin gene; NET, norepinephrine transporter; SERT, serotonin transporter; 5-HT, serotonin.

Initial transgenic studies of the mechanisms underlying the rewarding effects of cocaine found that deletion of the gene for the dopamine transporter (DAT) alone did not eliminate the rewarding effects of cocaine as assessed in either the conditioned place preference (CPP) or self-administration paradigms (Rocha et al., 1998; Sora et al., 1998). Subsequent studies found that combined elimination of the serotonin transporter (SERT) and DAT eliminated the rewarding effects of cocaine in the CPP paradigm (Sora et al., 2001). However, the effects of SERT knockout (KO) are rather complex and can also increase the rewarding effects of cocaine (Sora et al., 1998; Hall et al., 2002). This should not be surprising given the diverse effects that pharmacological treatments aimed at specific serotonin (5-HT) receptor subtypes have on drug reward, including both increases and decreases in the rewarding effects of diverse classes of addictive drugs (Carboni et al., 1989; Fadda et al., 1991; Higgins et al., 1992a,b; Bisaga et al., 1993; Kostowski et al., 1993; Lu et al., 1994; McMillen et al., 1994; Tomkins et al., 1994a,b, 1995; Rompre et al., 1995; Parsons et al., 1998; Wilson et al., 1998; Fletcher and Korth, 1999; Harrison et al., 1999; Maurel et al., 1999; Tomkins and O'Neill, 2000; Fletcher et al., 2004). Indeed, under some circumstances (e.g. DAT KO mice) the selective 5-HT reuptake inhibitor fluoxetine has been shown to have rewarding effects (Hall et al., 2002).

In monoamine transporter KO mice the rewarding and reinforcing effects of cocaine have been assessed primarily with the CPP paradigm. The effects of these KOs in other paradigms have not been extensively characterized, but should not be expected to be necessarily uniform. The different methods used to assess the rewarding properties of drugs of abuse have often been superficially treated as if they are all equivalent measures of a single unitary construct, in part based on early descriptions equating locomotor stimulant effects with drug reward (Wise and Bozarth, 1987), even though the diversity of reward mechanisms has long been recognized (Wise and Leeb, 1993), especially the role of conditioned responses in the maintenance of drug-seeking behavior and sensitization (Post et al., 1981, 1987; Stewart, 1983). In fact a critical, though often overlooked, distinction has been made between two factors that contribute to cocaine sensitization, the role of conditioned drug effects and the role of neuropharmacological alterations induced by the repeated exposure to drugs of abuse (Pert et al., 1990). These two factors are sometimes described as context-dependent and context-independent sensitization and have been shown to involve different neurobiological mechanisms (Wise and Leeb,

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1993). However, these types of effects involve administration of drugs after repeated treatment and sensitization is evinced by enhanced response to the drug compared to untreated animals or animals treated chronically with saline. However, context-dependent sensitization can be clearly shown to be a conditioned response. The increase in behavioral response in this circumstance is dependent on exposure to the conditioned stimuli and results in conditioned increases in locomotion (e.g. conditioned locomotion) even without any drug treatment. The relative importance of context-dependent and context-independent sensitization for the actual mechanisms underlying addiction is a matter of some debate, and although both are certainly important, it has certainly been argued that alterations in associative processes may play critical roles in addiction (Everitt et al., 2001). However, it is important to note that sensitization to cocaine can be observed independently of conditioned locomotion (Carey and Gui, 1998; Carey and Damianopoulos, 2006). Furthermore, multiple conditioned effects of drugs of abuse can be observed independently of each other, further indicating the non-unitary bases of drug reward and drug seeking behavior. For instance, conditioned locomotor activity can be observed independently from CPP (Kosten and Miserendino, 1998).

In the initial description of the elimination of the locomotor effects of cocaine in DAT KO mice they were described as "indifferent" to cocaine (Giros et al., 1996), the implication being that lack of locomotor stimulant effects should be equated with elimination of rewarding effects. This was proven to be incorrect (Rocha et al., 1998; Sora et al., 1998), but there often remains a tacit assumption that manipulations that affect one aspect of cocaine-mediated behavior should affect other behaviors in a similar manner. One way to directly address this issue is to evaluate gene KOs that produce a particular pattern of effects on one cocaine-associated behavior, and compare them to the consequences of those gene KOs on another cocaine-associated behavior. The effects of monoamine transporter KOs on cocaine CPP have been well characterized: Cocaine CPP is unaffected in DAT KO mice (Sora et al., 1998), but increased in SERT KO and NET KO mice (Sora et al., 1998; Xu et al., 2000). In addition to producing a place preference cocaine also induces conditioned locomotion (Post et al., 1987), which has not been examined for any of these gene KOs. Therefore, to further explore the role of these genes in the rewarding effects of cocaine, the ability of repeated injections of cocaine to induce conditioned locomotion was assessed in DAT, SERT and NET KO mice.

EXPERIMENTAL PROCEDURES

Subjects

DAT (Sora et al., 1998), SERT (Bengel et al., 1998) and NET (Wang et al., 1999) KO mice have been described previously. These KO lines were used to create DAT/SERT (Sora et al., 2001) and NET/SERT (Hall et al., 2002) double KO strains. In the present experiments DAT +/+, DAT +/− and DAT −/− mice were bred from the DAT/SERT line; SERT +/+, SERT +/− and SERT −/− mice were bred from the DAT/SERT line; and NET +/+, NET +/− and NET −/− mice were bred from the NET/SERT line. Male and female mice were used, and were tested at 12–18 weeks of age. Mice were bred from double heterozygote (e.g. DAT +/− SERT +/−×DAT +/− SERT +/−) or single heterozygote (e.g. DAT +/− SERT +/+×DAT +/− SERT +/+) crosses.

Wild-type (+/+), heterozygote KO mice (+/−) and homozygote KO mice (−/−) were genotyped by PCR, using two internal primers, one targeted at the KO insertion sequence and one targeted at the wild-type (WT) gene, and one external primer, which generated two products identifying the WT and KO genes. The DAT and SERT transgenic KO insertion sequences contained a neomycin gene (NEO), while the NET KO contained a green fluorescent protein gene insert (GFP). PCR using Takara DNA polymerase (Takara Bio, Japan) was performed on DNA that was released from tail tip fragments after overnight digestion with protease K. For DAT genotyping the external primer (5′ AGT GTG TGC AGG GCA TGG TGT A 3′) and the WT primer (5′ TAG GCA CTG CTG ACG ATG ACT G 3′) produced a 500 bp band, while the external primer and the NEO primer (5′ CTC GTC GTG ACC CAT GGC GAT 3′) produced a 600 bp band. For SERT genotyping the external primer (5′ GCT CTC AGT CTT GTC TCC ATA AC 3′) and the WT primer (5′ TGC TGA CTG GAG TAC AGG CTA G 3′) produced a 620 bp band, while the external primer and the NEO primer (5′ CTC GTC GTG ACC CAT GGC GAT 3′) produced an 800 bp band. For NET genotyping the external primer (5′ GCT CTG TCC CTG TGC TTC ACG 3′) and the WT primer (5′ TGA GGC CTA AGC TGG AGC TCG 3′) produced a 601 bp band, while the external primer and the GFP primer (5′ CGG TGA ACA GCT CCT CGC CC 3′) produced a 470 bp band.

Conditioned locomotion procedure

Homozygous and heterozygous DAT, NET and SERT KO mice and WT littermate controls were divided into three experimental groups: Paired, Unpaired and Control groups (DAT KO, N=8–12 per genotype per condition; NET KO, N=8–11 per genotype per condition; SERT KO, N=9–18 per genotype per condition). Mice in each group received two injections each day, one before being placed in a locomotor activity chamber and one later in the home cage. Locomotor testing was conducted using an Optovarimax locomotor activity testing apparatus (Columbus Instruments, Columbus, OH, USA) under dark conditions in sound attenuating chambers. Mice in the Paired group received an injection of cocaine HCl (20 mg/kg SC) prior to locomotor testing for 30 min. Subjects were then returned to their home cages and 2 h later they received an injection of saline (10 ml/kg). Mice in the Unpaired group received an injection of saline prior to locomotor testing and an injection of cocaine (20 mg/kg SC) in the home cage. Mice in the control group received saline injections before locomotor testing and in the home cage. This procedure was conducted each day for 5 days; on the day following the final injections, mice were placed in the locomotor activity chambers for 20 min without any injections to assess conditioned locomotion.

Statistics

Statistical comparisons were made with analysis of variance (ANOVA) followed by Scheffe's post hoc analyses using StatView V. 5.0 (SAS Institute, Inc.). Conditioning data were initially analyzed by an overall ANOVA with the between subjects factors of Genotype (+/+, +/− and −/−) and Conditioning Group (Paired, Unpaired and Control), and the additional within-subjects factor of Conditioning Trial (days 1–5). Subsequently, the data for each genotype (+/+, +/− and −/−) were analyzed separately with the between-subjects factor of Conditioning Group (Paired, Unpaired and Control), and the within-subjects factor of Conditioning Trial (day 1–5). Data from the post-conditioning test were analyzed with the between subjects factors of Conditioning Group and Genotype

(+/+, +/− and −/−). Post hoc comparison's were made with Scheffe's test ($P<0.05$ significance level).

RESULTS

Locomotion during conditioning trials in DAT KO mice

During the conditioning trials mice receiving injections of cocaine prior to testing (Paired Group) were significantly more active than mice treated with saline prior to testing (Unpaired and Control groups) as reflected by an overall significant effect of Conditioning Group ($F[2,87]=66.2$, $P=0.0001$; Fig. 1A–C). DAT −/− mice were significantly more active under all conditions compared to DAT +/− and DAT +/+ mice as reflected by a significant effect of Genotype ($F[2,87]=71.0$, $P=0.0001$), but did not exhibit increases in locomotor activity after acute cocaine administration so that there was also a significant Genotype× Conditioning Group interaction ($F[4,87]=4.9$, $P=0.0013$).

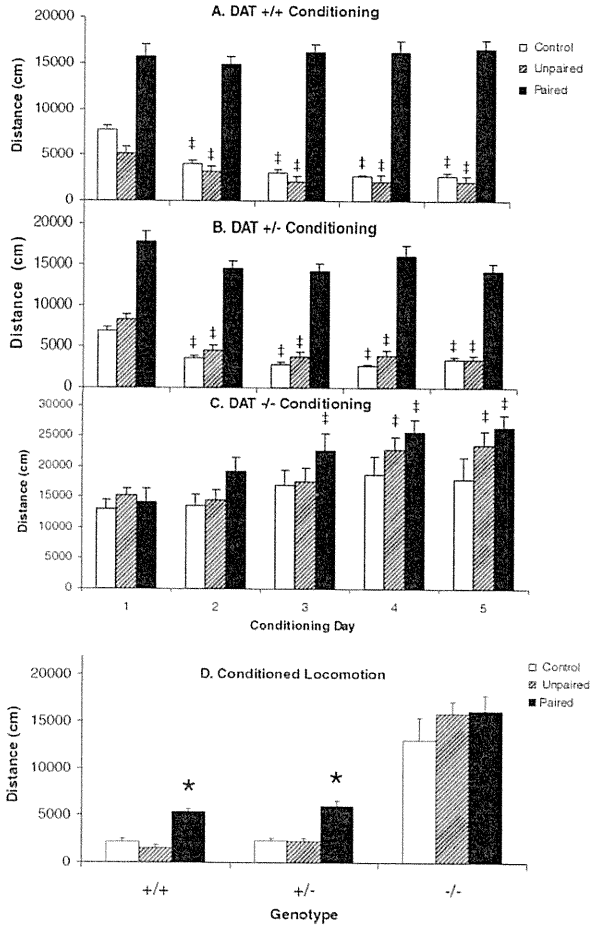

Fig. 1. Conditioned locomotion in DAT KO mice. Locomotor activity during conditioning sessions in DAT +/+ (A), DAT +/− (B) and DAT −/− (C) mice from each of the conditioning groups (Paired, Unpaired and Control) expressed in terms of distance traveled. Conditioned locomotion (D) in all groups. * Significant difference from Control conditioning group based on Scheffe's post hoc comparison ($P<0.05$). ‡ Significant difference from Trial 1 based on Scheffe's post hoc comparison ($P<0.05$). Data are represented as mean±the standard error of the mean.

Over the course of the conditioning trials locomotor activity decreased in DAT +/+ and DAT +/− saline-treated subjects, but not DAT +/+ and DAT +/− cocaine-treated subjects so that the relative magnitude of the cocaine effect increased over trials. In DAT −/− mice a different pattern of effects was observed. Unlike DAT +/+ and DAT +/− mice the activity of Control DAT −/− mice did not decrease. Furthermore, although there was no initial difference in locomotor activity between conditioning groups, over trials the activity of the cocaine-treated groups (Paired and Unpaired) increased. Note that only the Paired subjects received cocaine prior to this locomotor test, the Unpaired subjects were injected with saline. Thus, in the ANOVA there were significant effects of Conditioning Trial ($F[4,348]=8.1$, $P=0.0001$), Conditioning Trial×Conditioning Group ($F[8,348]=6.0$, $P=<0.0001$), Conditioning Trial×Genotype ($F[8,348]=30.5$, $P=0.0001$), and Conditioning Trial×Conditioning Group×Genotype ($F[16, 348]=2.0$, $P=0.012$). To further clarify the nature of these effects individual ANOVAs were performed on the data from each genotype.

DAT +/+ mice treated with cocaine prior to locomotor testing (Paired group) were significantly more active than mice treated with saline (Unpaired and Control groups) throughout all five conditioning trials (Fig. 1A; Conditioning Group: $F[2,31]=99.6$, $P=0.0001$). Over the course of the five conditioning trials the activity of mice in the Unpaired and Control groups decreased, but the activity of mice in the Paired group was unchanged compared to day 1 so that the relative difference between saline-injected and cocaine-injected animals was greater in later trials. Thus, there was a significant interaction between Conditioning Group and Conditioning Trial ($F[8,124]=5.6$, $P=0.0001$). Post hoc Scheffe's comparisons demonstrated significantly reduced locomotion in acute saline-treated groups (Unpaired and Control) for conditioning trials 2–5 compared to the first conditioning trial, but no differences between trials in the acute cocaine treated group (Paired).

A somewhat similar pattern was observed in DAT +/− mice (Fig. 1B), where there was a significant effect of Conditioning Group ($F[2,31]=147.6$, $P=0.0001$), but not a significant interaction between Conditioning Group and Conditioning Trial ($F[8,124]=1.4$, NS). In addition to decreases in locomotion in the Unpaired and Control groups, there was also a slight decrease in the activity of Paired subjects over trials. Post hoc one way ANOVA for each conditioning group revealed significant effects of Conditioning Trial in all three conditioning groups. Post hoc Scheffe's comparisons demonstrated significantly reduced locomotion in acute saline-treated groups (Unpaired and Control) for conditioning trials 2–5 compared to trial 1. In the Paired group the reduction in locomotion was much smaller than in Unpaired and Control subjects so that no individual comparisons were significant even though there was an overall effect in the ANOVA.

In contrast to the pattern of effects observed in DAT +/+ and DAT +/− mice, a completely different pattern was observed in DAT −/− mice. As has been observed previously, cocaine did not increase locomotor activity in

DAT −/− mice (Fig. 1C), although locomotion was substantially higher than the activity observed in DAT +/+ and DAT +/− mice (compare saline-treated subjects in Fig. 1A–1C). Nonetheless, there was an increase in locomotion on the second and subsequent days in cocaine-treated subjects (Paired group compared to the Control group). This increase in locomotion however was not limited to mice in the Paired group; the activity of mice in the unpaired group also increased over conditioning trials. Although there was not a significant overall effect of Conditioning Group (F[2,25]=2.6, NS), there was a significant effect of Conditioning Trial (F[4,100]=21.4, P=0.0001) and a significant interaction between Conditioning Group and Conditioning Trial (F[8,100]=2.6, P=0.013). In separate one way ANOVA performed on each conditioning group no effect of Conditioning Trial was found in Control subjects (F[4,28]=1.5, NS), but significant effects were observed in both Paired (F[4,36]=18.9, P=0.0001) and Unpaired (F[4,36]=9.2, P=0.0001) groups. Post hoc comparisons of activity versus the first testing day demonstrated significant increases in both Paired subjects on trials 3–5 compared to trial 1, and on trials 4–5 compared to trial 1 in Unpaired mice (Scheffe's post hoc comparisons).

Conditioned locomotion in DAT KO mice

In the post-conditioning test DAT +/+ and DAT +/− mice demonstrated a typical pattern consistent with conditioned locomotion (Fig. 1D): increased locomotor activity during the post-conditioning test in Paired mice compared to both Unpaired and Control mice. This test was conducted without any drug injection so it only reflects the ability of the conditioned associations of the environment to evoke locomotion. DAT −/− mice were much more active than DAT +/+ and DAT +/− mice independent of conditioning group. Thus, there were significant effects of both Conditioning Group (F[2,87]=12.7, P=0.0001) and Genotype (F[2,87]=126.5, P=0.0001). In post hoc Scheffe's comparisons in DAT +/+ and DAT +/− Paired subjects were significantly more active than either Unpaired or Control subjects. Locomotor activity during the post-conditioning test was slightly greater in both Paired and Unpaired DAT −/− mice, compared to Control subjects, but neither comparison was significant.

Locomotion during conditioning trials in NET KO mice

During the conditioning trials mice receiving injections of cocaine prior to testing (Paired Group) were significantly more active than mice treated with saline prior to testing (Unpaired and Control groups) as reflected by an overall significant effect of Conditioning Group (F[2,81]=373.6, P=0.0001; Fig. 2A–C). Over the course of the conditioning trials locomotor activity decreased in saline-treated subjects, but not in cocaine-treated subjects, so that there was a significant Conditioning Trial×Conditioning Group interaction (F[8,324]=6.7, P=0.0001). There was no effect of Genotype (F[2,81]=2.7, NS), nor any significant interactions with genotype: Conditioning Group×Genotype (F[4,81]=0.5, NS), Conditioning trial×Genotype (F[8,324]=1.6, NS), and Conditioning Trial×Conditioning Group×Genotype (F[16,324]=1.6, NS). Thus, for all genotypes individual post hoc ANOVA identified only the effects of Conditioning Trial, Conditioning Group, and their interaction.

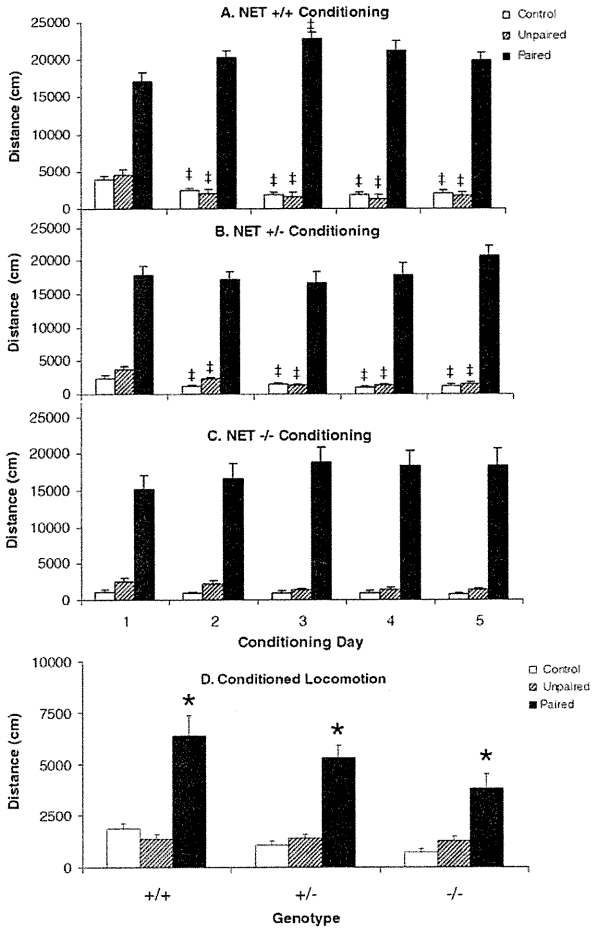

Fig. 2. Conditioned locomotion in NET KO mice. Locomotor activity during conditioning sessions in NET +/+ (A), NET +/− (B) and NET −/− (C) mice from each of the conditioning groups (Paired, Unpaired and Control) expressed in terms of distance traveled. Conditioned locomotion (D) in all groups. * Significant difference from Control conditioning group based on Scheffe's post hoc comparison (P<0.05). ‡ Significant difference from Trial 1 based on Scheffe's post hoc comparison (P<0.05). Data are represented as mean±the standard error of the mean.

NET +/+ mice treated with cocaine before testing (Paired group) were significantly more active than mice treated with saline (Unpaired and Control groups) over all conditioning trials (Fig. 2A, F[2,25]=128.5, P=0.0001). Over the course of the five conditioning trials the activity of saline-treated mice in the Unpaired and Control groups decreased, but the activity of mice in the paired group actually increased compared to day 1. These differential changes over conditioning trials resulted in a significant interaction between Conditioning Group and Conditioning Trial in the ANOVA (F[8,100]=9.6, P=0.0001). Post hoc one-way ANOVA for each conditioning group in NET +/+ mice revealed a significant effect of Conditioning Trial in Control mice (F[4,36]=17.1, P=0.0001), Unpaired mice

(F[4,36]=14.8, P=0.0001) and Paired mice (F[4,28]=4.2, P=0.0082). In both Control and Unpaired NET +/+ mice locomotor activity scores in trials 2–5 were all significantly lower than trial 1 (P<0.05 Scheffe's comparison). In Paired NET +/+ mice only trial 3 was significantly greater than trial 1 (P<0.05 Scheffe's comparison), but in no cases were decreases in activity observed in relation to trial 1.

A similar pattern was observed in NET +/− mice (Fig. 2B), where there was a significant effect of Conditioning Group (F[2,28]=141.1, P=0.0001), Conditioning Trial (F[4,112]=3.1, P=0.019) and a significant interaction between Conditioning Group and Conditioning Trial (F[8,112]=2.9, P=0.0060). Post hoc one-way ANOVA for each conditioning group in NET +/− mice revealed a significant effect of Conditioning Trial in Control mice (F[4,36]=6.6, P=0.0004), and Unpaired mice (F[4,36]=12.2, P=0.0001) but not Paired mice (F[4,40]=2.3, NS). In Control NET +/− mice locomotor activity was significantly reduced in conditioning trials 2, 4 and 5 compared to trial 1 (P<0.05, Scheffe's comparison), while in Unpaired NET +/− mice locomotor activity was significantly reduced in trials 2–5 compared to trial 1 (P<0.05, Scheffe's comparison). In Paired NET +/− mice no decreases in activity were observed.

In NET −/− mice administration of cocaine produced increases in locomotion across all conditioning trials but activity in NET −/− mice changed less across conditioning trials than activity in NET +/+ and NET +/− mice (Fig. 2C). Thus, there was a significant effect of Conditioning Group (F[2,28]=108.4, P=0.0001), but not Conditioning Trial (F[4,112]=0.3, NS), nor was there a significant interaction between Conditioning Group and Conditioning Trial (F[8,112]=1.3, NS).

Conditioned locomotion in NET KO mice

NET +/+, NET +/− and NET −/− mice demonstrated the typical pattern consistent with conditioned locomotion (Fig. 2D) as shown by a significant effect of Conditioning Group in the ANOVA (F[2,81]=73.0, P=0.0001). In addition, activity was slightly reduced in NET KO mice independent of conditioning group. Thus, there was a significant effect of Genotype (F[2,81]=5.9, P=0.0041) in the ANOVA, but not a significant Genotype×Conditioning Group interaction (F[4,81]=1.9, NS).

Locomotion during conditioning trials SERT KO mice

During the conditioning trials mice receiving cocaine prior to testing were significantly more active than mice treated with saline as reflected by an significant effect of Conditioning Group (F[2,104]=51.9, P=0.0001; Fig. 3A–C). SERT −/− mice were significantly less active under all conditions compared to SERT +/− and SERT +/+ mice as reflected by a significant effect of Genotype (F[2,104]=5.1, P=0.0078). The Genotype×Conditioning Group interaction was not significant overall (F[4,104]=1.9, NS), but there were differences between groups that emerged over repeated conditioning trials resulting in a significant Genotype×Conditioning Group×Conditioning Trial interaction (F[16,416]=2.0, P=0.012). Over the course of the conditioning trials locomotor activity decreased in saline-treated subjects of all genotypes. Locomotor activity did not decrease in SERT +/+ or SERT +/− acute cocaine-treated subjects so that the relative magnitude of the cocaine effect increased over trials, but the magnitude of locomotion in the SERT −/− mice treated with cocaine decreased so that the magnitude of the cocaine effect did not change over conditioning trials.

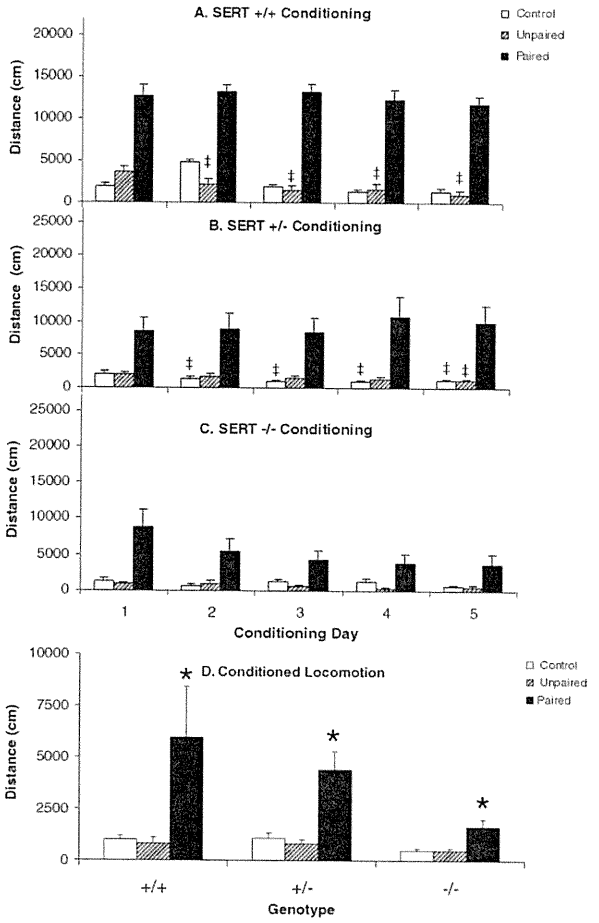

Fig. 3. Conditioned locomotion in SERT KO mice. Locomotor activity during conditioning sessions in SERT +/+ (A), SERT +/− (B) and SERT −/− (C) mice from each of the conditioning groups (Paired, Unpaired and Control) expressed in terms of distance traveled. Conditioned locomotion (D) in all groups. * Significant difference from Control conditioning group based on Scheffe's post hoc comparison (P<0.05). ‡ Significant difference from Trial 1 based on Scheffe's post hoc comparison (P<0.05). Data are represented as mean±the standard error of the mean.

SERT +/+ mice treated with cocaine prior to locomotor testing (Paired group) were significantly more active than mice treated with saline (Unpaired and Control groups) on the first and subsequent days (Fig. 3A). There was a significant effect of Conditioning Group (F[2,27]=22.5, P=0.0001), but the interaction between Conditioning Group and Conditioning Trial was not significant (F[8,108]=0.4, NS). Decreased locomotion across trials was observed in both saline-treated groups as confirmed in one-way ANOVA for the Unpaired (F[4,44]=14.8, P=0.0001) and Control (F[4,32]=3.9, P=0.011) groups. In post hoc Scheffe's comparisons in the Control group there

were no individual trials that were significantly different from trial 1, but in the Unpaired group trials 2–5 were all significantly lower than trial 1. There was no change in locomotion over trials in the Paired group ($F[4,32]=0.1$, NS).

A similar pattern was observed in SERT +/− mice (Fig. 3B), where there was a significant effect of Conditioning Group ($F[2,40]=14.1$, $P=0.0001$), but not a significant interaction between Conditioning Group and Conditioning Trial ($F[8,160]=1.5$, NS). Again, decreased locomotion across trials was observed in both saline-treated groups as confirmed in one-way ANOVA for the Unpaired ($F[4,40]=4.0$, $P=0.084$) and Control ($F[4,52]=9.2$, $P=0.0001$) groups. In post hoc Scheffe's comparisons for the Control group trials 2–5 were significantly lower than trial 1. For the Unpaired group only trial 5 was significantly lower than trial 1. There was no change in locomotion over trials in the Paired group ($F[4,68]=1.3$, NS).

In SERT −/− mice a different pattern of effects emerged. Administration of cocaine produced increases in locomotion on all conditioning trials (Fig. 2C), as shown by a significant effect of Conditioning Group ($F[2,37]=33.7$, $P=0.0001$). Locomotion decreased across trials, as shown by a significant overall effect of Conditioning Trial ($F[4,148]=5.6$, $P=0.003$), but this effect was due primarily to reductions in locomotion in the cocaine-treated group. Thus, there was a significant interaction between Conditioning Group and Conditioning Trial ($F[8,148]=3.3$, $P=0.0017$). Individual post hoc one-way ANOVA revealed a significant effect of Conditioning Trial in Paired SERT −/− mice ($F[4,40]=3.2$, $P=0.023$), but not Unpaired ($F[4,68]=1.3$, NS) or Control ($F[4,40]=2.1$, NS) SERT −/− mice.

Conditioned locomotion in SERT KO mice

SERT +/+, SERT +/− and SERT −/− mice demonstrated the typical pattern consistent with conditioned locomotion (Fig. 3D), as demonstrated by a significant overall effect of Conditioning Group ($F[2,104]=19.7$, $P=0.0001$). In addition, SERT −/− mice had reduced locomotion independent of conditioning group as demonstrated by a significant overall effect of Genotype ($F[2,104]=4.7$, $P=0.011$). This reduction in locomotor activity in SERT −/− mice compared to SERT +/+ mice was somewhat greater in Paired subjects than in Unpaired or Control subjects. The Conditioning Group×Genotype interaction was just statistically significant ($F[4,104]=2.5$, $P=0.050$). Nonetheless, for all genotypes Paired mice had significantly greater activity than Unpaired or Control mice ($P<0.05$, Scheffe's post hoc comparison).

DISCUSSION

The main conclusion that may be drawn from these experiments is that the ability of cocaine to produce conditioned locomotion is dependent on DAT, but not NET or SERT. This is consistent with a dopamine lesion study which found that 6-OHDA-induced lesions of the nucleus accumbens attenuated amphetamine conditioned locomotion (Gold et al., 1988). In addition, differences in context-independent sensitization and context-dependent sensitization were found in DAT KO, SERT KO and NET KO mice during the conditioning phase of the experiment. These are discussed below in detail but further emphasize the non-unitary structure of drug reward mechanisms, the polygenic basis of drug reward mechanisms, and the involvement of all three of these neurotransmitters in cocaine-mediated behavior, albeit to a different degree and in different circumstances.

The role of conditioned responses in drug-seeking behavior has long been recognized, including the role of conditioned responses in cocaine sensitization (Post et al., 1981, 1987; Stewart, 1983). Different underlying mechanisms are known to be involved in context-dependent sensitization and context-independent sensitization (Wise and Leeb, 1993), in particular, but this same argument can be applied to numerous cocaine-induced behaviors including acute locomotor responses, conditioned locomotion and CPP. For instance, differential sensitivity to cocaine sensitization across inbred strains of mice is not simply the result of differential acute sensitivity (Elmer et al., 1996). Different types of drug exposure experiences that enhance cocaine responses clearly have a different basis, including those relating to repeated drug exposure alone and those involving associative mechanisms. Enhanced responses after repeated cocaine treatments have both context-dependent and context-independent components, which can be dissociated, but specific conditioned responses can be further dissociated, including conditioned locomotion (Carey and Gui, 1998; Carey and Damianopoulos, 2006), which is not correlated with sensitization to cocaine (Hotsenpiller and Wolf, 2002; Tirelli et al., 2003) and persists for a longer time (Tirelli et al., 2005). Furthermore, conditioned responses can be dissociated from each other, including conditioned locomotion and CPP (Kosten and Miserendino, 1998).

Since many effects of cocaine and other psychostimulants have been thought to involve primarily dopaminergic mechanisms (Wise and Bozarth, 1987), much research has emphasized the importance of dopamine in these effects. This includes the first publication in DAT KO mice, in which these mice were described as "indifferent" to cocaine because they failed to exhibit locomotor stimulant responses after acute treatment (Giros et al., 1996). The presumption here was that all cocaine effects, including rewarding effects, could be represented in a unitary fashion by cocaine-stimulated locomotion. This was found to be incorrect by the demonstration that DAT KO mice can exhibit both cocaine CPP and cocaine self-administration (Rocha et al., 1998; Sora et al., 1998), although more recent evidence clearly demonstrates that the ability of cocaine to act as a reinforcer is substantially degraded in DAT KO mice (Thomsen et al., 2009). These and other studies also demonstrated the ability of gene KOs of other cocaine targets (e.g. SERT and NET) to modulate the rewarding effects of cocaine (Sora et al., 1998, 2001; Hall et al., 2002). Since there appears to be a somewhat differential involvement of these systems in different cocaine