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RAT BRAIN SEROTONIN NEURONES THAT EXPRESS NEURONAL NITRIC OXIDE SYNTHASE HAVE INCREASED SENSITIVITY TO THE SUBSTITUTED AMPHETAMINE SEROTONIN TOXINS 3,4-METHYLENEDIOXYMETHAMPHETAMINE AND *p*-CHLOROAMPHETAMINE

D. J. DE SILVA,[a1] S. J. FRENCH,[a] N. Y. CHEUNG,[a] A. K. SWINSON,[a] C. BENDOTTI[b] AND M. RATTRAY[a]*

[a]*Wolfson Centre for Age-Related Diseases, School of Biomedical Sciences, King's College London, Guy's Hospital Campus, London SE1 1UL, UK*

[b]*Department of Neuroscience, Mario Negri Institute, via Britrea 62, Milan 20157, Italy*

Abstract—Substituted amphetamines such as *p*-chloroamphetamine and the abused drug methylenedioxymethamphetamine cause selective destruction of serotonin axons in rats, by unknown mechanisms. Since some serotonin neurones also express neuronal nitric oxide synthase, which has been implicated in neurotoxicity, the present study was undertaken to determine whether nitric oxide synthase expressing serotonin neurones are selectively vulnerable to methylenedioxymethamphetamine or *p*-chloroamphetamine. Using double-labeling immunocytochemistry and double *in situ* hybridization for nitric oxide synthase and the serotonin transporter, it was confirmed that about two thirds of serotonergic cell bodies in the dorsal raphé nucleus expressed nitric oxide synthase, however few if any serotonin transporter immunoreactive axons in striatum expressed nitric oxide synthase at detectable levels. Methylenedioxymethamphetamine (30 mg/kg) or *p*-chloroamphetamine (2×10 mg/kg) was administered to Sprague–Dawley rats, and 7 days after drug administration there were modest decreases in the levels of serotonin transporter protein in frontal cortex, and striatum using Western blotting, even though axonal loss could be clearly seen by immunostaining. *p*-Chloroamphetamine or methylenedioxymethamphetamine administration did not alter the level of nitric oxide synthase in striatum or frontal cortex, determined by Western blotting. Analysis of serotonin neuronal cell bodies 7 days after *p*-chloroamphetamine treatment, revealed a net down-regulation of serotonin transporter mRNA levels, and a profound change in expression of nitric oxide synthase, with 33% of serotonin transporter mRNA positive cells containing nitric oxide synthase mRNA, compared with 65% in control animals. Altogether these results support the hypothesis that serotonin neurones which express nitric oxide synthase are most vulnerable to substituted amphetamine toxicity, supporting the concept that the selective vulnerability of serotonin neurones has a molecular basis.

Key words: 5-hydroxytryptamine, nitric oxide synthase, ecstasy, 5,7-DHT, serotonin transporter.

Serotonin-selective substituted amphetamines including the abused drug, methylenedioxymethamphetamine (MDMA) and *p*-chloroamphetamine (PCA) bind to the serotonin transporter (SERT) and induce an elevation of the levels of serotonin (5-hydroxytryptamine, 5-HT) in the synaptic cleft, by blocking reuptake of 5-HT and inducing 5-HT efflux via reversal of SERT (Green et al., 2003; Fuller, 1992; Rudnick and Wall, 1992a,b). In animals, administration of a single or multiple doses of MDMA or PCA has been consistently associated with a neurotoxicity characterized by a long-term loss of biochemical markers of 5-HT nerve terminals and the appearance of broken and swollen 5-HT axons (Green et al., 2003; Schmidt et al., 1986; O'Hearn et al., 1988). Because of the widespread abuse of MDMA, this 5-HT neurotoxicity is of considerable importance. Although at first glance the doses of MDMA administered to animals to produce 5-HT neurotoxicity appear much higher than those by humans, the use of inter-species dose scaling suggests some individuals who are taking multiple doses are consuming doses that have been shown to demonstrate neurotoxicity in animals. Evidence for 5-HT neurotoxicity in man stems from the measurement of 5-HT metabolites in the cerebrospinal fluid and positron emission tomography neuroimaging of ecstasy users (McCann et al., 1994, 1998). In addition a variety of corroborative evidence suggests MDMA induces a persistent alteration in 5-HT function. These studies include assessment of functional change such as cognition, memory, sleep, and personality (Green et al., 2003).

The exact mechanisms underlying the toxicity of MDMA or PCA are largely unknown. Toxicity appears to be critically dependent on SERT activity, and it is thought that a toxin—either derived from the drug itself or a biogenic amine released as a result of MDMA administration—is taken up selectively via SERT into 5-HT nerve terminals whereby it causes damage through a variety of mecha-

[1] Present address: Western Eye Hospital, Marylebone Road, London, NW1 5YE, UK.

*Corresponding author. Tel: +44-020-7848-6172; fax: +44-020-7848-6145.

E-mail address: marcus.rattray@kcl.ac.uk (M. Rattray).

Abbreviations: DRd, dorsomedial subdivision of the dorsal raphé nucleus; DRl, lateral subdivision of the dorsal raphé nucleus; DRv, ventromedial subdivision of the dorsal raphé nucleus; MDMA, methylenedioxymethamphetamine; MR, median raphé nucleus; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PCA, *p*-chloroamphetamine; PCPA, *p*-chlorophenylalanine; SERT, serotonin transporter; TBS-X, 20 mM Tris, 500 mM NaCl, pH 7.4 containing 0.1% Triton X-100; T-TBS, TBS containing 0.5% Tween-20; 5-HT, 5-hydroxytryptamine.

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nisms, most likely including generation of intracellular oxidative stress (Colado and Green, 1995; Cadet et al., 1995; Murray et al., 1996; Yeh, 1997; Colado et al., 1997, 1999; Shankaran et al., 2001; Camarero et al., 2002; Green et al., 2003). Not all 5-HT neurones are affected equally. Studies suggest that fine 5-HT axons originating in the dorsal raphé nucleus, such as those which innervate the striatum and frontal cortex, are most sensitive while 5-HT axons with more beaded varicosities, such as those originating in the median raphé nucleus (MR) that innervate the hippocampus are selectively spared (O'Hearn et al., 1988; Mamounas and Molliver, 1988; Wilson et al., 1989; Mamounas et al., 1991; Brown and Molliver, 2000). However this selective vulnerability is not completely supported by biochemical data, where typically the losses of tissue levels of 5-HT are similar in hippocampus compared with striatum and cerebral cortex. It is not known whether selective vulnerability of 5-HT axons is due to biochemical differences between 5-HT axons, such as expression of enzymes that may contribute to neurotoxicity, for example nitric oxide synthase (NOS).

Nitric oxide (NO) may be involved in MDMA and PCA toxicity, since it has been strongly implicated as a contributor to neurodegeneration of dopamine neurones following exposure to amphetamines or MPTP (Schulz et al., 1995; Imam et al., 2001; Sanchez et al., 2003). NO is a gaseous neuromodulator with a range of effects in the CNS that is synthesized on demand by NOS. One NOS isoform, neuronal nitric oxide synthase (nNOS) is expressed by a subset of 5-HT neurones in the dorsal raphé as visualized by NADPH diaphorase staining or by immunocytochemistry (Johnson and Ma, 1993; Wotherspoon et al., 1994; Xu and Hokfelt, 1997; Tagliaferro et al., 2001), therefore NO might be produced directly within 5-HT cell bodies and axons to contribute to MDMA- or PCA-induced 5-HT axonal damage. NO can promote oxidative damage of neurones by the generation of reactive nitrogen species and reactive nitrogen species, including the peroxynitrite anion (Lipton et al., 1993; Dawson and Dawson, 1998). It is not yet known to what extent activation of nNOS contributes to the production of reactive oxygen or reactive nitrogen species that are involved in 5-HT neurotoxicity observed after MDMA or PCA administration. No studies have clearly shown whether or not NO has a role in MDMA- or PCA-produced toxicity in rats. A NOS inhibitor, L-NAME was shown to block MDMA-induced cell death in JAR cells (Simantov and Tauber, 1997). However, *in vivo* studies in rats do not unequivocally support a role for NO in MDMA-induced 5-HT system damage. Zheng and Laverty (1998) used the NOS inhibitor, *N* omega-nitro-L-arginine, and found only partial protection in some brain regions, for example in frontal cortex but not striatum. Taraska and Finnegan (1997) found the nNOS inhibitor, L-NAME was protective against MDMA induced 5-HT depletion, but two other NOS inhibitors, *NG*-monomethyl-L-arginine or *NG*-nitro-L-arginine, were ineffective in preventing MDMA-induced 5-HT toxicity. More recently Darvesh et al. (2005) showed that the NOS inhibitor *S*-methyl-L-thiocitrulline prevented MDMA-induced 5-HT depletion in striatum. Thus the exact role of NO in 5-HT neurotoxicity is unclear at the present time.

While NO may contribute to MDMA- or PCA-induced 5-HT neurotoxicity, it may also be neuroprotective. It is clear that NO can have a protective role, depending on the exact cellular conditions into which it is released (Lipton et al., 1993). For example, the selective sparing of nNOS containing neurones in areas of infarction suggests nNOS containing neurones may be more resistant to ischemic insult (Zhang et al., 1994). NO may afford protection by vasodilation following ischemia, the inhibition of calcium influx through NMDA receptors and scavenging of reactive oxygen species (e.g. Dawson et al., 1994). Thus, it is possible that 5-HT neurones which express nNOS may be more resistant to the toxic effects of MDMA or PCA compared with non-nNOS expressing neurones.

In summary, there is a body of evidence describing the neurotoxic effects of substituted amphetamines on 5-HT neurones in rats that suggest NO may be a modulatory factor of this neurotoxicity. The aim of this study was to further investigate the role of NO in substituted amphetamine induced 5-HT neurotoxicity, in particular to determine whether NO serves a neuroprotective or neurotoxic role. Since nNOS is an endogenous enzyme found in a subpopulation of 5-HT neurones in the dorsal raphé nucleus, we postulated that, if nNOS contributed to substituted amphetamine-induced 5-HT toxicity, we would see selective vulnerability of nNOS positive 5-HT axons compared with nNOS negative 5-HT axons following treatment with MDMA or substituted amphetamines. To address this question, we carried out a detailed characterization of nNOS expression in relation to a marker of 5-HT neurones, SERT, using immunocytochemical and *in situ* hybridization techniques. We used immunocytochemical methods, Western blotting and *in situ* hybridization to assess the response of nNOS-expressing 5-HT neurones to PCA or MDMA.

EXPERIMENTAL PROCEDURES

Animals and drug treatments

All reagents were supplied by VWR International (Poole, UK), unless detailed otherwise below. Male Sprague–Dawley rats (225–250 g, Harlan, UK) were used in all experiments. Animals were housed two per cage in a temperature-controlled room (23–25 °C) with 12-h alternating light/dark cycle (lights on 09:00 h). Animals were allowed free access to standard laboratory food and water. All procedures were carried out under UK Home Office procedures for the care and use of laboratory animals, therefore conforming with international standards to minimize animal use and suffering. Groups ($n=6$ in each case) of rats received either PCA (Sigma, Poole, UK; 10 mg/kg, i.p. daily for 2 consecutive days), or a single injection of ±MDMA (Sigma, 30 mg/kg, i.p.). This dose was chosen since a single dose of MDMA (20–40 mg/kg) has been consistently reported to cause significant striatal 5-HT depletion in rats (Nichols and Nash, 1991; Nash et al., 1990; Schmidt et al., 1990a,b; Zheng and Laverty, 1998). In some experiments, rats were administered 5,7-DHT (150 μg), i.c.v. (described by Bendotti et al., 1993). In all cases, vehicle injections were used as controls.