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GENETIC INTERDEPENDENCE OF ADENOSINE AND DOPAMINE RECEPTORS: EVIDENCE FROM RECEPTOR KNOCKOUT MICE

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Abstract—Dopamine and adenosine receptors are known to share a considerable overlap in their regional distribution, being especially rich in the basal ganglia. Dopamine and adenosine receptors have been demonstrated to exhibit a parallel distribution on certain neuronal populations, and even when not directly co-localized, relationships (both antagonistic and synergistic) have been described. This study was designed to investigate dopaminergic and purinergic systems in mice with ablations of individual dopamine or adenosine receptors. *In situ* hybridization histochemistry and autoradiography was used to examine the level of mRNA and protein expression of specific receptors and transporters in dopaminergic pathways. Expression of the mRNA encoding the dopamine D_2 receptor was elevated in the caudate putamen of D_1, D_3 and A_{2A} receptor knockout mice; this was mirrored by an increase in D_2 receptor protein in D_1 and D_3 receptor knockout mice, but not in A_{2A} knockout mice. Dopamine D_1 receptor binding was decreased in the caudate putamen, nucleus accumbens, olfactory tubercle and ventral pallidum of D_2 receptor knockout mice. In substantia nigra pars compacta, dopamine transporter mRNA expression was dramatically decreased in D_3 receptor knockout mice, but elevated in A_{2A} receptor knockout mice. All dopamine receptor knockout mice examined exhibited increased A_{2A} receptor binding in the caudate putamen, nucleus accumbens and olfactory tubercle. These data are consistent with the existence of functional interactions between dopaminergic and purinergic systems in these reward and motor-related brain regions. © 2006 Published by Elsevier Ltd on behalf of IBRO.

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Abbreviations: CPu, caudate putamen; DAT, dopamine transporter; d.p.m., disintegrations per minute; DPMA, *N*6-[2-(3,5-dimethoxyphenyl)-2-(methylphenyl)ethyl]adenosine; NAcc, nucleus accumbens; OT, olfactory tubercle; PBS, phosphate-buffered saline; SKF 77434, (±)-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; SSC, saline sodium citrate; VP, ventral pallidum; VTA, ventral tegmental area; [^{3}H]CGS 21680, [^{3}H]-2-[4-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamido]adenosine; [^{3}H]-NBTI, [^{3}H]-*S*-(4-nitrobenzyl)-6-thioinosine; [^{125}I]-NCQ 298, (*S*)-3-iodo-*N*-[(1-ethyl-2pyrrolidinyl) methyl]-5,6-dimethoxysalicylamide; [^{125}I]-SCH 23982, (5*R*)-8-iodo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepine-7-ol.

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Dopamine is a neurotransmitter with a role in diverse neuronal functions, including cognitive, motor, mood and reward processing (Gingrich and Caron, 1993; Jaber et al., 1996; Drago et al., 1998). Molecular cloning techniques have led to the identification of at least five distinct dopamine receptor subtypes, D_1–D_5, and these receptors have been further separated into receptor families; D_1- (D_1 and D_5) and D_2- (D_{2L} (long) and D_{2S} (short), D_3 and D_4) like receptor subtypes (Sibley and Monsma, 1992). Functionally, D_1-like receptors are generally stimulatory, while D_2-like receptors are considered to be inhibitory. Interestingly, the analysis of dopaminergic function becomes more complex with significant evidence describing interactions between D_1 and D_2 receptors, both antagonistic and synergistic, at a variety of levels (Clark and White, 1987; Nestler, 1994). The opposing effects of dopamine receptors upon adenylyl cyclase may in part explain the antagonistic interactions of D_1- and D_2-like receptors; however, the cooperative effect of activating both receptor subtypes, for example the 'enabling effect' of D_1-like receptors upon many D_2 receptor-mediated responses (e.g. stereotypy, climbing and turning behaviors) is more complicated (Robertson and Robertson, 1986; Clark and White, 1987; White, 1987; Fetsko et al., 2003).

Of interest is the substantial overlap in A_{2A} and dopamine receptor expression. The adenosine A_{2A} receptor is localized in brain regions rich in dopamine D_1 and D_2 receptor expression, notably, the caudate putamen (CPu), nucleus accumbens (NAcc) and olfactory tubercle (OT) (Schiffmann et al., 1991b; Johansson et al., 1993b). There is a wide body of literature describing functional, negative interactions between dopamine and adenosine receptors; receptors that are extensively co-localized within brain regions involved in movement and reinforcement processing (Schiffmann et al., 1991a; Fink et al., 1992; Augood and Emson, 1994; Svenningsson et al., 1997). Direct interactions between dopamine D_2 and A_{2A} receptors have been described (these receptor subtypes are mainly expressed upon the same striatal neuronal population, therefore this interaction may be occurring receptor–receptor, at the membrane level) (Ferré et al., 1991, 1993; Dasgupta et al., 1996). Interactions between D_1 and A_{2A} receptors have also been demonstrated, this interaction presumably occurring at the network level via the modulation of output pathways (Short et al., 2005), and thus the A_{2A} receptor is considered a potential target for drug development or therapeutic interventions intended to alter dopaminergic func-

tion within the basal forebrain (Pollack and Fink, 1996; Ferré et al., 1997).

Due to the well-described functional interactions between dopamine and adenosine receptors, in concert with substantial co-localization of specific receptor subtypes, we hypothesize that these receptor systems will be subject to perturbation in independently generated dopamine D_1, D_2 and D_3 and adenosine A_{2A} receptor knockout mice. In the present study, therefore, techniques such as *in situ* hybridization histochemistry and autoradiography were used to examine the level of mRNA and protein expression of specific receptors and transporters within reward-related areas of the brain, across adenosine and dopamine receptor knockout mice, concurrently. These studies therefore question whether there is genetic interdependence between adenosine and dopamine receptors.

EXPERIMENTAL PROCEDURES

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986, under the guidelines of the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. Formal approval for all experiments was granted by the Department of Pharmacology Animal Ethics Committee (Monash University). All efforts were made to minimize the number of animals used and their suffering, and all experiments were completed on adult mice. A_{2A} receptor knockout mice (developed on a CD-1 background) were provided by Ledent and associates (1997), and were then backcrossed with C57/Bl6J mice for four generations in our laboratory. D_1 and D_3 receptor knockout mice were developed by Drago et al. (1994) and Accili et al. (1996), respectively, with the D_2 receptor knockout mouse produced by Baik et al. (1995). Appropriate wild-types used in experiments were obtained from litters generated by heterozygous breeders. Animals received laboratory mouse chow and water *ad libitum*, and were kept in a constant 12-h light/dark cycle (light 7 a.m. to 7 p.m.). The genotype of experimental mice was confirmed via polymerase chain reaction and Southern blotting, as previously described (Drago et al., 1994; Snell et al., 2000).

Mice ($n=4-6$ per genotype, plus littermate wild-types) were killed via cervical dislocation and decapitation. Whole mouse brains were frozen over liquid nitrogen, and stored at −80 °C until further processing. Cryostat (Cryocut 1800; Leica, Wetzlar, Germany) -cut coronal sections (14 μm) were collected onto poly-l-lysine- (100 μg/mL) or gelatin (0.5% w/v)/chrome alum (0.05% w/v) -coated microscope slides for *in situ* hybridization histochemistry and autoradiography processing, respectively. Brain tissue was sectioned through the NAcc, ventral pallidum (VP) and ventral tegmental area (VTA), with the appropriate anatomic levels determined according to the atlas of Franklin and Paxinos (1997).

In situ hybridization histochemistry

The experimental procedures described herein were developed from previous studies (McLean et al., 1996; Chen et al., 1998). Slide-mounted sections were placed in ice-cold de-polymerized paraformaldehyde (pH 7.0) for 5 min. The slides were then transferred to a phosphate-buffered saline (PBS: 130 mM NaCl, 7 mM $Na_2HPO_4.2H_2O$, 3 mM $NaH_2PO_4.2H_2O$, pH 7.4) solution at room temperature for 3 min. Sections were dehydrated in a series of 2 min ethanol washes (70, 95 and 100%). Brain sections then underwent delipidation in chloroform for 20 min, before a final rinse in 100% ethanol for 2 min. Sections were placed in PBS for 2 min, before being transferred to a chamber containing triethanolamine (100 mM), 0.9% saline buffer (pH 8.0) and 0.25% acetic anhydride for 10 min. Brain slices were dehydrated in 70% ethanol (2 min) and 95 and 100% ethanol baths for 1 min each. Brain sections were then delipidated in chloroform for 5 min, and rinsed in 100 and 95% ethanol for 1 min and allowed to dry at room temperature.

Oligonucleotide probes were synthesized commercially and diluted to a working solution of 0.3 pmol/μL in diethyl pyrocarbonate–treated milliQ water. Probes were 3′ end labeled at 37 °C for 30 min with [α-^{33}P]dATP using terminal deoxynucleotidyl transferase (Boehringer Mannheim, Germany), to a final radioactivity of between 1.0 and 3.0×10^5 disintegrations per minute (d.p.m.)/μL. Labeled probe was separated from unincorporated nucleotides after elution through a Sephadex G-25 spin column, centrifuged for 1 min at 2000 r.p.m. (GS200 Centrifuge; Clements, Sydney, Australia). The collected oligonucleotide probe was then diluted in hybridization buffer (50% deionized formamide, 4× saline sodium citrate (SSC: 150 mM NaCl, 15 mM sodium citrate; pH 7.0) 10% dextran sulfate), applied to sections (1 pg/μL, 100 μL) and overlaid with Parafilm coverslips to minimize dehydration of the tissue.

Hybridization was completed in a humidified atmosphere, overnight at 42 °C. Non-specific hybridization was determined in the presence of a 100-fold excess of unlabeled oligonucleotide (relative to the molar concentration of the labeled probe). The absence of signal in brain slices hybridized with unlabeled oligonucleotide, and the reproducibility between replicates demonstrated the consistency and specificity of the probe labeling. The following day slides were rinsed in SSC buffer at room temperature before being washed in SSC for 1 h at 55 °C. Finally, brain sections were rinsed in SSC and 10% SSC, dehydrated in 70 and 95% ethanol (30 s each) and dried at room temperature. Hybridized sections were apposed to X-ray film in the presence of standard ^{14}C microscales to allow for densitometric quantification. The time periods of film exposures were dependent on the particular probe and labeling.

Probe sequences: dopamine D_1 receptor mRNA (Drago et al., 1994): 5′-GACAGGGTTT-CCATTACCTG-TGGTGGTCTG-GCA-GTTCTTG-GCATGGAC-3′; dopamine D_2 receptor mRNA (Le Moine et al., 1990): (1) 5′-CCCATTGAAG-GGCCGGCTCC-AGT-TCTGCCT-CTCCAGATCA-TCATCGTA-3′, (2) 5′-GGCGATCAT-G-ACAGTAACTC-GGCGCTTGGA-GCTGTAGCGT-GTGTTATA-3′; adenosine A_{2A} receptor mRNA (Ledent et al., 1997): 5′-CTG-TTGATC-CACACGGCC-CAGCACACA-AGCACGTTA-CCCAGG-ATG-3′; dopamine transporter (DAT) mRNA: 5′-AGTTATTGGT-GAACTTATTG-TAACTGGAGA-AGGCAATCAG-CAC-3′.

Receptor and transporter autoradiography

D_1 receptor autoradiography was completed according to a published protocol (Djouma and Lawrence, 2002). Sections were incubated for 30 min at room temperature in a Tris–HCl (50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$; pH 7.4) buffer containing (5*R*)-8-iodo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepine-7-ol ([^{125}I]-SCH 23982) (0.01 nM) and ketanserin (50 nM), to prevent binding to 5-HT_2 receptors. Non-specific binding was defined as that remaining in the presence of (±)-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SKF 77434) (10 μM). Sections were rinsed briefly and then washed (2×5 min) in fresh ice-cold buffer, before a final rinse (1 s) in ice-cold distilled water.

NCQ 634 (*N*-[(1-ethyl-2pyrrolidinyl)methyl]-5,6-dimethoxysalicylamide) was iodinated to (*S*)-3-iodo-*N*-[(1-ethyl-2pyrrolidinyl) methyl]-5,6-dimethoxysalicylamide ([^{125}I]-NCQ 298) using the oxidizing agent chloramine-T, and then purified by paper chromatography. This procedure and the methodology for $D_{2/3}$-dopamine receptor autoradiography are described elsewhere (Lawrence et al., 1995). Tissue sections were pre-incubated for 30 min at room temperature before incubation with [^{125}I]-NCQ 298 (0.35 nM) for 60 min, again at room temperature (incubation buffers: 170 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$,