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Topographical Assessment of Ethological and Dopamine Receptor Agonist-Induced Behavioral Phenotype in Mutants with Congenic DARPP-32 'Knockout'

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Congenic (10 backcrosses into C57BL/6J) mutants with targeted gene deletion of DARPP-32, a neuronal phosphoprotein regarded as an essential mediator of the biological effects of dopamine (DA), were assessed phenotypically using an ethologically based approach that resolves all topographies of behavior in the mouse repertoire. Over initial exploration, female, but not male, DARPP-32 mutants evidenced increased locomotion and decreased grooming, while a decrease in rearing seated was evident in mutants of both genders; continuing assessment over several hours did not reveal additional phenotypic effects. Following challenge with the nonselective DA receptor agonist apomorphine, low doses were associated with reduced levels of sniffing, grooming, total rearing, and rearing seated in DARPP-32 mutants relative to wildtypes; this would suggest some role for DARPP-32 in mediating the biological effects of presynaptic D_2-like autoreceptor or inhibitory postsynaptic D_2-like receptor activation. Following challenge with higher doses, while stereotyped sniffing and locomotion with chewing was largely unaltered, the additional murine response of Straub tail was essentially abolished in DARPP-32 mutants, indicating some specific involvement of DARPP-32 in mediating this topography of behavior; additionally, there were overall reductions in levels of sniffing, total rearing, rearing seated, and grooming in DARPP-32 mutants that were unrelated to the dose of apomorphine administered, indicating broader topographical effects following the stress of the injection procedure relative to more naturalistic conditions. The developmental absence of DARPP-32 following targeted gene deletion appears to be associated with compensatory processes that maintain certain topographies of spontaneous and agonist-induced DAergic function, while other topographies remain impaired.

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INTRODUCTION

While dopamine (DA) is recognized to be a fundamental regulator of multiple aspects of mammalian behavior, relating specific aspects thereof to individual DA receptor subtypes and to components of their associated transduction mechanisms has proved difficult (Waddington *et al*, 1995, 2001; Di Chiara, 2002; Sidhu *et al*, 2003). Given the lack of both selective ligands for influencing individual DA receptor subtypes and of specific pharmacological tools for manipulating components of DAergic cellular transduction, targeted gene deletion ('knockout') of these entities now offers an alternative approach to functional parcellation. This technique is being applied to construct and phenotype mutant mice with deletion of individual members of the D_1-like (D_{1A}/D_1, D_{1B}/D_5) and D_2-like (D_2, D_3, D_4) families of DA receptor subtypes (Sibley, 1999; Waddington *et al*, 2001). However, to understand more completely the sequence of events by which these subtypes regulate behavior, it is also necessary to clarify the roles therein of the cellular mechanisms by which such receptor events are transducted.

Among these, DA and adenosine 3′,5′-monophosphate-regulated phosphoprotein-32 kDa (DARPP-32) is a neuronal phosphoprotein which in response to DA, is converted into a potent inhibitor of protein phosphatase-1 (PP-1) (Hemmings *et al*, 1984), a critical determinant of the state of

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phosphorylation and hence the physiological activity of a wide array of neuronal phosphoproteins, including neurotransmitter receptors, ion channels, and transcription factors. At this cellular level, D_1-like receptors activate the phosphorylation of DARPP-32, via adenylyl cyclase and protein kinase A (PKA), to inhibit PP-1, while D_2-like receptors dephosphorylate DARPP-32, both via inhibition of PKA and through an adenylyl cyclase-independent pathway, to disinhibit PP-1; thus, DARPP-32 is regarded as an essential mediator of the biological effects of DA (Greengard *et al*, 1999). To investigate further its functional role in the absence of specific pharmacological tools, DARPP-32-null mice have been constructed (Fienberg *et al*, 1998). The cellular phenotype of these mutants confirms deletion of functional DARPP-32 in the absence of changes in the number of D_1-like or D_2-like receptors (Fienberg and Greengard, 2000; Svenningsson *et al*, 2000); however, their behavioral phenotype has yet to receive comparable breadth or depth of examination. For example, previous studies have noted no apparent differences in spontaneous horizontal or vertical photobeam interruptions in DARPP-32-null mice; however, cage climbing induced by the nonselective DA agonist apomorphine, the acute stimulatory response to amphetamine, and catalepsy induced by lower but not higher doses of the D_2-like antagonist raclopride appeared reduced in DARPP-32 mutants (Fienberg *et al*, 1998; Fienberg and Greengard, 2000).

Recently, it has become more fully appreciated that a number of important methodological factors influence the phenotypic characterization of DA-related (and potentially other) 'knockouts': (i) assessment of otherwise undifferentiated 'activity' in terms of photobeam interruptions, or observational assessments restricted to operational definitions of gross elements of behavior, over limited timeframes, can obscure critical phenotypic effects; these can be addressed by application of ethologically based approaches that resolve all topographies of behavior within the mouse repertoire (ie specification of its *ethogram*) over the prolonged time-course of exploration of and subsequent habituation to its environment (Waddington *et al*, 2001; McNamara *et al*, 2003). (ii) Failure to make systematic comparisons between the genders can obscure important sex-related differences in phenotypic expression (McNamara *et al*, 2002). (iii) The mixed (129/Sv × C57BL/6) genetic background, on which essentially all DA-related 'knockouts' have been constructed and examined to date, leaves open the possibility that phenotypic effects might reflect not only the entity deleted but also variations in that genetic background (Gerlai, 1996; Crawley *et al*, 1997; Kelly *et al*, 1998; Phillips *et al*, 1999; Waddington *et al*, 2001); this potential problem can be overcome in substantial part by repeated backcrossing onto a single strain, usually C57BL/6, to attain essential congenicity (Tomiyama *et al*, 2002; McNamara *et al*, 2002, 2003). (iv) It is recognized that there exist important, if poorly understood, differences between what are notionally 'similar' experimental paradigms applied in different laboratories (Crabbe *et al*, 1999).

Here, experiments are described to resolve topographically, for the first time, the phenotypic *ethogram* of congenic DARPP-32 'knockout' mice and how this is influenced by the DA receptor agonist apomorphine. These studies utilize procedures that address both the above methodological concerns and, additionally, allow systematic comparison with the phenotype of congenic D_{1A}, D_2, and D_3 'knockouts' that we have determined using the same procedures (Clifford *et al*, 2001; McNamara *et al*, 2002, 2003).

MATERIALS AND METHODS

Targeted Gene Deletion

The generation of DARPP-32 'knockout' mice was as reported previously (Fienberg *et al*, 1998). In outline, the targeted gene deletion was constructed in 129/Ola-derived embryonic stem cells and male chimeras mated with C57BL/6J females to produce heterozygous mutants (DARPP-32$^{+/-}$); these were then backcrossed into C57BL/6J for 10 generations to create a congenic DARPP-32-null line. Congenic, homozygous (DARPP-32$^{-/-}$) and wild-type (DARPP-32$^{+/+}$) breeding pairs were then transported to Dublin, where homozygous mutants were generated from homozygous mutant breeding pairs ($n = 10$), while wild-types were generated from wild-type breeding pairs ($n = 10$); the genotype of all progeny was confirmed using PCR of isolated tail DNA. Animals were housed in groups of four to five, with food and water available *ad libitum*, and maintained at $21 \pm 1^{\circ}$C on a 12/12 h (0800 on; 2000 off) light/dark schedule. Young adult mice from litters of the same generational age were used in all studies. These studies were approved by the Research Committee of the Royal College of Surgeons in Ireland and were conducted under license from the Department of Health & Children in accordance with Irish legislation and the European Communities Council Directive 86/609/EEC for the care and use of experimental animals.

Behavioral Assessment

On experimental days, mice were removed from their home cage and placed individually in clear glass observation cages ($36 \times 20 \times 20$ cm). Behavioral assessments were carried out in a manner similar to that described previously for D_{1A}, D_2, and D_3 mutants (Clifford *et al*, 2001; McNamara *et al*, 2002, 2003) using a rapid time-sampling behavioral checklist technique. For this procedure, each of 10 randomly allocated mice was observed individually for 5 s periods at 1 min intervals over 15 consecutive minutes, using an extended, ethologically based behavioral checklist. This allowed the presence or absence of the following individual behaviors (occurring alone or in combination) to be determined in each 5 s period: sniffing (flaring of nostrils with movement of vibrissae); locomotion (coordinated movement of all four limbs producing a change in location); total rearing (rearing of any form); rearing seated (front paws reaching upwards with hind limbs on floor in sitting position); rearing free (front paws reaching upwards away from any cage wall while standing on hind limbs); rearing to wall (front paws reaching upwards onto or towards a cage wall while standing on hind limbs); sifting (characteristic sifting movements of the front paws through bedding material on cage floor); grooming (of any form); intense grooming (characteristic pattern of grooming of the snout

and then the face with the forepaws, followed by vigorous grooming of the hind flank or anogenital region with the snout); chewing (chewing movements directed onto cage bedding and/or fecal pellets without consumption); stillness (motionless, with no behavior evident). This cycle of assessment by behavioral checklist over a 15 min period (0–15 min) was repeated twice (20–35 and 40–55 min): thereafter, 8×10-min cycles of otherwise identical assessments were repeated at 80–90, 120–130, 160–170, 200–210, 240–250, 280–290, 340–350, and 360–370 min. Under these conditions, each animal was observed on one occasion only, with all assessments made by an observer who was unaware of genotype for each animal.

An independent group of female mice was examined for apomorphine-induced behavior using procedures similar to those used for the evaluation of spontaneous behavior; however, in these experiments animals were habituated to identical observation cages for a period of 3 h, so that baseline activity was reduced before agonist challenge. Immediately following challenge with one of four doses of apomorphine or vehicle, each of 10 randomly allocated mice was observed individually for 5 s period at 1 min intervals over 15 consecutive minutes, with the behavioral checklist supplemented to include: ponderous locomotion, a 'plodding' variant induced in mice by D_2-like agonists that differs from normal, fluid ambulation (Clifford *et al*, 1999, 2000, 2001; McNamara *et al*, 2002, 2003); Straub tail, whereby the tail is lifted from the cage floor and inclined towards the vertical (Zarrindast *et al*, 1993); and hind limb abduction. After these 15 min assessments using the checklist, each animal was evaluated over a 30 s period using a conventional 0–6 point stereotypy scale: 0 = asleep or inactive; 1 = episodes of normal activity; 2 = discontinuous activity with bursts of prominent sniffing or rearing; 3 = continuous stereotyped activity such as sniffing or rearing along a fixed path; 4 = stereotyped sniffing or rearing fixated in one location; 5 = stereotyped behavior with bursts of licking or gnawing; 6 = continuous licking or gnawing. This cycle of assessment by behavioral checklist followed by stereotypy scale was repeated on two further occasions over a total period of 1 h. For evaluation of agonist-induced behavior, mice were used on two occasions only, separated by a drug-free interval of at least 1 week; on each occasion mice were allocated randomly to one of the various treatments. All assessments were made by an observer who was unaware of genotype and treatment for each animal.

Drugs

Apomorphine (as the hydrochloride; Sigma, UK) was dissolved in 1% sodium metabisulfite and made up to volume with distilled water. Drug and vehicle were injected subcutaneously into the flank in a volume of 2 ml/kg.

Data Analysis

For determination of *ethograms* over the phase of initial exploratory activity, the total 'counts' for each individual behavior was determined as the number of 5 s observation windows in which a given behavior was evident, summed over the initial 3×15 min (0–15, 20–35, 40–55 min) cycle periods; these were expressed as means $\pm$ SEM. Data for individual behaviors were analyzed using analysis of variance (ANOVA) following square-root transformation, to allow examination of interaction effects in the absence of nonparametric techniques for interaction terms. For determination of the habituation profiles of these ethograms, total counts for each individual behavior were summed as above over each of the following periods: 0–10, 20–30, 40–50, 80–90, 120–130, 160–170, 200–210, 240–250, 280–290, 340–350, 360–370 min; these were also expressed as means $\pm$ SEM and analyzed using repeated-measures ANOVA following square-root transformation.

For agonist-induced behaviors, the total 'counts' for each individual behavior was determined as the number of 5 s observation windows in which a given behavior was evident, summed over the initial 3×15 min (0–15, 20–35, 40–55 min) cycle periods, and expressed as means $\pm$ SEM; stereotypy scores were averaged over the 1 h period and expressed similarly. 'Counts' for individual behaviors in relation to agonist dose were analyzed using ANOVA following square-root transformation and followed by Student's *t*-test; stereotypy scores were analyzed using the Kruskal–Wallis nonparametric ANOVA followed by the Mann–Whitney *U*-test.

RESULTS

General Parameters

On examining 40 (20 females and 20 males) congenic DARPP-32-null mice, mean body weight (males: 27 $\pm$ 1 g, mean age 185 $\pm$ 11 days; females: 21 $\pm$ 1 g, mean age 171 $\pm$ 9 days) did not differ relative to 40 (20 females and 20 males) wildtypes (males: 27 $\pm$ 1 g, mean age 182 $\pm$ 6 days; females: 21 $\pm$ 1 g, mean age 171 $\pm$ 6 days). On qualitative inspection of posture, reactivity to handling, and general activity, no gross motor phenotype was apparent.

Ethogram of Spontaneous Behavior Over Exploratory Phase

On comparison with wildtypes, congenic DARPP-32-null mice were characterized over the initial 60 min exploratory phase (Figure 1) by increased locomotion (effect of genotype, $F_{1,76} = 4.21$, $p < 0.05$), which occurred essentially in female but not in male 'knockouts' (effect of gender, $F_{1,76} = 5.50$, $p < 0.05$; gender $\times$ genotype interaction, $F_{1,76} = 3.83$, $p = 0.05$). DARPP-32-null mice were also characterized by reduction in rearing seated for both genders (effect of genotype, $F_{1,76} = 4.63$, $p < 0.05$; no effect of gender or gender $\times$ genotype interaction). Total grooming was decreased in female, but not in male DARPP-32-null mice (gender $\times$ genotype interaction, $F_{1,76} = 4.18$, $p < 0.05$). For sniffing, total rearing, rearing free, rearing to wall, intense grooming, sifting, chewing, and stillness there were no effects of genotype or gender, or gender $\times$ genotype interactions.

Ethogram of Spontaneous Behavior Over Habituation Phase

Locomotion habituated readily over the total period of 370 min (effect of time, $F_{10,760} = 112.37$, $p < 0.001$)

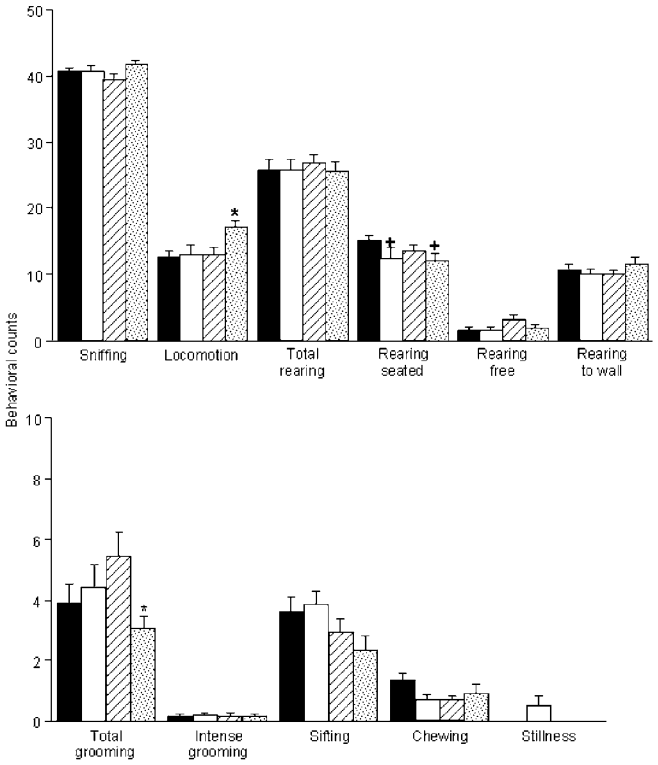

Figure 1 Topography of spontaneous behavior over an initial 60 min exploratory period. Data are mean behavioral counts ± SEM for sniffing, locomotion, total rearing, rearing seated, rearing free, rearing to wall, total grooming, intense grooming, sifting, chewing, and stillness for wild-type male (filled columns; $n=20$) and female (hatched columns; $n=20$) and DARPP-32-null male (open columns; $n=20$) and female (dotted columns; $n=20$) mice. *$p<0.05$ *vs* wildtype of same gender; $^{+}p<0.05$ *vs* wildtype.

(Figure 2a); the increase in locomotion among female DARPP-32-null mice over the initial 60 min period of exploration was sustained over 120 min thereafter, before declining to a level similar to that of wildtypes (effect of genotype, $F_{1,76}=3.12$, $p=0.08$; no time × genotype × gender interaction) (Figure 2a and b). Total rearing also habituated readily over the total period (effect of time, $F_{10,760}=106.69$, $p<0.001$) (Figure 2b); a decrease in total rearing in DARPP-32-null mice (effect of genotype, $F_{1,76}=4.89$, $p<0.05$) occurred primarily over intermediate time-bins (time × genotype interaction, $F_{1,76}=1.16$, $p<0.05$). Rearing seated showed an initial increase followed by ready habituation over the total period (effect of time, $F_{10,760}=57.20$, $p<0.001$); this initial increase was attenuated in DARPP-32-null mice, and their lower levels of rearing seated were prolonged such that they attained prematurely the level to which wildtypes declined (effect of genotype, $F_{1,76}=9.53$, $p<0.01$; time × genotype interaction, $F_{10,760}=1.16$, $p<0.05$).

Total grooming was more labile; the decrease in grooming among female, but not male, DARPP-32-null mice over the initial 60 min period of exploration was followed by transient increases among females and transient reductions among males (effect of time, $F_{10,760}=8.73$, $p<0.001$; genotype × gender interaction, $F_{1,76}=4.81$, $p<0.05$; time × gender × genotype interaction, $F_{10,760}=2.41$, $p<0.01$). Sniffing, sifting, and rearing to wall each habituated readily in a manner that did not differ between the genotypes (effects of time, $F_{10,760}>75.09$, $p<0.001$; no time × genotype interaction). Habituation effects for the above topographies of behavior were reflected in increasing levels of stillness with time for both genotypes, attaining higher overall levels in DARPP-32 mutants (effect of genotype, $F_{1,76}=5.74$, $p<0.05$; effect of time, $F_{10,760}=87.38$, $p<0.001$; no time × genotype interaction). Other than those instances noted above, isolated effects of gender and time × gender interactions were unrelated to genotype. Levels of rearing free, intense grooming, and chewing were too low for meaningful analysis.

Ethogram of Responsivity to Apomorphine

Following habituation, apomorphine (0.03–3.0 mg/kg) induced sniffing and a shift from fluid to ponderous locomotion (effects of dose, $F_{4,70}>12.79$, $p<0.001$), with increases in chewing and hind limb abduction (effects of dose, $F_{4,70}>11.51$, $p<0.001$) for both genotypes (no dose × genotype interactions) (Figure 3a and b); increased stereotypy scores indicated higher doses of apomorphine to stimulate these behaviors in a stereotyped fashion (effect of dose, $H=39.72$, $p<0.001$), for both genotypes, though scores reflecting normal behaviors in vehicle-treated 'knockouts' were lower ($p<0.05$) than in their wild-type counterparts. These actions were accompanied by decreases in grooming and total rearing (effects of dose, $F_{4,70}>2.92$, $p<0.05$) and by a marginal decrease in rearing seated (effect of dose, $F_{4,70}=2.18$, $p=0.08$), for both genotypes; independent of these effects of apomorphine, overall levels of grooming, total rearing, rearing seated, and sniffing were lower in DARPP-32-null mice (effects of genotype, $F_{1,70}>7.89$, $p<0.01$; no dose × genotype interactions). Rearing to wall evidenced no effects, while levels of rearing free were too low for meaningful analysis. Stillness reflected this complex interplay of behavioral topographies (effect of dose, $F_{4,70}=23.28$, $p<0.001$; dose × genotype interaction, $F_{4,70}=3.68$, $p<0.01$). It was notable that in DARPP-32-null mice, the lowest dose of apomorphine was associated with significantly ($p<0.05$) lower levels of sniffing, total grooming, total rearing, and rearing seated, and with a higher level of stillness, than in wildtypes. The action of apomorphine to induce Straub tail was markedly reduced in DARPP-32-null mice (effect of dose, $F_{4,70}=14.64$, $p<0.001$; effect of genotype, $F_{1,70}=6.93$, $p<0.01$; dose × genotype interaction, $F_{4,70}=7.89$, $p<0.001$).

DISCUSSION

Using an ethologically based approach to resolve and quantify all topographies of behavior within the natural repertoire of the mouse over the prolonged time-course of exploration of and subsequent habituation to its environment, phenotypic effects of congenic DARPP-32 deletion were identified.

Over an initial period of exploration, female DARPP-32-null mice evidenced increased locomotion with decreases in rearing seated and total grooming, while males evidenced only a decrease in rearing seated; other topographies of behavior over exploration were unaltered. One explanation for these decreases in sedentary rearing and grooming in

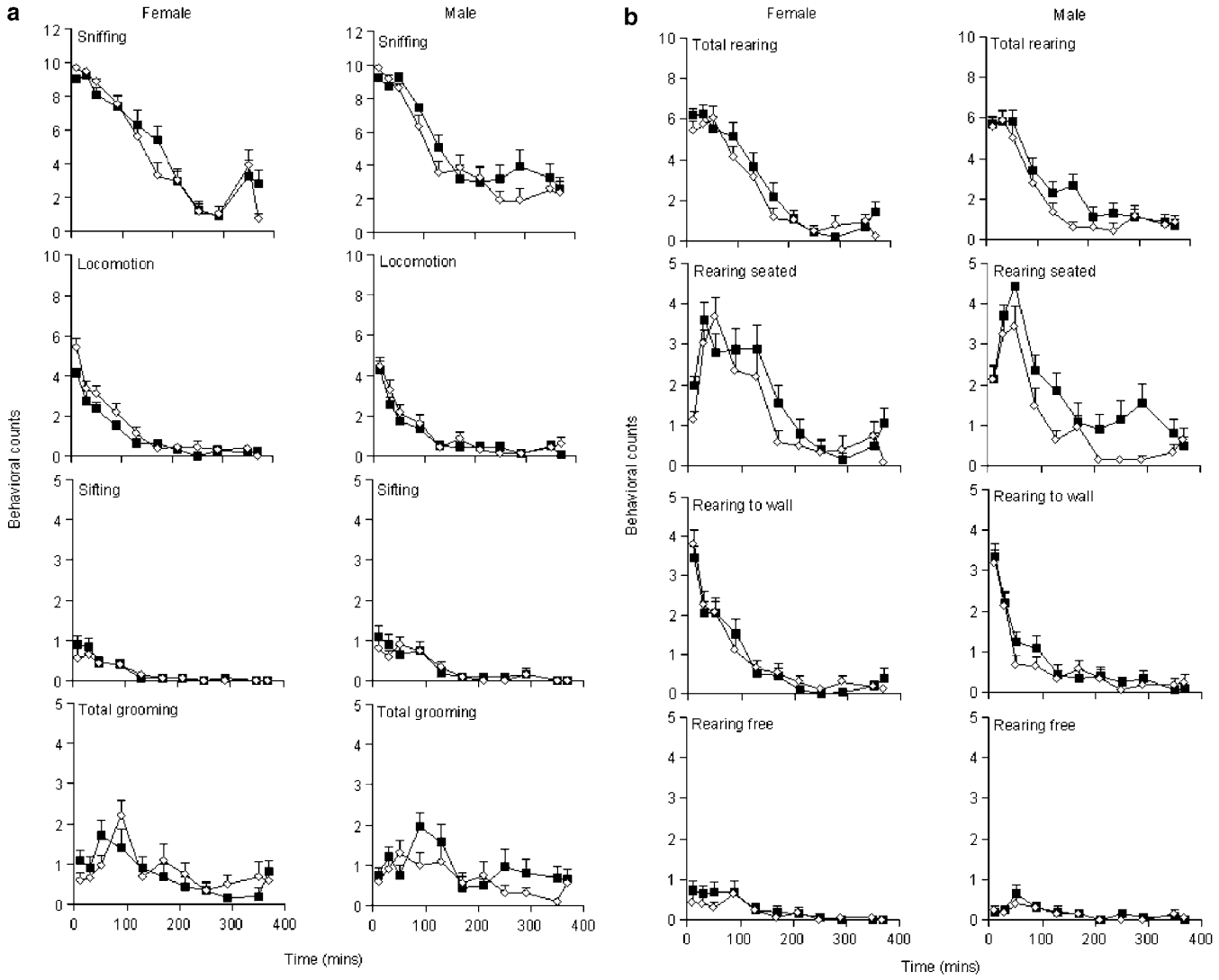

Figure 2 Topographical assessment of spontaneous behavior over a 370 min phase of habituation. Data are mean behavioral counts ± SEM for $n = 20$ per group per 10 min period for (a) sniffing, locomotion, sifting, and total grooming and (b) rearing total, rearing seated, rearing to wall and rearing free, in wild-type (closed squares) *vs* DARPP-32 mutants (open diamonds) for female (left column) and male (right column) mice.

association with increased locomotion would be some shift away from localized, self-directed behaviors to exploration of the surrounding environment. Although a functional role for DARPP-32 in exploration has not been investigated comprehensively, evidence suggests that the DA-DARPP-32-PP-1 cascade (see Introduction) may influence the induction and maintenance of long-term potentiation in the hippocampus, a mechanism putatively involved in exploratory learning (Frey *et al*, 1991; Huang and Kandel, 1995; Otmakhova and Lisman, 1996; Kusuki *et al*, 1997; Greengard *et al*, 1999; Crusio, 2001); therefore, impaired exploratory learning due to DARPP-32 deletion might favor externally directed behaviors. However, increased locomotion was not accompanied by increases in other exploratory behaviors such as sniffing, rearing to wall, or sifting; this could suggest an increase in locomotor drive, with sedentary rearing and grooming being reduced due to their physiological incompatibility with locomotion. Irrespective of such considerations, these findings suggest a role for DARPP-32 in the regulation of specific rather than generic topographies of behavior.

Continuing assessments, through subsequent habituation towards quiescence over a total period of 370 min resolved the time-course of those phenotypic effects identified over the initial 60 min period of exploration, but did not reveal either fundamentally new aspects of phenotype for additional topographies of behavior or any marked delay in the time-course of habituation. These profiles of phenotypic effect, in terms of *ethograms* over initial exploration and subsequent habituation, are distinct from those that we have reported recently for congenic D_{1A}, D_2, and D_3 'knockouts': D_{1A}—marked increase in locomotion and rearing topographies, with reduced sifting, due to profoundly delayed habituation (McNamara *et al*, 2003); D_2—modest reduction in locomotion, with shifts in rearing topographies, without alteration in habituation (Clifford *et al*, 2001); D_3—slight increase in rearing due to delayed habituation (McNamara *et al*, 2002).

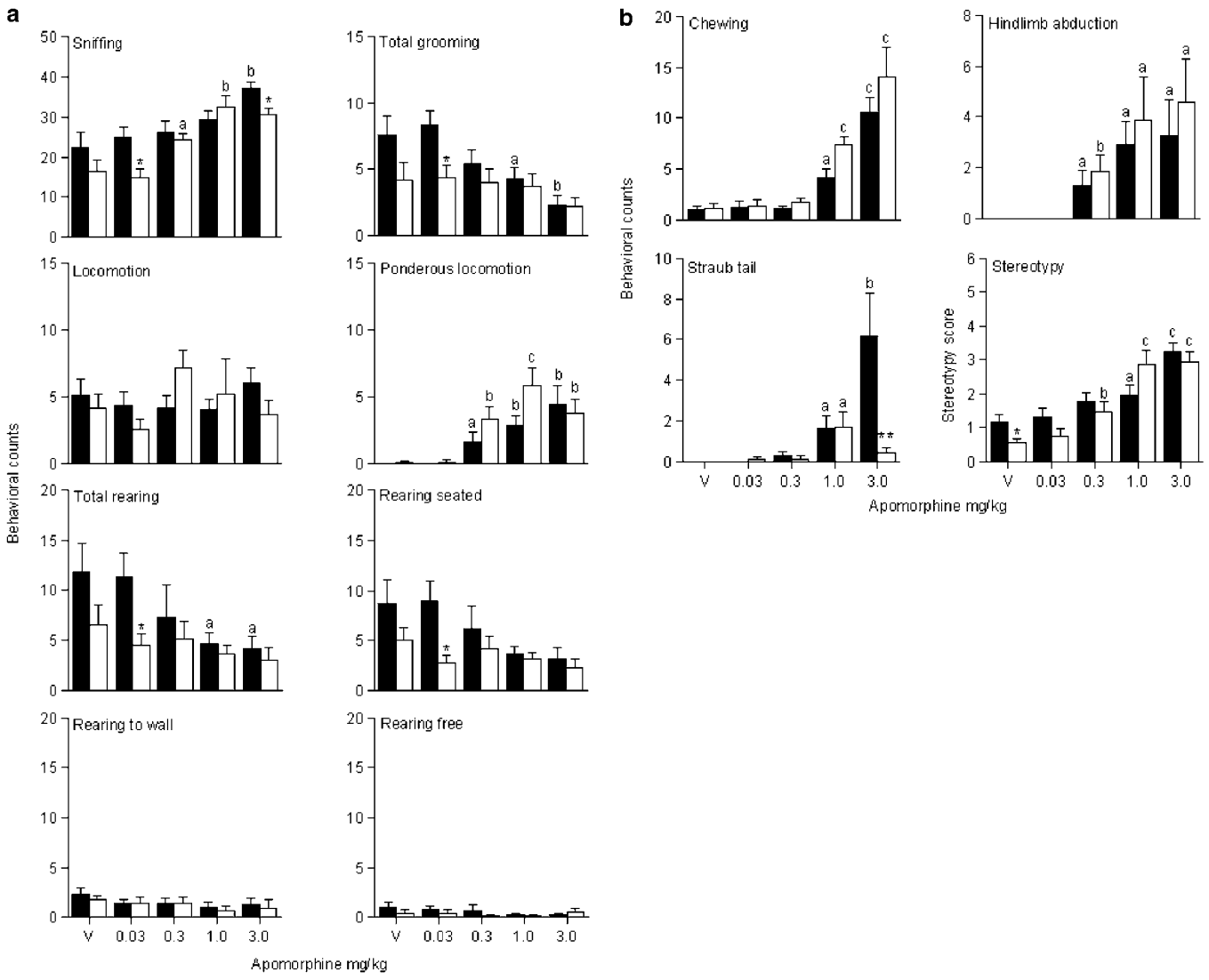

Figure 3 Topographical effects of pretreatment with 0.03–3.0 mg/kg apomorphine or vehicle (V) following 3 h of habituation. Data are mean behavioral counts ± SEM over a 60 min period for $n = 8$ per group for (a) sniffing, total grooming, locomotion, ponderous locomotion, rearing total, rearing seated, rearing to wall, rearing free, and (b) hind limb abduction, chewing, and Straub tail, with stereotypy scores, in wild-type (closed columns) *vs* DARPP-32-null (open columns) female mice. $^{c}p < 0.001$, $^{b}p < 0.01$, $^{a}p < 0.05$ *vs* vehicle-treated control of the same genotype; $^{**}p < 0.01$, $^{*}p < 0.05$ between genotypes receiving the same dose.

Explanation of these phenotypic effects is complicated by increased locomotion and decreased grooming over initial exploration being evident essentially in female, but not in male, DARPP-32-null mice. Interestingly, we have recently reported delayed habituation of rearing in congenic D_3-null mice to also be evident essentially in female, but not in male, mutants (McNamara *et al*, 2002); no comparable or alternative gender-specific effects were noted in congenic D_{1A} (McNamara *et al*, 2003) or D_2 (Clifford *et al*, 2001) 'knockouts'. At a methodological level, these findings indicate that the phenotype of one gender cannot be assumed to apply to the other gender in the absence of systematic comparison between them, and that collapsing across the genders or using mutants of unspecified gender may obscure phenotypic effects (Waddington *et al*, 2001).

At a mechanistic level, there are several precedents in non-DAergic systems for gender-specific expression of mutant phenotypes at the level of behavior, for example mice expressing the Δ-168 human insulin transgene (Douhet *et al*, 1997), or lacking the *Mas* proto-oncogene (Walther *et al*, 2000) or the gene encoding phosphodiesterase 1B (Reed *et al*, 2002).

In relation to the present gender-specific phenotypic effects, it should be noted that females evidence estrogen concentrations considerably higher than those in males and DAergic function is influenced by sex hormone status (Diaz-Veliz *et al*, 1994; Becker, 1999), such that estradiol increases the phosphorylation of DARPP-32 (Auger *et al*, 2001) via D_1-like receptors. Such factors relating to interactions between DARPP-32 deletion and female hormones, over development or in the mature nervous system, may contribute to the present gender-specific aspects of phenotype; for example, decreased grooming, a D_1-like-mediated behavior (Molloy and Waddington, 1984;

Waddington *et al*, 1995), in female DARPP-32-null mice might reflect hormonally mediated disruption of the D_1-like-DARPP-32 pathway.

In relation to putative effects of estrous cycle status in females, spontaneous locomotion, rearing, and grooming in rodents, unlike motivational, goal-directed behaviors, do not vary substantially during the estrus cycle (Steiner *et al*, 1980; Kazandjian *et al*, 1987). However, DAergic function and responsivity to agonists have been shown to vary over the estrus cycle, with gender-dependent levels of basal extracellular DA in the striatum and nucleus accumbens thought to reflect variations in synthesis, release, and metabolism (Becker and Cha, 1989; Castner *et al*, 1993; Diaz-Veliz *et al*, 1994; Becker, 1999; Sell *et al*, 2000). Thus, the presence of DARPP-32 in the normal striatum and nucleus accumbens (Hemmings *et al*, 1992) might indicate a basis for hormonally modulated variations in DAergic transduction in DARPP-32 mutants over the estrus cycle. However, any such effect is offset by the housing of five to six females per cage; females housed together tend to go into anestrus and not cycle (Ma *et al*, 1998). Nevertheless, future studies might usefully assess hormonal levels as well as behavior over the estrus cycle in DARPP-32 mutants *vs* wildtypes to clarify these issues further.

Apomorphine is the archetype DA receptor agonist, acting at all members of the D_1-like and D_2-like families (Seeman, 1980; Waddington *et al*, 1995; Di Chiara, 2002). Lower doses have been reported to inhibit motor activity in rats, due to a preferential action either at presynaptic D_2-like autoreceptors that attenuate endogenous DAergic activity, or at a putative population of postsynaptic D_2-like receptors that exert an inhibitory role on DA-mediated function (Waters *et al*, 1993; Clifford and Waddington, 1998); such a profile may be less evident in mice and the present studies utilized only a single low dose, so as to accommodate also higher, stimulatory doses, following habituation to optimize the resolution of stimulatory effects (McNamara *et al*, 2002). However, it was notable that relative to wildtypes, low-dose apomorphine in DARPP-32 mutants was associated with reduced levels of sniffing, total grooming, total rearing, and rearing seated, with higher levels of stillness. This could suggest some role for DARPP-32 in mediating the biological effects of presynaptic D_2-like autoreceptor or inhibitory postsynaptic D_2-like receptor activation. It should be noted that for practical reasons, that is, the requirement for multiple groups of 'knockouts' so as to include a range of challenge doses, studies with apomorphine in DARPP-32 mutants had to be confined to a single gender, in this instance, females. Thus, given the above consideration of gender-related phenotypic effects, it cannot be assumed that the apomorphine phenotype in females applies similarly to males until this is assessed directly.

At higher doses, apomorphine stimulates motor activity and induces stereotyped behavior, due to predominant actions at postsynaptic D_1-like and D_2-like receptors; other than a modest reduction in sniffing, topographies of behavior within the classical stereotypy syndrome were unaltered in DARPP-32 mutants. In rats, Straub tail is associated primarily with serotonergic activation, whereas in mice it can be induced by apomorphine (Zarrindast *et al*, 1993); this topography of response to higher doses of apomorphine was markedly reduced in DARPP-32 mutants, suggesting some particular involvement of DARPP-32 in those aspects of DAergic function that regulate Straub tail in the mouse. We have not yet evaluated the effects of apomorphine in congenic D_{1A} (McNamara *et al*, 2003), D_2 (Clifford *et al*, 2001) or D_3 (McNamara *et al*, 2002) 'knockouts'.

An unexpected finding was an overall reduction in levels of sniffing, total rearing, rearing seated, and grooming in DARPP-32-null mice that was unrelated to the dose of apomorphine administered, including vehicle-injected animals; also, stereotypy scores reflecting normal behaviors were lower in vehicle-treated mutants than in their wildtype counterparts. While these reductions in total rearing, rearing seated, and grooming would appear to reflect those evident in the *ethogram* over initial exploration and/or subsequent habituation, they were of larger magnitude; this, with additional reduction in sniffing, could reflect an interaction between targeted gene deletion and the stress of handling/subcutaneous injection, such that the effect of DARPP-32 deletion on topographies of behavior is accentuated under stressful relative to nonstressful conditions.

Although phenotypic effects were readily identified in DARPP-32-null mice, some conceptual challenge is apparent. It should be recognized that this phenotype is modest *vis-à-vis* the status of DARPP-32 in DAergic regulation; specifically, absence of DARPP-32 was associated with some preservation in essentially all topographies of behavior that are known to be mediated via DAergic neuronal systems and are abolished readily by treatment with DA receptor antagonists or DA-depleting agents (Waddington *et al*, 1995, 2001). How might this be explained?

It must first be considered whether the assessment techniques applied are sensitive enough to detect the phenotype; however, we have shown repeatedly that this ethologically based approach is of high sensitivity and capable of identifying phenotypic effects in hybrid D_{1A} (Clifford *et al*, 1998) and α_4 nicotinic (Ross *et al*, 2000), and in congenic D_{1A} (McNamara *et al*, 2003), D_2 (Clifford *et al*, 2001), and D_3 (McNamara *et al*, 2002) 'knockouts', and in mice transgenic for the *Huntingtin* gene (Clifford *et al*, 2002), that are missed using other, more conventional approaches (Waddington *et al*, 2001). Such effects may not have been identified initially in DARPP-32-null mice (Fienberg *et al*, 1998; Fienberg and Greengard, 2000) because of the application of techniques that composite the diversity of behavior into automated measures, such as photobeam interruptions over limited time-frames. Also, previous studies have involved DARPP-32 mutants on a mixed (hybrid 129/Ola × C57BL/6J) genetic background or following two to six backcrosses into C57BL/6J, while here we have utilized a congenic line following 10 backcrosses into C57BL/6J; it has been of enduring concern that genetic background may influence apparent phenotype independent of the entity deleted (Gerlai, 1996; Crawley *et al*, 1997; Kelly *et al*, 1998; Phillips *et al*, 1999; Waddington *et al*, 2001), and we have shown recently that the phenotype of D_{1A}-null mice is qualitatively similar but quantitatively much more pronounced on a congenic relative to a hybrid background (McNamara *et al*, 2003).

Secondly, it must be considered whether the physiological role of DARPP-32 in DAergic regulation has been over-

estimated, such that its deletion results only in subtle effects on behavior. However, a wealth of molecular and cellular studies indicating DARPP-32 to be an essential mediator of the biological effects of DA (Greengard *et al*, 1999) would contradict any such explanation. It could be argued that DARPP-32 has some involvement in mediating the actions of opposing signaling pathways, for example those transducted by D_1 *vs* D_2 or DA *vs* glutamate receptors, such that the phenotype expected for DARPP-32 mutants might be less dramatic than for a D_1 or a D_2 mutant; however, using the same assessment approach the phenotypes of congenic D_1 and D_2 mutants are not reflective of acute treatment with antagonists of those receptors (Clifford *et al*, 2001; McNamara *et al*, 2003). These and related issues might be illuminated by phenotypic studies with selective D_1-like *vs* D_2-like agonists and antagonists in DARPP-32-null mice.

A third perspective is that these essential mediating effects of DARPP-32 at a molecular and cellular level are real, such that 'removal' of DARPP-32 would indeed produce a profound DAergic phenotype in terms of behavioral dysfunction, but that DARPP-32-targeted gene deletion does not constitute such a situation. It should be emphasized that conventional 'knockouts' are *not* mice in whom the entity at issue was once present but has been 'removed'; rather, the entity has never been present, due to deletion of its encoding gene. Such developmental absence makes it likely that compensatory processes will be recruited to sustain functions usually subserved by the entity deleted (Waddington *et al*, 2001). For example, we have reported recently that D_2 mutants fail to respond to a D_2-like agonist, indicating the absence of D_2 receptor-mediated function; however, they show substantial preservation in the topography of spontaneous DAergic behaviors that in wildtypes are attenuated readily by a D_2-like antagonist (Clifford *et al*, 2000, 2001; McNamara *et al*, 2003).

In summary, the present studies define, for the first time, the *ethogram* and DA agonist-induced behavioral topography of congenic DARPP-32 'knockouts' in comparison with congenic D_{1A}, D_2, and D_3 counterparts; they thus indicate those topographies of spontaneous and DA agonist-induced behavior in which DARPP-32 may play a particular regulatory role. In relation to the magnitude of the phenotype resolved, the most parsimonious explanation would be that, following its normal developmental trajectory, DARPP-32 functions as an essential cellular mediator of the biological effects of DA; however, the developmental absence of DARPP-32 following targeted gene deletion is associated with the emergence of compensatory processes that are able to subserve in substance many DAergic functions that would otherwise have been mediated via DARPP-32. The nature of such compensatory processes is poorly understood, but their elucidation could reveal critical neuronal processes and identify novel therapeutic targets.

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