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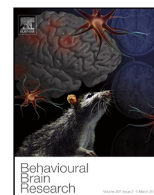

Short communication

Cage-induced stereotypic behaviour in laboratory mice covaries with nucleus accumbens FosB/ΔFosB expression

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HIGHLIGHTS

- Stereotypic behaviour (SB) occurs in certain human disorders (e.g. autism), and animals in impoverished conditions, including laboratory mice in standard cages.
- Dysfunctional cortico-basal ganglia pathways have been implicated, but for cage-induced SB, the precise regions had not been ascertained.
- We show that spontaneously highly stereotypic mice show the most elevated FosB/ΔFosB activity in the nucleus accumbens.
- No such patterns occurred in the caudate-putamen.
- This suggests that the SB common in standard-housed mice has an aetiology akin to compulsive gambling and drug-seeking.

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ABSTRACT

Stereotypic behaviour (SB) occurs in certain human disorders (e.g. autism), and animals treated with stimulants or raised in impoverished conditions, including laboratory mice in standard cages. Dysfunctional cortico-basal ganglia pathways have been implicated in these examples, but for cage-induced forms of SB, the relative roles of ventral versus dorsal striatum had not been fully ascertained. Here, we used immunohistochemical staining of FosB and ΔFosB to assess long-term activation within the nucleus accumbens and caudate-putamen of C57BL/6 mice. Housed in typical laboratory cages, these mice spontaneously developed different degrees of route-tracing, bar-mouthing and other forms of SB (spending 0% to over 50% of their active time budgets in this behaviour). The most highly stereotypic mice showed the most elevated FosB/ΔFosB activity in the nucleus accumbens. No such patterns occurred in the caudate-putamen. The cage-induced SB common in standard-housed mice thus involves elevated activity within the ventral striatum, suggesting an aetiology closer to compulsive gambling, eating and drug-seeking than to classic amphetamine stereotypies and other behaviours induced by motor loop over-activation.
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Stereotypic behaviour (SB) is common in some human neurodevelopmental disorders (e.g. autism), where it seems to reflect dysfunctional basal ganglia circuitry [1,2]. It can also be experimentally induced in animals by manipulating these circuits [e.g. via stimulants [2,3]]. SB is common, too, in farm, zoo and laboratory animals raised and housed in impoverished enclosures [1,2,4]. Here, SB often covaries with generalised signs of impaired behavioural control [1,4,5], again suggesting cortico-striatal dysfunction. Furthermore, the types of enclosure that promote SB induce diverse structural and biochemical changes within the basal ganglia [2,6,7]. However, more direct evidence for basal ganglia involvement, based on neurological differences that correlate with SB at the individual level, is rare, and comes from just two species: the deer mouse (*Peromyscus maniculatus*) and horse (*Equus caballus*). The deer mouse studies found that animals spontaneously developing high levels of SB, compared to those displaying little or

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none, have reduced cytochrome oxidase (CO) activity in the ventromedial caudate-putamen (CPu), reduced CO activity in subthalamic nuclei, and altered CPu dynorphin/encephalin ratios consistent with reduced inhibition in cortico-striatal 'motor loops' [2,6,7]. Complementary studies highlighted changes in the frontal cortex, including deficient glutathione systems [8]. Equine research, focussing on dopamine receptor densities, has instead revealed elevated D1 and D2 densities within the nucleus accumbens (NAc) of highly stereotypic horses [1], alongside unexpectedly lower D1 densities in the dorso-medial striatum.

Over 30 million mice of the *Mus* genus are used annually in research worldwide, and in standard housing many display SB [9]. Despite this, the neurological bases of laboratory mouse SB are essentially unknown. In one study, the tendencies of two strains, C57BL/6 (henceforth 'C57') and DBA/2 (henceforth 'DBA'), to develop SB were contrasted, and strain-typical predispositions to high levels suggested to reflect strain differences in the upregulation of NAc dopamine receptors [3,10]. Consistently, a second experiment drew parallels between the high SB of C58BL/6 mice compared to other strains, and its enhanced locomotor responses to amphetamine [11]. Neither study, however, looked at within-strain individual differences or obtained strong evidence of causality. A third study revealed distinct individual behavioural correlates of SB in C57s: elevated response repetition in two-choice 'gambling' tasks in the most stereotypic mice [5] (something other behavioural studies failed to replicate in another strain, CD-1 [12,13]). This effect in the stereotypic C57s was interpreted as reflecting dorsal striatal dysfunction [5]. This summarises what little is known about cage-induced SB in *Mus*. We therefore compared spontaneously high and low SB C57 mice, using immunohistochemistry to assay FosB and ΔFosB. Fos family proteins are transcription factors that regulate gene expression, ΔFosB being a highly stable FosB variant that accumulates over time with repeated stimulation, and that mediates long-term neuronal plasticity [e.g. [14–16]]. ΔFosB thus reflects long–term neuronal activation, in a manner perhaps likely to covary with CO [7]. We investigated whether, within the basal ganglia, the caudate-putamen and/or the nucleus accumbens show evidence of sustained activation in highly stereotypic mice.

Protocols were approved by the University of Guelph's Animal Care Committee. 30 C57 females (from Charles River, Quebec) were housed from 4 weeks of age in mixed strain triplets (for the purposes of another study: two C57s plus one DBA/2; see [17] for validation), in 15 standard woodchip-bedded laboratory cages (12 cm H × 27L × 16W), each provided with Shepherd Envirodri© nesting material and a UDEL polysulfone plastic mouse house shelter, plus *ad libitum* food (Harlan® Teklad Global Diet) and water. One C57 per cage was ear-notched for identification. Cages were maintained at 21 °C, on a 12:12 reversed dark/light cycle (lights off at 1000 h). After five months, behavioural observations were conducted. Mice were live scan-sampled *in situ*, using red room lights/headlamps, to assess activity budgets. Scans were taken every 20 min, for four hours per session (1130–1530 h or 1730–2130 h), repeated over four days (thus eight sessions): a method based on [17]. Scans were split between two experimenters (LH and KR; inter-observer reliability: >95%). Table 1 provides the ethogram (modified from [18]). SB was calculated as a percentage of both overall activity and observations. However, these covaried tightly ($F_{1,\,15.2}$ = 914.38, P < 0.0001, R^2 > 0.99) and gave near identical results, so only results for the former are presented.

Subjects were killed by cervical dislocation when 7 months old. Brains were extracted immediately and drop-fixed in cold 4% paraformaldehyde, PFA (cf. e.g. [19]), the PFA being refreshed twice within the first 24 h to aid fixation. After c. 4 weeks in PFA (stored at 4°), brains were then transferred into cryoprotective 30% sucrose in phosphate buffered saline (PBS) for 48 h, and tissue was then sliced using a Leica Cryostat into 30 μm coronal sections collected in series, mounted onto gelled slides, and stored at −80°C until staining.

In six of the cages, both C57s were clearly stereotypic (spending 3.5–55.5% of their active time budget in SB). In the remaining nine cages, they performed negligible SB (0–1.5% of the active time budget). Data from cagemates are not statistically independent [20], and correspondingly, the SB levels of the two C57s per cage tightly covaried ($F_{1,\,13}$ = 111.89, P < 0.0001). The brain of one mouse was therefore chosen from each of the six stereotypic cages, and one subject randomly chosen from five of the others (by GM, to ensure the researcher performing staining and quantification [DP] was blind). One slide was chosen per mouse, from which two adjacent sections were selected (using landmarks [cf. [14,21]] ensuring staining of both caudate-putamen and nucleus accumbens).

To develop the FosB/ΔFosB immunohistochemical procedure (the antibody used [Sc-48, Santa Cruz Biotechnology, CA] was anti-ΔFosB but cross-reacted with FosB), protocols were obtained from authors of relevant studies [14,22,23,24]. These varied, so were combined and optimised via pilots on spare tissue. In the final protocol, all conducted at room temperature, slides were post-fixed in 4% PFA for 10 min, rinsed in PBS, then exposed to H_2O_2 (1% in PBS) for 10 min. The samples were blocked (PBS, 1.5% Triton X and 3% goat serum) for 1 hour, incubated in primary antibody (diluted 1:500 in PBS with 0.3% Triton X and 0.03% sodium azide) for 22 h, then incubated in biotinylated goat anti-rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, CA) for 2 h. The amplification step used an avidin/biotin peroxidase complex (Vectastain ABC Kit, Vector Laboratories) for 90 min. Immunoreactivity was revealed by incubating slides for 12 min in 0.06% DAB and 0.1% H_2O_2, diluted in PBS. Sections then were rinsed in PBS, dehydrated in increasing concentrations of ethanol (50% for 1 min, 70% for 1 min, 95% for 3 min, 100% for 3 min), dipped in xylene for 2 h, then coverslipped using DPX Mountant (Fisher Scientific). Additional control slides underwent each step except the primary antibody, to ensure background staining was absent.

Images were taken using the x10 objective of a Leica DMR HC Brightfield microscope, and a sample of 200 × 200 pixels selected from each of five regions: the NAc, and four CPu areas (dorsal, ventral, medial and lateral; cf. e.g. [6,14,21]; see Fig. 1). These images were taken bilaterally from the two consecutive sections, resulting in four samples per region per mouse. Stained nuclei were identified by dark nuclear coloration surrounded by lighter stained cytoplasm (Fig. 2), published images of successful FosB/ΔFosB immunolabeling being used as guides [23]. Dead cells were also counted (because these could act as confounds by preventing positive staining), easily identified via nuclear condensation (see Fig. 2). Both were counted manually.

Because of weak staining compared to published studies [e.g. [14,23]] and concerns over cell deaths, we first ran extensive checks for data quality and consistency of staining. This included assessing the scorer (DP)'s internal consistency, a subset of 25 regions being randomly selected for a blind re-count of cells positively stained for FosB/ΔFosB and dead cells. Here, as throughout, data were analysed using General Linear Models (GLMs) in JMP 12.0, and Box-Cox transformed when needed to meet the assumptions of parametric statistics (Spearman's tests being used when this was unsuccessful). First counts strongly predicted blind re-counts (P < 0.0001 for both cell types) indicating high intra-rater reliability. Next, relationships between positively stained and dead cell counts between the two consecutive sections per hemisphere were assessed. Both positive counts and dead cells counts from successive sections positively correlated (P < 0.05 in all tests), save for the right hemisphere's NAc (P > 0.05 in both tests). Because successive sections were thus generally similar, values from each pair were averaged for subsequent analyses. A series of GLMs then assessed whether positive counts for each region covaried between

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