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Neurophysiological correlates of stereotypic behaviour in a model carnivore species

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Abstract

Stereotypic behaviour (SB) is common in animals housed in farm, zoo or laboratory conditions, including captive Carnivora (e.g. wild ursids and felids). Neurobiological data on housing-induced SBs come from four species (macaques, two mouse species, and horses), and suggest basal ganglia (BG) dysfunction. We investigated whether similar patterns occur in Carnivora via a model, American mink, because their SB is distinctive in form and timing. We raised 32 males in non-enriched (NE) or enriched (E) cages for 2 years, and assessed two forms of SB : 1) Carnivora-typical locomotor-and-whole-body ('loco') SBs (e.g. pacing, weaving); 2) scrabbling with the forepaws. Neuronal activity was analysed via cytochrome oxidase (CO) staining of the dorsal striatum (caudate; putamen), globus pallidus (externus, GPe; internus, GPi), STN, and nucleus accumbens (NAc); and the GPe:GPi ratio (GPr) calculated to assess relative activation of direct and indirect pathways. NE mink stereotyped more, and had lower GPr CO-staining indicating relatively lower indirect pathway activation. However, no single BG area was affected by housing; and nor did GPr values covary with SB. Independent of housing, elevated NAc CO-staining predicted more loco SB; while scrabbling, probably because negatively correlated with loco SB, negatively covaried with NAc CO-staining in NE subjects. These results thus implicate the NAc in individual differences in mink SB, but because they cannot explain why NE subjects showed more SB, they provide limited support for the BG dysfunction hypothesis for housing-induced SB. More research is therefore needed to understand how barren housing causes SB in captive Carnivora.

Key words: basal ganglia; stereotypic behaviour; environmental enrichment; dorsal striatum; ventral striatum; mink
Declaration of interests: none

1. Introduction

Stereotypic behaviour (SB) occurs in millions of animals kept in farm, laboratory and zoo conditions [1]. It is particularly prevalent and time-consuming in subjects exposed to adverse experiences, especially sub-optimal parental care (e.g. [2, 3]), repeated stressors such as acute isolation or repeated research procedures (e.g. [4, 5]), and small, barren cages rather than larger more enriched enclosures (e.g. [6-10]). However, only recently have researchers tried to understand the neurobiological bases of such housing-induced SB [11]. Instead, evidence for the neural bases of SB and other abnormal repetitive behaviours primarily comes from pharmacological studies on animals, along with imaging and Deep Brain Stimulation studies of human disorders (e.g. autism and obsessive-compulsive disorder). These bodies of research typically implicate structural and functional changes in the basal ganglia (e.g., [12-14]): subcortical nuclei that sit within complex circuits, both cortical and sub-cortical (e.g., [15-17]), and play a crucial role in the regulation of behaviour. Alterations in basal ganglia function and structure are thus involved in the abnormally repetitive behaviour of individuals with Obsessive Compulsive Disorder, Tourette's syndrome and autism [12, 18-21], as well as in the SB elicited in animals by psychostimulant drugs (e.g. [22-23]). One specific type of malfunction involves imbalances in the activity of two anatomically and functionally distinct neural pathways within "cortico-striato-thalamo-cortical circuitry" (henceforth, cortico-basal ganglia loops; reviewed in e.g. [15, 17, 24-26]). These are a direct pathway, which relays striatal input to the thalamus through the internal segment of the globus pallidus (GPi) and the substantia nigra (pars reticulata, SNpr); and the indirect pathway (e.g. [27-29]), which includes an additional relay between the striatum and the GPi/SNpr via the external segment of the globus pallidus (GPe) and subthalamic nucleus (STN). Several such cortico-basal ganglia loops operate in parallel, largely passing through the same BG regions but differing in the parts of the striatum they involve (reviewed in e.g. [17, 24]). For example, some of these loops travel through the dorsal striatum (e.g. the "motor loop" crossing the putamen), while a “limbic” or “motivational” loop travels

through the ventral striatum (nucleus accumbens, NAc). Imbalances between the direct and indirect pathways of such loops have been linked with movement disorders (reviewed in e.g. [14, 25, 27]), as well as with perseverative responding [30, 31]. For example, pharmacologically inhibiting the GPe induces SB in non-human primates [32], while stimulating the activity of the STN decreases them [33], highlighting the importance of the indirect pathway.

In captive animals not subject to pharmaceutical treatments, the few relevant studies have linked their SB with neurotransmitters that are central to basal ganglia function, albeit not exclusive to it (e.g. dopamine, serotonin) (rhesus monkeys *Macaca mulatta*, [34]; horses, *Equus caballus*, [35]; deer mice *Peromyscus maniculatus*, [36]). Caged animals' SBs are also often linked with recurrent perseveration (repetition of learned motor responses that are no longer required, [37]; e.g. in voles [38]; horses, [39]; American mink *Neovison vison* [40, 41]). Such effects might be caused by being reared in adverse or suboptimal conditions. For example, compared to mother-reared individuals, isolation-reared primates show reduced BG receptor densities for leucine-enkephalin, substance P, somatostatin, calbindin and tyrosine hydroxylase (mainly in dorsal striatum, GP and SN, and to a lesser extent in NAc; [42]). Impoverished housing conditions similarly affect basal ganglia [38, 43-45]. For example, barren-housed deer mice also show lower neuronal metabolic activity (as assessed via cytochrome oxidase, CO, an indicator of oxidative metabolism shown to correlate with long-term dendritic activity: [46-48]) in the striatum, STN and SN compared to enriched-housed, low-stereotypic individuals ([6, 49, 50; although cf. [51] in laboratory mice *Mus musculus*). Thus, similar to pharmacologically-induced and human clinical SB, SB in confined animals may reflect alterations in basal ganglia functioning.

Evidence on which specific basal ganglia regions and circuitry are involved is mixed, perhaps reflecting that SBs are a heterogeneous group of behaviours, both within (e.g. [52]) and between species [53]. As described above, housing differences have been found to be correlated with neuronal CO-staining of the striatum: enriched-housed, low-stereotypic deer mice show higher levels of neural activation in both dorsal and ventral striatum [6], and in the dorsal striatum, also more dendritic arborization and increased expression of neurotrophic factors [54]. In addition, consistent with under-activation of the indirect pathway at the striatal level, deer mice with high levels of SB had lower levels of neural activity in caudate/putamen, STN and SN, with individual levels of SB negatively correlating with neuronal metabolic activity in the STN

and SN [49, 55], and positively correlating with the ratio of two opiate neurotransmitters in the dorsal striatum: dynorphin (specific to neurons of the direct pathway) and enkephalin (specific to neurons of the indirect pathway) [56]. Consistent with indirect pathway under-activation specifically, the ratio difference was driven by decreased enkephalin ([56], although cf. [57] for a different interpretation). Other studies of individual differences in SB in confined animals also implicate the nucleus accumbens (and therefore possibly the limbic loop). Highly stereotypic horses had elevated densities of dopamine (D1 and D2) receptors in the NAc (along with decreased D1 receptor density and D2 receptor affinity in the caudate: [35]) compared to horses with little or no SB. In laboratory mice, using markers of long-term neuronal activity (ΔFosB: [58]), highly stereotypic standard-housed individuals show evidence of greater NAc activation; while some of the deer mouse studies [49, 55] found significant *negative* relationships between SB and CO activity in the NAc.

Here, we investigated the role of the BG in the SB of a carnivore: the American mink. Carnivora such as tigers, lions and bears often perform SB in captive situations like zoos and conservation breeding centres (e.g. [53]), yet this behaviour potentially has negative implications for the captive breeding of endangered species, as well as for the public perception of zoos (cf. [59-61]). Understanding Carnivora SB is therefore important. However, Carnivora SB typically differs from that of the rodents and equids studied to date (potentially reflecting both true taxonomic differences, and differences in how different species are typically fed, whether via *ad libitum* versus discrete meals, [62]). Carnivora favour route-tracing and body movements (e.g. pacing, body-bobbing) that peak just before the arrival of food (e.g. [63]). In contrast, the mice studied to date have largely bar-mouthed and jumped (e.g. [55, 58]), while the equine subjects showed a form of oral SB, crib-biting, that seems triggered by food ingestion (e.g., [35, 62]). Research on American mink, a behaviourally well-studied stereotypic carnivore, could thus better yield findings more relevant to captive wild felids, ursids and other Carnivora. Further making mink a useful research subject, they show clear within species heterogeneity in SB, allowing us to test whether the correlates of distinct SB forms within one model have similar or distinct neurological correlates. Thus, they show carnivore-typical locomotor-and-whole body SB, as mentioned, such as pacing and head twirling (sometimes abbreviated to 'loco' SB; [59]). This correlates with recurrent perseveration [41], predicts failure in mating competitions [60], and is reduced long-term by enriched-rearing, even in animals transferred to barren conditions

125 [59]. But mink also show an idiosyncratic scrabbling or digging with the forepaws. Unlike loco
126 SB, this sub-type of SB is not Carnivora-typical; is unrelated to perseveration [42]; is elicited by
127 the proximity of neighbours [64]; does not predict failure in mating competitions [65]; and is not
128 reduced long-term by enriched-rearing, if enriched-raised mink are transferred to barren
129 conditions [59].

130 To investigate whether either of these forms of mink SB resemble or differ from the SB
131 of other taxa, we tested four hypotheses about their neurobiological bases: i) that they reflect the
132 relative under-activity of the indirect pathway; ii) that they involve the dorsal striatum; iii) that
133 they involve the ventral striatum; and, iv), that the two different sub-types of SB differ in their
134 neurobiological correlates, or even the extent to which they reflect any long-term neurobiological
135 changes at all. These hypotheses were tested by regressing regional neuronal metabolic activity
136 (assessed using CO metabolism: see Methods) in six brain regions against individual differences
137 in the two sub-types of SB ('loco' SB and scrabbling), and by comparing groups of subjects
138 raised in barren versus enriched environments that should induce, as previously shown [10, 40],
139 respectively high and low levels of SB. We predicted that, if either mink SB is like that of deer
140 mice [e.g. 43, 55], it would correlate with changes in neuronal metabolic activity in the striatum.
141 If instead either sub-type of mink SB is like that of laboratory rodents or horses, then we
142 predicted changes in this SB to correlate with neuronal metabolic activity in the ventral striatum.
143 If either SB involves alterations in direct or indirect pathway activity, we predicted altered
144 neuronal metabolic activity of two regions involved in the indirect pathway (STN and GPe), as
145 well as an altered ratio in the activity of the two segments of the GP. Specifically, here we
146 predicted a lower ratio of neuronal metabolic activities in the externus to internus globus pallidus
147 (GPe:GPi) in barren-housed, highly stereotypic animals, since this would indicate a relatively
148 less active indirect pathway (cf. e.g. [56, 66]).

149 Finally, if any changes reflected the causal bases of housing-induced SB, we should see
150 them both correlating with individual differences in SB *and* being affected by NE versus EE
151 housing. In testing these hypotheses, we also used statistical approaches that could assess
152 whether SB was best explained by the combined contribution of several of the sampled regions
153 (rather than just each singly).

154

2. Methods and Materials

All housing conditions and experiments were approved by the University of Guelph's Animal Care Committee (AUP #07R033) and Michigan State University's Institutional Animal Care and Use Committee (AUF #04/07-041-00).

2.1. Subjects and housing

Subjects were 32 adult unrelated black male American mink housed indoors at the Michigan State University Experimental Fur Farm. Only males were used as they were the subjects of a larger study investigating phenotypic indicators of male reproductive success [60]. The facility was artificially lit with fluorescent lights, controlled by a digital timer which automatically adjusted on/off times to keep natural daylight hours; and was heated to ~10-15ºC during winter months, and kept at ambient temperatures the rest of the year. Mink had continuous access to drinking water via nipple drinkers; and, as typical for captive carnivores, they were fed a single, well signaled meal daily (c.12pm).

Our subjects were reared for two years from birth in non-enriched (NE; n = 16) or enriched (E; n = 16) conditions (as detailed in [40, 41, 60]). Specifically, mink from both groups were singly housed after weaning (as adolescent and adult mink are naturally solitary in the wild [67]), and visually screened from their immediate neighbours. Briefly, the NE environments consisted of a wire mesh home cage (61x76x46cm) with an attached nestbox (bedded year round). E environments consisted of this basic home cage with a series of overhead structures (including ramps to climb up and down and a 2.5m tunnel) providing each animal with free access to an extra compartment double the size of his home cage, and containing an extra nestbox, a swing, circulating water in which to wade, and several manipulable objects, some familiar, some new each month [60, 65]. These E conditions are preferred by mink [10] and reduce their physiological stress [59].

2.2. Stereotypic behaviour

SB was recorded in the subjects' NE and EE housing environments when they were *c*.23 months old (i.e. at the end of the study) using a combination of live scan and focal sampling (see [59] for details). The two sub-types – locomotor forms (e.g. pacing) combined with whole-body forms (e.g. head-bobbing) (together 'loco' for short), and scrabbling (see Introduction) – were always distinguished. Behavioural data were collected for 4 hours in the morning of 8 consecutive days.

Both forms of SB were recorded as percentage of active time budget, since compared with scores as a percentage of total observations, this measure correlates better with recurrent perseveration [40, 41, 68] and success in mating competitions [60].

2.3. Regional metabolic activity within the basal ganglia

Brains were collected from 30 subjects, killed as humanely as possible less than 24h after the last day of behavioural data collection (SB in this species is stable across time [69], and the time course for CO changes to reflect behaviour is slow, taking hours to days or even weeks [70, 71]). The killing and extraction methods are described elsewhere [65]. Brains were cut at the caudal end of the medulla, extracted, flash frozen and preserved in dry ice for transport from the farm and then stored at -80°C until further processing (sample sizes for each recorded variable are given in Table 1). CO activity was assessed in the following basal ganglia regions: caudate, putamen, GPe, GPi, STN and NAc (Fig. 1), using methods described elsewhere [6, 55]. Briefly, CO histochemistry was performed in ten batches to stain all relevant tissue. For each batch, equal numbers of E and NE brains were removed simultaneously from the - 80° C freezer. Each was coronally cryosectioned (-20 degrees C) at 20μm, until the whole basal ganglia region was captured. Due to the absence of a stereotaxic mink brain atlas, gross anatomical regions were determined with the help of cat [72] and dog [73] atlases, as well as photographs of a polecat brain from the Michigan State University Brain Biodiversity Bank [74]. Sections were then mounted on slides which were kept in the -80° C freezer overnight before staining the following day with diaminobenzidine following a published protocol [6, 75]. CO activity was quantified by optical densitometry (OD; where higher densities indicate higher CO activity [76]) using Fiji (v.2.0.0; used for the NAc) and ImagePro (Media Cybernetics; other BG regions) on selected rectangular areas of digitized images by an experimenter blind to treatment conditions and SB levels. An average of eight such samples per BG region (one sample per hemisphere per section) of the basal ganglia was obtained (brains with regions that could not be sampled at least eight times being excluded from analyses pertaining such regions; see Table 1 for final sample sizes for each region). The reliability of each staining batch was checked by correlating OD readings for a standard (a homogenate whole brain processed for even staining) smeared at different thicknesses (from 10 to 100μm), to check that staining density increased linearly with slice

thickness in every batch. This method identified four brains (2E:2NE) as having sub-optimal staining of the STN. Therefore, those brains were excluded from any analyses involving this region (again, Table 1 gives final sample sizes per region). Because this region does not have anatomical boundaries, to locate the NAc in our images we referred to published papers on ferrets (e.g. [77]) and consulted Dr. Susanne Radtke-Schuller, compiler of the first stereotaxic ferret brain atlas [78].

2.4. Statistical analyses

Statistical analyses were performed using General Linear Models (GLMs) in JMP statistical software [79]. When data did not meet the assumptions of parametric testing, they were transformed to do so (e.g. proportions were arcsine square root transformed). Alpha was set at 0.05, and 2-tailed results are presented. Where models were non-orthogonal, we used the sequential (Type I) sum of squares [80], placing the factor of interest as the last main effect [81]. GLMs were first used to assess the effect of housing on behaviour, and the relationships between the two sub-types of SB, before analysis of the CO data.

Because of variation between staining batches, there were strong correlations between the values obtained for each region and the density of staining in the corresponding 20μm standard from the same batch (for caudate: $F_{1,28}=5.36$, $p<0.05$; putamen: $F_{1,28}=5.97$, $p<0.05$; GPe: $F_{1,24}=9.86$, $p<0.01$; GPi: $F_{1,21}=12.2$, $p<0.01$; STN: $F_{1,23}=24.7$, $p<0.0001$; NAc: $F_{1,23}=10.2$, $p<0.001$). Therefore, all analyses involving CO activity did not use raw data, but instead controlled for these batch differences in degrees of staining by using the residuals of the correlation between the CO activity values for each BG region and the CO values from the same batch's standard homogenates (with large positive residuals thus meaning strong staining for that batch, thence high relative CO activity; see Fig. 2). To check for internal validity, measures taken from both left and right hemispheres for each region were also regressed against each other. Values for both hemispheres were highly positively correlated ($R^2>0.55$; $p<0.001$) for all regions sampled, indicating good internal consistency. Values from left and right hemispheres were then averaged for all subsequent analyses.

Relationships between CO activity and SB were analysed by means of GLMs in which each form of SB (loco or scrabbling) was the dependent variable, and treatment, basal ganglia metabolic activity (residuals of each region -- see above – and also the GPe:GPi GP ratio "GPr"),

and their interaction were independent variables. To assess whether the BG sub-regions (plus GPr) might act in concert to predict SB more strongly than any individual relationship, we also ran two forward stepwise regressions (with no restrictions on predictive variables), where behaviour - either loco or scrabbling - was the dependent variable, and treatment plus CO activity for each BG region plus the GPr were the predictors (all as main effects with no interactions). Best model fit was assessed using the Akaike Information Criterion (AIC). *Post hoc*, we also ran two further tests to further understand the implications of our results. We ran GLMs with SB as the dependent variable, NAc activity, housing and their interactions as independent variables, and adding respectively GP ratio or STN values as additional terms in order to assess whether including these indices of inhibitory pathway function would increase the degree of variance (the R^2) explained by the model. In addition, for effects on NAc CO values, we ran GLMs with both loco SB and scrabbling in the model, to assess whether controlling for one sub-type of SB would reveal otherwise masked correlates of the other to emerge.

3. Results

3.1. Enrichment effects on basal ganglia activity and stereotypic behaviour

Enrichment reduced loco SB ($F_{1,29}$=13.866, p=0.0008; see Fig. 3), and tended to reduce scrabbling (see Fig. 3; the housing effect on scrabbling did not reach statistical significance here, but had been significant in these same males when they were juvenile, at 7-8 months old [59]). In terms of the inter-relationships between the two sub-types, 'loco' and scrabbling SB typically inversely correlate, at least in non-enriched mink, such that individuals with high levels of one show little or none of the other (unpubl. analyses of mink in [10]; unpubl. analyses of male mink in [41, 52]; unpubl. analyses of female mink in [59]). Checking for this pattern in these subjects revealed that in the NE mink, the two SB sub-types negatively co-varied as expected ($F_{1,13}$=42.78, p<0.0001). However, they unexpectedly positively co-varied in E mink (note that although only 4 mink showed both loco and scrabbling, this was enough to make the two forms of SB unexpectedly positively covary in this group: $F_{1,13}$= 5.65, p<0.05).

Our prediction that enrichment would increase the metabolic activity of the basal ganglia regions implicated in the indirect pathway was only partly supported. Enriched mink had greater GP ratios of CO activity (GPe:GPi) than non-enriched mink ($F_{1,24}$=4.723, p=0.003; Figs. 4 and 5). However, no individual basal ganglia region was significantly affected by environmental

enrichment (see Table 3). In addition, GP ratio, the one housing-induced basal ganglia change, did not correlate with SB (see Table 4).

3.2. Correlations between neural activity of the basal ganglia and stereotypic behaviour

Dorsal striatal CO-staining did not correlate with either SB sub-type, and nor did any measure involving the indirect pathway. However, ventral striatal function was implicated: loco SB positively co-varied with activity in the NAc (see Table 3, Fig. 6). Scrabbling also covaried with CO activity in the NAc, but in a pattern that differed between housing groups: in NE mink the relationship was negative, while in E mink it tended to be positive (see Table 4 and Fig. 3). This difference between housing groups reflected the way in which loco SB and scrabbling covaried negatively in NE mink, but positively in EE mink.

Stepwise regressions (summarised in Table 4a) confirmed the importance of the NAc and the apparent irrelevance of the dorsal striatum and other BG regions. They thus identified that loco SB was best explained by a combination of NAc activity and an independent housing effect (in a model that was also itself significant: $F_{3,23}=3.14$; $p<0.05$). Consistent with this, when we ran *post hoc* GLMs to assess whether the degree of variance in loco SB explained (i.e. the R^2 of the model) would be improved by adding GP ratio or STN values to a model including NAc activity and housing as independent variables, we found no increase. Thus these indices of indirect pathway function did not even have additive effects on SB acting in concert with the NAc. Scrabbling, in contrast, was best explained by the relative underactivation of the indirect pathway alone, although note that consistent with our previous lack of effects, this model was not significant (see Table 4b).

4. Discussion

Our results yielded limited evidence for a role of altered indirect pathway functioning in mink stereotypic behaviour, and also no clear evidence for dorsal striatal involvement. Instead, as in laboratory mice and horses [35, 58], individual differences in measures of nucleus accumbens (NAc) activity seemed to positively covary with our subjects' carnivore-typical pacing, bobbing and repetitive head movements ('loco' SB). Such apparent consistency across these three species is interesting because, as outlined in the Introduction (Section 1), the forms of SB involved are

very diverse. A similar positive trend also emerged between NAc CO activity and scrabbling in the E mink. However, this seemed merely to be an artefact of the way that in these subjects, scrabbling and loco SBs positively covaried (in turn likely just a chance effect, since dependent on just 3 loco mink, and also not found in other cohorts of E mink [e.g. those studied by [10, 41, 52], nor in the E females raised alongside the males of the current study [59]). Instead, in NE animals (where both sub-types of SB were more prevalent and time-consuming, and where scrabbling and loco SB showed the inverse correlation expected from other cohorts of mink), scrabbling inversely correlated with NAc CO activity. This resembles the pattern found in deer mice, where high SB is sometimes negatively related to NAc activity [49, 82]. Thus in stereotypic NE mink, individual differences in SB reflect individual difference in NAc CO activity: animals with high loco SB but little or no scrabbling showed relatively high NAc CO activity, while those with little loco SB but much scrabbling showed relatively low NAc CO activity.

At first sight, such results might suggest that mink SBs involve altered reward processing, cautiously suggesting aetiologies related to some forms of OCD (e.g. [15]), 'hyper-motivated' compulsive gambling, drug-taking and eating, and stimulant-induced hyper-activity (reviewed in [58, 83]). However, our data came from differentially reared subjects, and these two housing groups did *not* differ in NAc activity, despite the elevated performance of both forms of SB (especially the loco sub-type) by non-enriched (NE) animals. This lack of housing effect on NAc CO is important because it reveals that changes in NAc activity are not the primary causes of housing-induced SB. Instead, individual differences in NAc CO activity are merely correlates of individual variation in SB that are unrelated to housing conditions. Furthermore, nor could any of our other CO data explain why SB is more time-consuming and prevalent in NE subjects. We thus could not replicate in mink what has previously been found in the dorsal striatum and subthalamic nucleus of differentially housed deer mice [6, 49], where NE animals show lower levels of CO staining in these regions. Our one significant housing effect was that NE animals had lower GPe:GPi ratios, revealing, as predicted, reduced indirect pathway activity in their basal ganglia relative to enriched animals. However, this effect was subtle (individually, neither GPe nor GPi CO values differed between the groups), and also unrelated to the behavioural effects of housing, failing to correlate with SB.

The apparent lack of treatment effect in the present study thus indicates that other factors likely underlie the impact of rearing and housing on mink SB, in turn suggesting new directions for future work. Given the complexity of the cortico-basal ganglia circuitry, one possibility is that enrichment affects mink basal ganglia in ways that we did not measure. For example, measuring neuronal activity per unit area might fail to detect changes in regional volume (cf. the striatal volumetric changes linked with SB in autistic individuals [12]); or perhaps we should have also assessed substantia nigra activity (as decreases in stereotypic and barren-housed deer mice [6, 49]), or striosome:matrix activation (as implicated in stimulant-induced SB: [22]). Alternatively, more focussed analyses could have regionally separated limbic involvement in all other BG nuclei, not just the striatum (e.g. inhibiting the limbic loop region of the GPe induced SB in primates: [32]), to more thoroughly assess limbic loop involvement; and/or have analysed NAc core and shell separately. Looking *beyond* the basal ganglia also seems a logical next step, however. Environmental enrichment alters prefrontal cortex function, for instance [45, 84-86]: a region that interacts with the basal ganglia (e.g. through the striatal 'direct' and 'indirect' pathways, but also bypassing the striatum relay through the 'hyperdirect' pathway, [87, 88]), is crucial for behavioural flexibility, and is also implicated in SB [89]. Alternatively, other cortical and sub-cortical regions could be critical. For example, environmental enrichment increases neurogenesis in the hippocampus (e.g. [45, 90]) and increases behavioural signs of increased hippocampal function in mink [40]; while, in rodents, hippocampal lesions can also induce stereotypic behaviours [91, 92]. Finally, imaging studies have recently also implicated volumetric changes in the cerebellum to the stereotypic behaviour of autistic humans (reviewed in [93]): another region to now investigate in mink.

Overall, our results thus implicate the nucleus accumbens in individual variation in the performance of different forms of SB in mink (the direction of the relationship varying with SB sub-type). But they also suggest that this region is unaffected by housing, and so unlikely to explain the effects of housing on SB. Further, our results show that barren housing affects the balance of the direct and indirect pathways, but without this accounting for the housing effects on SB. Using this CO-staining approach, we thus found no evidence that a relatively underactive indirect pathway contributes to barren housing-induced SB in mink, nor any clear support for an association between basal ganglia dysfunction and SB in this species. However, as well as suggesting further research directions for Carnivora, as outlined above, our results have four

important methodological implications for future work on all housing-induced SBs, regardless of species. First, in showing that individual differences in SB can have different neurological correlates depending on the sub-type considered, they highlight the heterogeneity of SB, and the importance of not pooling sub-types whose causes, correlates and triggers may well differ (cf. [52, 64]). Second, they show that different behaviours can inter-correlate, and that not taking this into account can influence conclusions. They thus build on previous work linking SB with elevations in general activity and/or decreased levels of abnormal inactivity [38, 94], and confirm the importance of both measuring multiple aspects of behaviour and using statistical tools that can separate out inter-correlated variables. Third, they show that individual neurological correlates of SB may reveal nothing about the effects of housing on SB, and thus little or nothing about "altered function" let alone "impairment" or "dysfunction" (an extrapolation sometimes erroneously made in studies relying on individual differences alone, e.g. [38, 57]). Thus meaningful research into housing-induced SB must utilise subjects that come from known, varying housing treatments. Fourth and finally, our results suggest that previous studies may have too narrowly focused on the BG, to the exclusion of other regions. Future studies should now therefore re-test and extend the basal ganglia dysfunction hypothesis by not only looking at more diverse measures of neural function in the basal ganglia, and always including diverse treatment groups, but also by broadening scope to consider other regions including areas external to the cortico-basal ganglia circuitry.

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645

646 **Table 1**. Sample sizes for the different variables recorded. STN: subthalamic nucleus; GPe:
647 globus pallidus externus; GPi: globus pallidus internus; NAc: nucleus accumbens. Square
648 brackets indicate brains for which data were not reliable and so not used in analyses (see main
649 text).

Variable		Enriched raised	Non-enriched raised	Total
Stereotypic behaviour		16	16	**32**
Cytochrome oxidase activity in:	Caudate	15	15	**30**
	Putamen	15	15	**30**
	STN	12 [+ 2]	13 [+ 2]	**25 [+ 4]**
	GPe	13	13	**26**
	GPi	12	12	**24**
	NAc	13	14	**27**

650

651 **Table 2**. Effects of enrichment on basal ganglia metabolic activity (optical density scores). E:
652 enriched; NE: non-enriched; STN: subthalamic nucleus; GPe: globus pallidus externus; GPi:
653 globus pallidus internus; NAc: nucleus accumbens. Significant results in bold. Means are least
654 squared means; SE is the standard error. All analyses corrected for staining batch effects (see
655 text).
656

	E		NE		Statistic	p
	Mean	SE	Mean	SE		
Caudate	0.119	0.007	0.111	0.007	$F_{1,27}$=0.670	0.210

Putamen	0.103	0.007	0.097	0.007	$F_{1,27}$=0.429	0.258
STN	0.198	0.008	0.194	0.008	$F_{1,26}$=0.102	0.376
GPe	0.108	0.008	0.107	0.008	$F_{1,23}$=0.01	0.455
GPi	0.185	0.016	0.197	0.015	$F_{1,19}$=0.310	0.584
GPe:GPi	**0.596**	**0.013**	**0.552**	**0.011**	**$F_{1,20}$=5.840**	**0.012**
NAc*	-1.301	2.400	1.208	2.313	$F_{1,25}$=0.566	0.458

* Data on CO activity for the NAc were acquired using a different software (see Methods), which may explain the difference in orders of magnitude between NAc values and the rest of the sampled BG.

Table 3. Correlations between regional CO activity and two sub-types of stereotypic behaviour (controlling for housing effects). For loco SB, effects of housing was still significant (see text) in all models; for scrabbling, effect of housing was still non-significant (see text) in all models. All analyses again corrected for staining batch effects (see text).

	Loco SB	Scrabbling
Caudate	$F_{1,26}$=0.029, p=0.865 (+ve)	$F_{1,26}$=0.275, p=0.604 (+ve)
Putamen	$F_{1,26}$=0.693, p=0.413 (+ve)	$F_{1,26}$=0.199, p=0.659 (+ve)
GPe:GPi	$F_{1,22}$=0.730, p=0.401 (+ve)	$F_{1,22}$=0.00, p=0.995 (-ve)
GPe	$F_{1,22}$=0.197, p=0.662 (-ve)	$F_{1,22}$=1.051, p=0.316 (+ve)
GPi	$F_{1,19}$=1.009, p=0.328 (-ve)	$F_{1,19}$=2.434, p=0.135 (+ve)
STN	$F_{1,25}$=2.428, p=0.132 (+ve)	$F_{1,25}$=0.125, p=0.727 (+ve)
NAc	**$F_{1,23}$=6.132**	There was an interaction with housing: $F_{1,23}$=6.989, p=0.0145

p=0.021: +ve	Data were therefore split by housing to reveal the following: NE: $F_{1,12}$=5.013, p=0.045: -ve E: $F_{1,11}$=3.64, p=0.082 (+ve)

665

666 **Table 4.** Models from forward stepwise regressions: a) Loco; b) Scrabbling.

667 The best fitting models (according to AICs) are presented, and the best explanatory model is

668 presented in bold font. Cd: caudate; Pt: putamen; STN: subthalamic nucleous; GPe: globus

669 pallidus externus; GPi: globus pallidus internus; GPr: ratio of GPe/GPi; NAc: nucleus

670 accumbens; H: housing

671 a)

Number of factors in the model	Terms in the best predictive model	Associated AIC value
1	NAc	130.113
2	***NAc + H ** **	***128.724 ** **
3	GPr + Pt + NAc	130.271
4	GPr + GPi + Pt + NAc	133.715
5	GPr + GPi + Pt + NAc + H	138.393
6	GPr + GPe + GPi + Cd + Pt + NAc	144.070
7	GPr + Gpe + GPi + Cd + Pt + NAc + H	151.142
8	GPr + Gpe + GPi + STN + Cd + Pt + NAc + H	160.195

672

673 b)

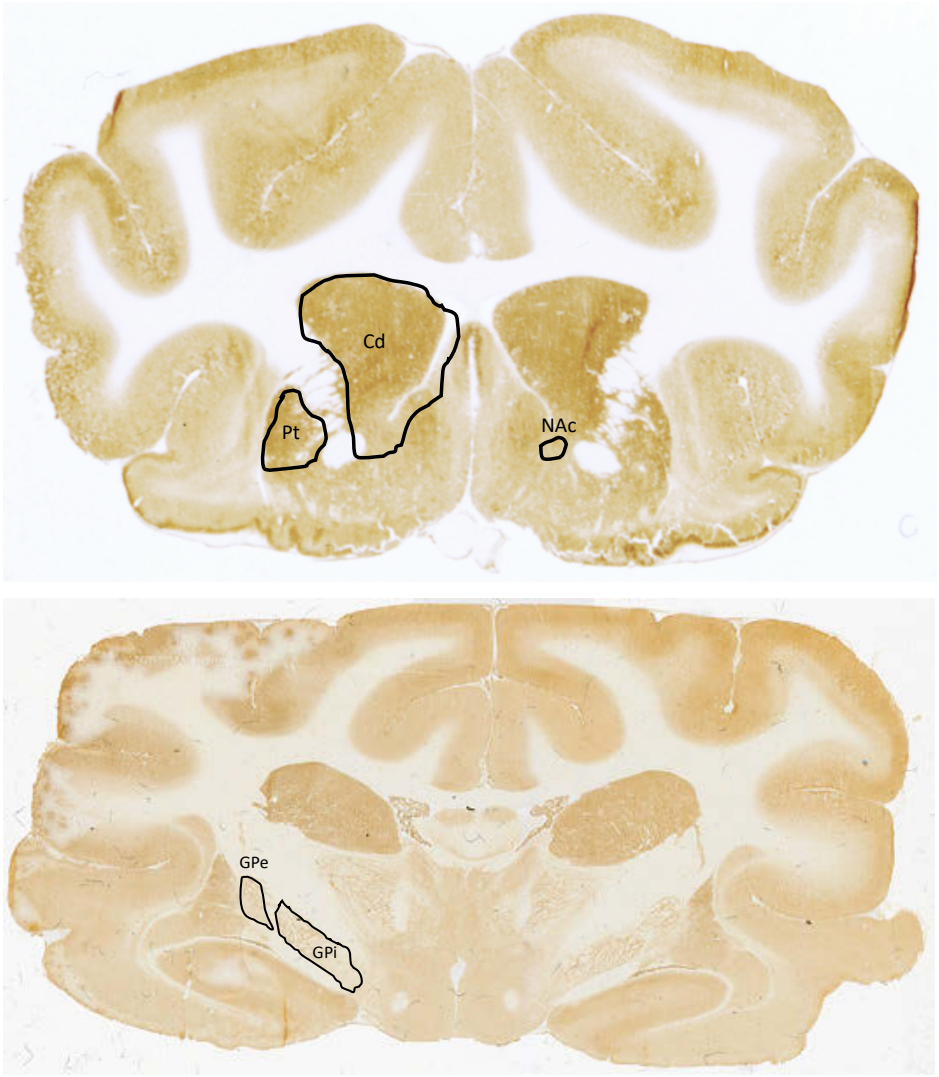
Number of factors in the model	Terms in the best predictive model	Associated AIC value
1	***GPr ** **	***154.594 ** **
2	GPi + Cd	155.626
3	GPi + STN + Cd	157.925
4	GPe + STN + Cd + H	161.080

5	STN + Cd + Pt + NAc + H	164.858
6	GPi + STN + Cd + Pt + NAc + H	169.590
7	GPi + GPe + STN + Cd +Pt + NAc + H	176.916
8	GPi + GPe + GPr + STN + Cd + Pt + NAc + H	186.390

674

675 **Figure 1**. Cytochrome oxidase histochemical staining of the basal ganglia of a mink brain. From
676 top to bottom (rostral to caudal): Cd - Caudate; Pt - Putamen; NAc - Nucleus accumbens; GPe -
677 Globus pallidus externus; GPi - Globus pallidus internus; STN - Subthalamic nucleus; VHp -
678 ventral hippocampus (not sampled, as not part of the basal ganglia, but see Discussion).

679



5	STN + Cd + Pt + NAc + H	164.858
6	GPi + STN + Cd + Pt + NAc + H	169.590
7	GPi + GPe + STN + Cd +Pt + NAc + H	176.916
8	GPi + GPe + GPr + STN + Cd + Pt + NAc + H	186.390

674

675 **Figure 1**. Cytochrome oxidase histochemical staining of the basal ganglia of a mink brain. From
676 top to bottom (rostral to caudal): Cd - Caudate; Pt - Putamen; NAc - Nucleus accumbens; GPe -
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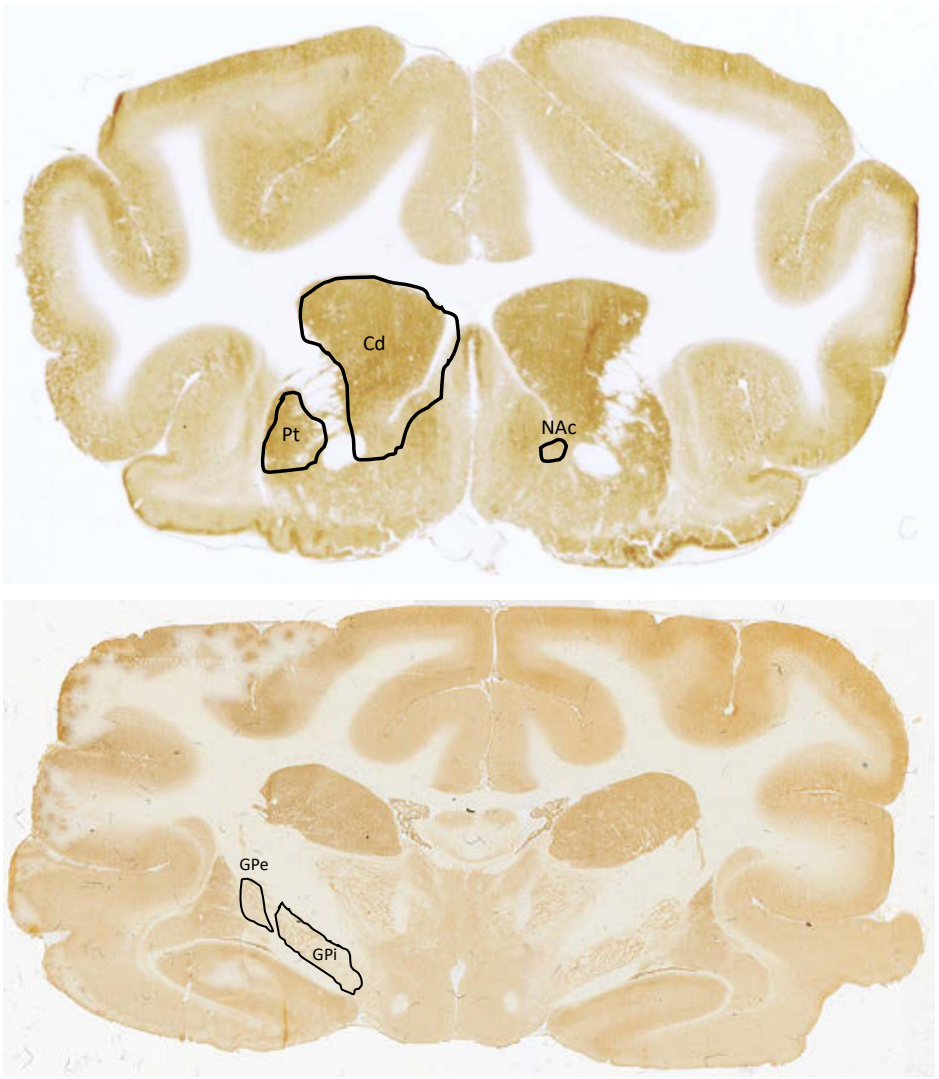
678 ventral hippocampus (not sampled, as not part of the basal ganglia, but see Discussion).

679



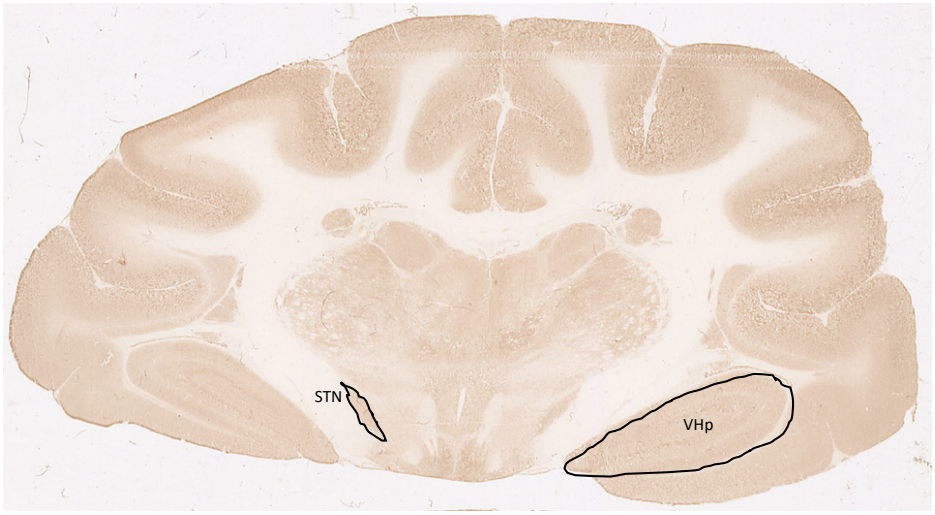


Figure 2. Sample graph showing the correlation between the CO value in the region of interest (here for the STN, the same process being repeated for each region) and the CO value of the corresponding standard. This correlation reflects differential staining intensity across batches. To correct for this in subsequent analyses, residuals are calculated for each data point as the distance from the line of best fit. For example, [A] is a mink with less regional CO activity in the STN than expected given his level of standard staining; while [B] is a mink with more regional CO activity in the STN than expected given his level of standard staining.

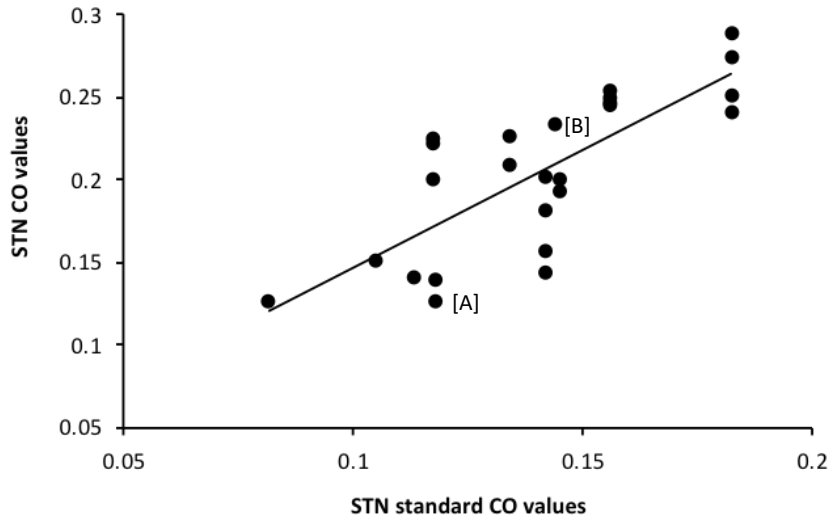


Figure 3. Effects of enrichment on the two subtypes of stereotypic behaviour recorded: loco (solid bars) and scrabbling stereotypies (open bars).

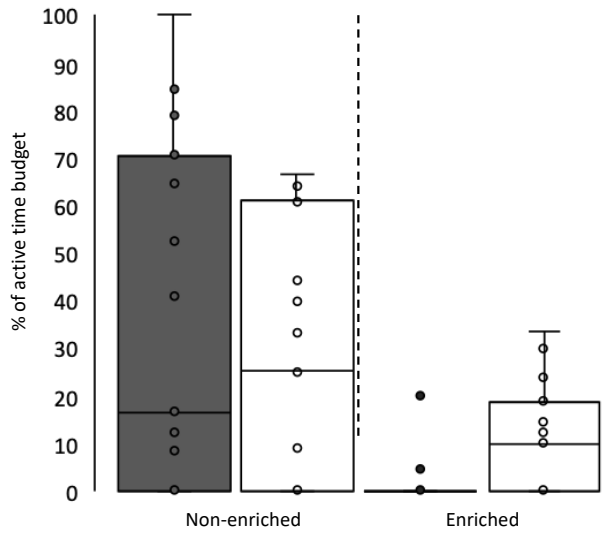


Figure 4. Differences in CO staining between GPe and GPi in an enriched (left) and a non-enriched (right) brain. Pictured brains were stained in the same batch.

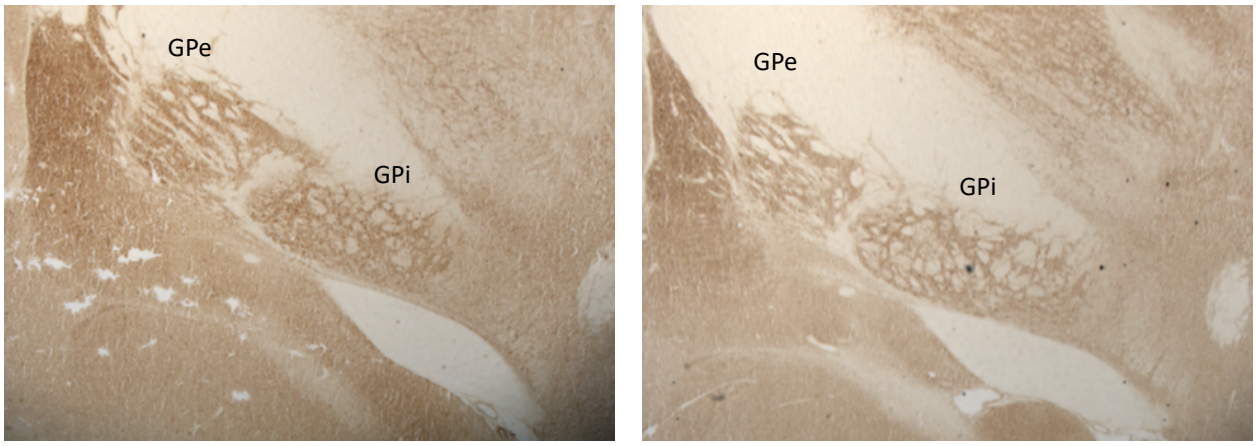


Figure 5. Effects of enrichment on GP ratio (GPe:GPi) of CO staining.

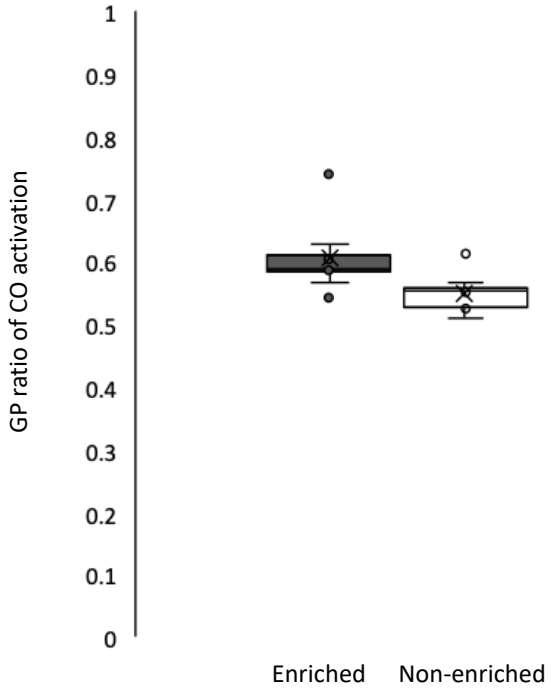
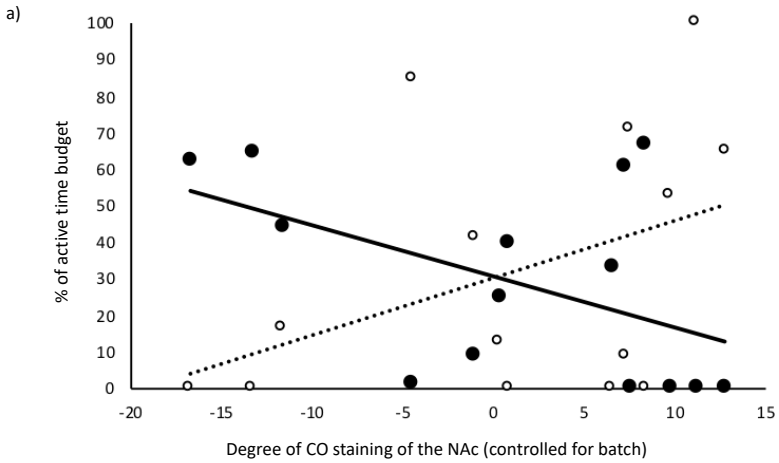
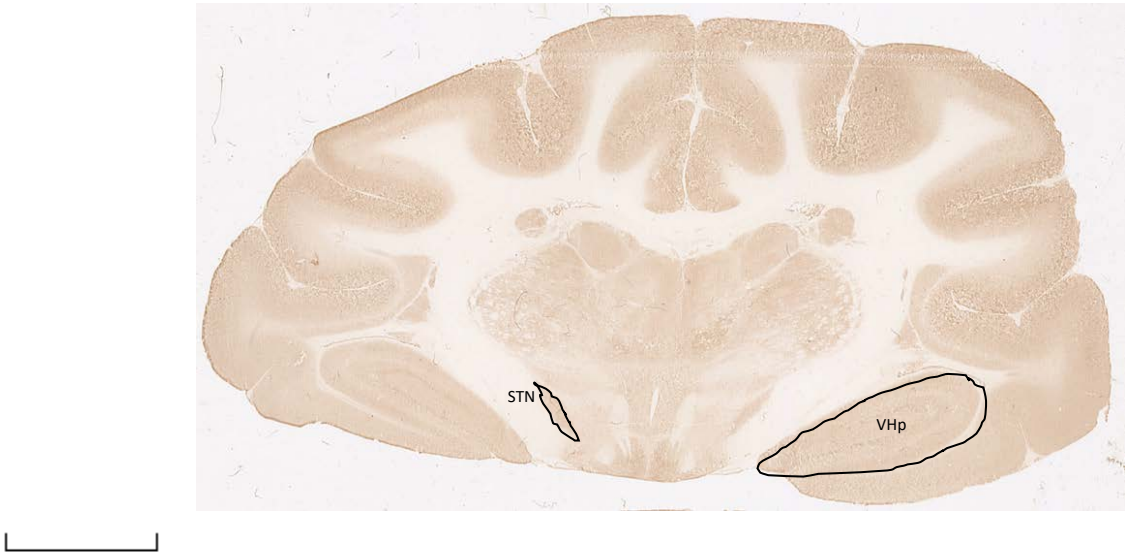


Figure 6. Relationship between CO staining in the Nucleus accumbens (NAc) and stereotypic behaviour. a) non-enriched individuals; b) enriched individuals. Open dots and dotted line: loco SB; black dots and solid line: scrabbling SB.





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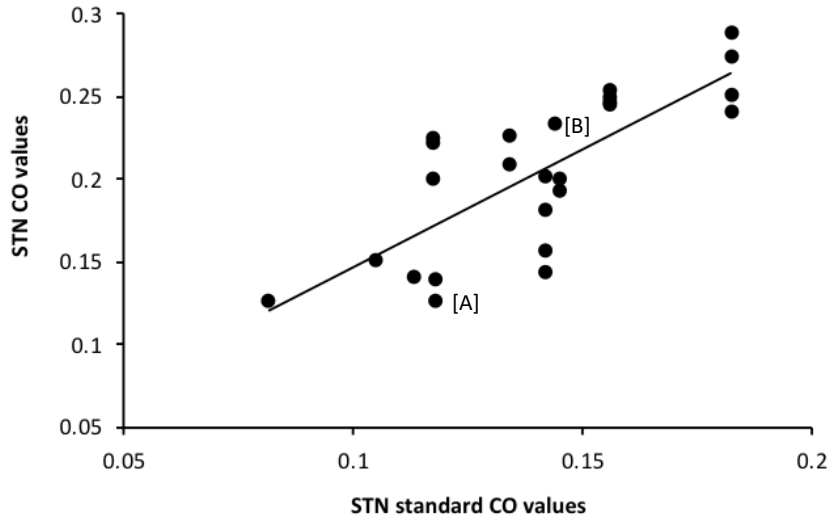

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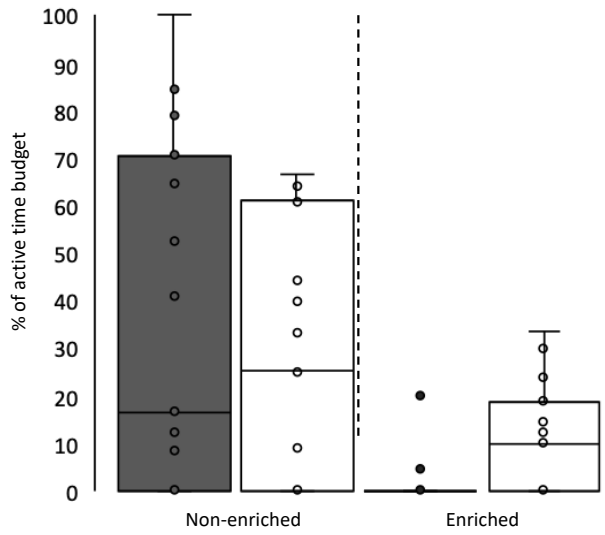

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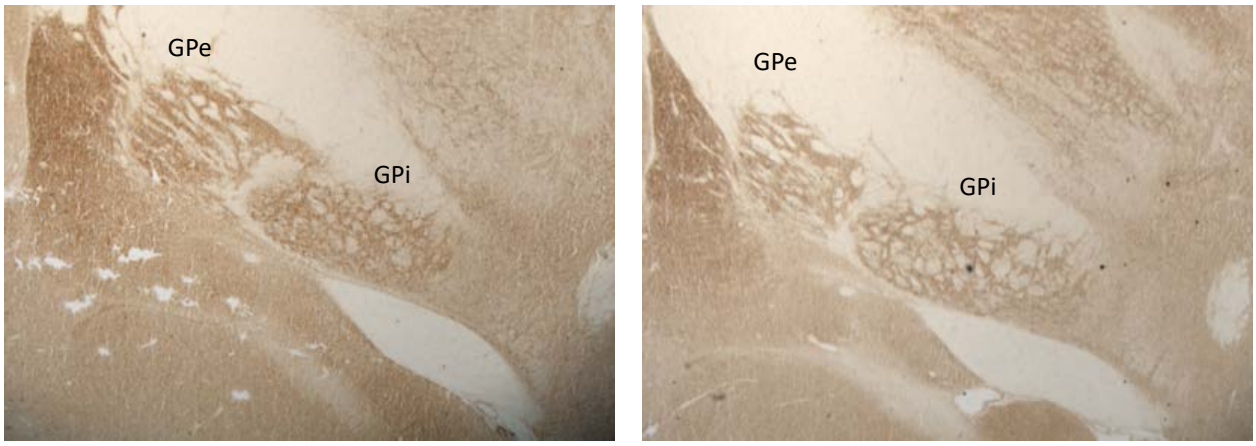

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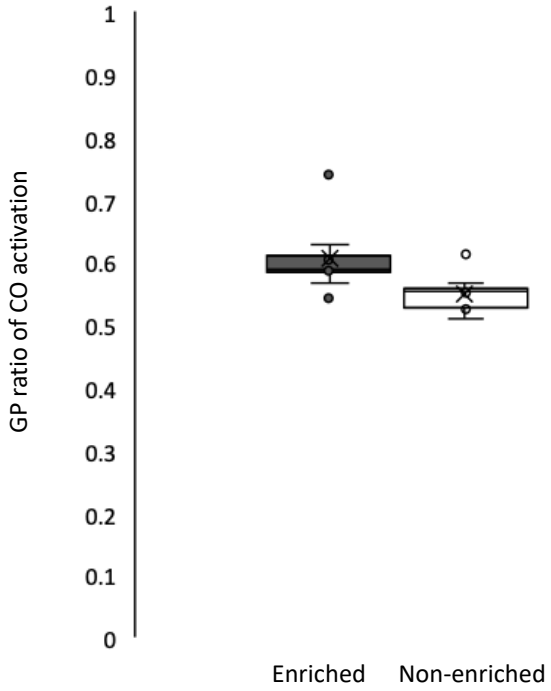

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