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Research report

Causal and functional interpretation of mu- and delta-opioid receptor profiles in mesoaccumbens and nigrostriatal pathways of an oral stereotypy phenotype

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ABSTRACT

Spontaneous stereotypic behaviours are repetitive, compulsive, topographically invariant response patterns commonly observed in captive or domestic animals, which have been linked to dysfunction of basal ganglia input/output pathways. There is evidence that endogenous opioids play a key regulatory role in basal ganglia direct and indirect pathways, but their precise role, both causally and functionally, in spontaneous stereotypic behaviour is unclear. Here we examined the profile of mu- and delta-opioid receptors (density [Bmax] and affinity [Kd]) of basal ganglia structures in stereotypy (n = 10) and non-stereotypy (n = 10) animals using a competitive ligand binding approach. Mu receptor densities were significantly higher in the nucleus accumbens ($p < 0.001$), ventral tegmentum area ($p < 0.001$) and caudate nuclei ($p < 0.001$) of stereotypy compared to control animals. No differences were observed for delta *B*max values in any of the brain regions studied ($p > 0.15$). Receptor binding affinity was only found to be significantly different between control and stereotypy animals for mu receptors on the caudate region; ($p < 0.001$). Our findings suggest that increased inhibition (via mu-opioid receptors) of the indirect (dorsal striatopallidal) pathways are associated with spontaneous stereotypy development. Data also suggested that different types of spontaneous stereotypies (e.g. oral versus locomotor) within or a cross species may have a different neurological basis. This may have important implications for understanding the aetiology and function of these behaviours. In some instances (oral stereotypy), the behaviour may be associated with allostasis, a process that could enhance the reward value of appetitive behaviour performance (as a starting point of stereotypy development).

1. Introduction

Spontaneous stereotypic behaviours are characterised as repetitive, rigid, idiosyncratic and topographically invariant response patterns that can either be environmentally [1] or pharmacologically [2] induced. Although dopamine and dopaminergic pathways have been identified as the primary underlying substrates of stereotypy development and maintenance [2–4], evidence also suggests endogenous opioids may have a substantial role to play in the causal and functional aspects of this behavioural condition. From a causal perspective, administration of predominantly mu opioid receptor antagonists to a range of species (dogs, pigs, cats, chickens, horses and bank voles) has been observed to significantly reduce the performance of environmentally-induced stereotypy [5–14]. More recently, work on stereotypic back-flipping in deermice has shown a significant reduction in enkephalin (mu and delta opioid receptor agonist) release in the anterior dorsolateral striatum (putamen) in animals performing high levels of stereotypy compared to low stereotypy counterparts [15]. This was interpreted as an over-activation of behavioural output to produce uninhibited motor sequences i.e. stereotypy [15]. However, mu and delta receptors act to dampen the indirect pathway of the striatum [16,17]. Thus the results of Presti and Lewis [15] in fact suggest reduced inhibition of the indirect pathway (and thus decreased behavioural output) associated with stereotypy performance. Other research, measuring opioid receptor levels in different brain regions of stereotypy pigs, reported a negative correlation of mu opioid receptors with stereotypy performance in the prefrontal cortex but no changes in the caudate nucleus [15]. In summary, evidence suggests that CNS opioid physiology is critical in

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mediating spontaneous stereotypy performance, but the exact role of endogenous opioids in this process is still unclear.

From a functional perspective, it has been proposed that stereotypy may be a way of increasing endogenous opioids in barren or stressful environments. This 'coping' hypothesis originated from studies reporting opiate antagonist administration reduced stereotypy performance (e.g [18].). This was interpreted as stereotypy increasing levels of central endogenous opioid thereby allowing the animal to 'cope' with stressful environments and events [12]. However, subsequent testing of this hypothesis by looking at direct and inferred measures of opioid release post-stereotypy have produced inconclusive results [14,19–22]. Similarly, studies examining stress levels in the animal before, during and after stereotypy performance have also produced contradictory and inconclusive data [14,23–27].

In summary, studies assessing the role of opioid physiology from both a causal and functional perspective of stereotypy development have to date been contradictory or incomplete. Some studies have suggested that spontaneous stereotypy is mediated via opioid inhibition of the indirect pathway, whereas other studies have suggested opioids are not involved in spontaneous stereotypy either at the level of the basal ganglia or at all.

The aim of the present study, therefore, was to re-assess opioid physiology in a spontaneous stereotypy phenotype with specific focus on mu and delta opioid receptor activity of the mesoaccumbens and nigrostriatal pathways (putamen, caudate and nucleus accumbens, ventral tegmentum area and substantia nigra) to help gain additional understanding about the putative role of opioids in mediating oral stereotypy from both a functional and a causal perspective. Activation of mu and delta receptors appear to be critical for the hedonic sensation elicited by natural reward substrates and behaviours, whereas agonists of kappa receptors produce aversion, hallucination and malaise (see [28] for review). In addition, chronic stimulation with natural reward substrates upregulates opioid receptors within the striatum [29]. Thus, analysing up- and down-regulation of mu and delta receptors profiles in specific brain regions associated with hedonic control (e.g. nucleus accumbens) in stereotypy and non-stereotypy phenotypes, may provide additional insight into the putative function of these behaviours. This aim was achieved by analysing opioid receptor density (Bmax) and affinity (Kd) (as indicators of neural pathway activation [30]) in the basal ganglia regions of horses peforming or not performing the spontaneous stereotypy of crib-biting. This stereotypy is well defined both behaviourally and neurophysiologically [31], and is considered a valid representative of stereotypies in other species given the commonality in underlying causal factors (e.g. restricted feeding, stress) [31].

2. Methods

2.1. Animals

Ten control and 10 stereotypy (crib-biting) horses of similar breed (thoroughbred and thoroughbred cross e.g. Irish draught) and sex (see Table 1) designated for abattoir slaughter (for the purposes of meat production) were used in the study. Horses were behaviourally screened for a 24 h period to confirm them as either control or stereotypy (crib-biting) animals. Crib-biting was defined as the horse gripping onto a fixed object with its incisor teeth, usually at chest level, leaning back onto its hindquarters and contracting the muscles of the neck to bring its head into an arched position [32]. Depending on the individual horse, air may or may not be drawn into the esophagus, producing a grunting sound; this is known as windsucking. Crib-biting can reliably be induced after ingestion of a small quantity of palatable food substrate (e.g. cereal-based concentrate) [31]. As part of the screening process for each animal, stereotypy intensity was measured as the mean crib-biting rate (crib-bites per minute) over 5 min of crib-biting in response to 30 g of cereal-based concentrate over two separate sessions (one hour apart). Stereotypy behaviour was recorded by a human observer and also captured on video. Measurements were carried out two hours after the animals' normal morning feeding time (~11am) (see Table 1.)

Table 1
Characteristics and stereotypy intensity of horses recruited for homologous competition assay. *TB = Thoroughbred, TBx = Thoroughbred cross.

Stereotypy subject	Sex	Breed	Approximate Age (yrs)	Stereotypy Intensity (crib-bites / 5 min., mean of 2 trials)
1	F	TBX	7	11.5
2	M	TB	12	5.5
3	M	TB	5	6
4	M	TBX	15	4
5	M	TBX	18	25
6	M	TB	5	15
7	F	TBX	15	7.5
8	F	TBX	4	5
9	F	TB	16	11.5
10	M	TBX	9	13.5
		Mean ± SEM	**10.60 ± 1.65**	**10.45 ± 2.02**

Control subject	Sex	Breed	Approximate Age (yrs)	Stereotypy Intensity (crib-bites / 5 min., mean of 2 trials)
1	M	TB	3	–
2	M	TB	7	–
3	M	TB	18	–
4	M	TB	22	–
5	M	TB	17	–
6	F	TB	6	–
7	F	TB	4	–
8	F	TB	6	–
9	F	TB	5	–
10	F	TB	9	–
		Mean ± SEM	**9.70 ± 2.13**	–

2.2. Measurement of opioid receptor densities (Bmax) and affinities (Kd)

Brains were removed and sliced into 15 mm coronal sections before being frozen (−40 °C) until required for assay. All brain regions (nucleus accumbens, caudate nucleus, putamen, substantia nigra and ventral tegmentum) were dissected, weighed, and homogenised individually in 15 volumes of Tris buffer (50 mM, pH 7.4, 4 °C). Homogenate was then aliquoted into 1.5 ml plastic tubes and centrifuged at 30,000 × *g* for 35 min at 4 °C. Resultant pellets were then re-suspended in 1 ml of fresh Tris buffer and incubated at 37 °C to remove endogenous ligand. After a second identical centrifugation stage, pellets were re-suspended in 100 volumes of 50 mM Tris buffer (pH 7.4, room temperature). Additionally, for all of the studied brain regions, a 4 ml quantity of tissue suspension was reserved for protein quantification using the Lowry procedure adjusted for Tris. For quantification of mu receptors, 33 × 750ul aliquots of the tissue suspension were incubated at room temperature for 1 h in the presence of 2 nM DAMGO [*N*-methyl-3*H*] (Perkin-Elmer Life Sciences, UK; specific activity 37 kBq/μl) and 1 of 10 cold DAMGO (Sigma, UK) concentrations ranging from 0.5 to 0.6 − 3uM. Prior to incubation each tube was made up to 1 ml using 250ul of 50 mM Tris (pH 7.4).

The use of 33 reactions enabled triplication of each cold DAMGO concentration, leaving two reactions for assessment of non-specific binding, performed in the presence of 10 u M naloxone (Sigma, UK). Binding to Delta receptors proceeded in a similar fashion, except that tissue was incubated in 0.25 nM Naltrindole [benzene ring-3*H*] (Perkin-Elmer Life Sciences, UK; specific activity 37 kBq/μl). Binding was terminated by rapid filtration through GF/B filter paper disks, using a cell harvester (2020B Midi-Harvester, AQS Manufacturing Ltd., UK) followed by 2 × 4 ml washes in ice-cold Tris buffered water (50 mM, pH 7.4). Filter paper disks were transferred individually to 4 ml of

scintillant (Fluaronsafe XE; VRR Life Sciences, UK), and were allowed to stand overnight before counting using a beta counter.

Counts per minute (cpm) were plotted against a logarithmic scale of cold ligand concentration to establish a homologous competitive binding curve in Graphpad Prism (Version 3.0, Graphpad Software, Incorporated, San Diego, USA). In order to establish whether the binding reaction had taken place where only one type of binding site was available, the Hill slope coefficient was established also using Graphpad Prism. Results (Mu : 0.94 ± 0.5; Delta: 0.1 ± 0.02) confirmed one available binding site. *K*d and *B*max values were determined using non-linear regression curve fit (Graphpad Prism) using the equation for a homologous competitive binding curve (one class of binding site).

2.3. Statistical analysis

A normalcy test for 2 sample means (Smirnof-Kolmogorov) suggested agreement with the null hypothesis of skewed distribution ($P > 0.05$) for all density and affinity variables and thus non-parametric statistics (Mann-Whitney U) were used to compare for statistical differences between opioid receptor data obtained from stereotypy and control horses. In order to reduce the chances of a Type 1 statistical error, a Bonferroni correction was implemented to set the level of significance to 0.0025 (0.05/20 tests). Statistical analyses were carried out in SPSS v23 for Macintosh.

2.4. Ethical note

This study was approved by the Aberystwyth University Research Ethic Committee and was carried out in accordance with UK laws relating to the Animals (Scientific Procedures) Act, 134 1986.

2.5. Data availability statement

All data generated from this study will be made freely available via the Aberystwyth University online repository site.

3. Results

Using non-linear regression curve fit and the equation for a homologous competitive binding curve (one class of binding site), log competitive binding curves were used successfully to generate Bmax (receptor density) and Kd (receptor affinity) values for mu and delta receptors in five regions (for examples see Fig. 1).

Mu receptor densities, as indicated by the *B*max values, were significantly higher in the nucleus accumbens (mean rank 15.5 [stereotypy] vs 5.5 [control]; $U = 0$, $p < 0.001$), ventral tegmentum area (mean rank 15.5 [stereotypy] vs 5.5 [control]; $U = 0$, $p < 0.001$) and caudate nuclei (mean rank 15.5 [stereotypy] vs 5.5 [control]; $U = 0$, $p < 0.001$) of stereotypy compared to control animals (using Mann Whitney tests [see Table 2]) (Fig. 2). No differences were observed for delta *B*max values in any of the brain regions studied ($p > 0.15$). Mean *K*d values (nM) were only found to be significantly different between control and stereotypy animals for mu receptors on the caudate region; binding affinity was significantly higher in the stereotypy versus the control animals (mean rank 15.4 [stereotypy] vs 5.6 [control]; $U = 0$, $p < 0.001$; all other regions, $p > 0.21$) (Fig. 2).

4. Discussion

Brains from horses that performed oral stereotypy displayed increases in mu opioid receptor density in both the dorsal and ventral striatal regions. These data add to the growing body of evidence [15,31,33,34] that suggest that alterations in striatal function underlie the development of spontaneously emitted stereotypy in a range of species. More specifically our data strongly indicate increased inhibition (via mu opioid receptors) of the indirect (dorsal striatopallidal) pathway in stereotypy animals, and suggest that the neurobiological basis of oral stereotypy in horses may be different from bar-biting in sows [35] and back-flipping in deermice [36]. We found that inhibition of the dorsal striatopallidal pathways was limited to the caudate and not the putamen region of the striatum (the reverse of that observed in back-flipping deermice by Presti and Lewis [36]). This may reflect differences in the form of stereotypy (oral versus locomotor) or the species (horse vs mouse) between the two studies. Interestingly, differences between the dorsomedial and dorsolateral striatum have previously been reported for equine D1 dopamine receptors [3] whereby crib-biting horses displayed significant reductions in receptor density compared to controls in the caudate, but not the putamen. The data reported here (with specific reference to elevation of caudate MOR's) supports the idea of altered function of the dorsomedial, but not the dorsolateral, striatum being associated with oral stereotypy performance. Overall, the data from this and previous studies suggest that different stereotypies within or between species may have different neurological bases. This is an important point in our understanding of spontaneous stereotypy as it suggests that the causal factors and putative function may also differ within or between species depending the nature of the repetitive behaviour. This notion is also supported by the findings of [37] who reported that horses performing locomotor stereotypy did not demonstrate habitual response patterns in an extinction learning paradigm as compared to those performing oral stereotypy. Given that extinction learning paradigms have been used by previous authors to probe dorsal striatal function [38–40], behavioural evidence points towards differential dorsal striatal activity in the oral versus locomotor stereotypy phenotype. Further research neurophysiologically profiling different spontaneous stereotypies within and between species will further elucidate differences and commonality of the underlying mechanism.

The direct/indirect pathway design of the basal ganglia is limited to the dorsal striatum, with the neurocircuitry of the ventral striatum ordered differently [41]. Mu opioid receptor activation within the nucleus accumbens leads to significantly increased dopamine levels within this ventral part of the striatum [42]. This potentially explains some of the driving mechanisms underpinning our previous observations of

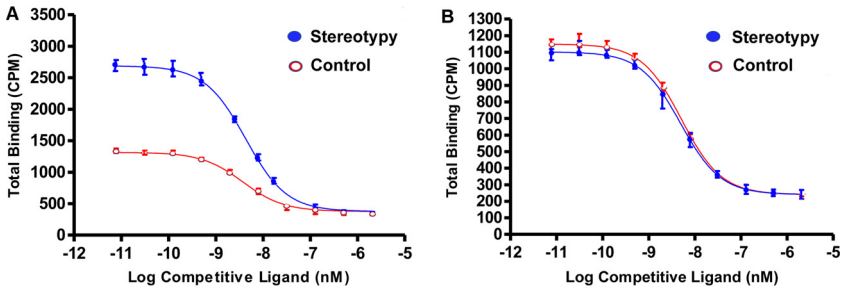

Fig. 1. Mean ± S.E.M. (n = 10) log homologous competitive binding curves for A) mu receptor (DAMGO) binding (cpm) within the nucleus accumbens brain region for control and stereotypy horses; and B) Delta receptor (Naltrindole) binding (cpm) within the nucleus accumbens brain region for control and stereotypy horses.

Table 2
Median (IQR) mu and delta receptor density (Bmax, fmol/mg) and binding (Kd, nM), for control (n = 10) and stereotypy (n = 10 [SB]) animals in striatal and mid-brain regions (Mann-Whitney U [SB vs Control]: ***p < 0.001).

Brain region	Nucleus Accumbens		Caudate		Putamen		Ventral tegmentum area		Substantia Nigra	
	SB	Control	SB	Control	SB	Control	SB	Control	SB	Control
Mu density (Bmax, fmol/mg)	533.9 (424–622)***	158.5 (117–222)***	774.3 (669–900)***	240.5 (211–262)***	627.3 (472–837)	780.3 (578–1136)	244.5 (212–317)***	123.9 (82–130)***	315.6 (215–344)	208.6 (177–363)
Mu affinity (Kd, nM)	2.5 (1.6–4.7)	1.6 (.7–3.5)	4 (3.1–4.7)***	.6 (.4–1)***	2.8 (1.5–4.3)	4 (2–5)	.75 (.4–2)	.7 (.3–.9)	3.5 (2.5–4)	2.9 (2.4–4.5)
Delta density (Bmax, fmol/mg)	463.3 (343–699)	379 (313–648)	623.9 (476–782)	657 (471–799)	625 (278–1360)	554.7 (290–847)	287.6 (227–675)	340.8 (277.5–571.5)	606.9 (339–752)	369 (300–749)
Delta affinity (Kd, nM)	2.2 (1.7–5.2)	3 (1.7–4.4)	3.6 (2.6–4)	3.8 (2–5.7)	4.7 (.9–11.5)	4.3 (1.8–9.5)	1 (.4–3.5)	1.2 (1–3)	4 (2–6)	2 (.8–4.7

increased dopamine receptor density and affinity (D1 and D2 receptor) in the nucleus accumbens of oral stereotypy horses [3] as well as other species [34]. Collectively, the opioid and dopamine data suggest that although dopamine projections into the striatum may be the predominant force in determining and altering basal ganglia mechanics to produce stereotypy behaviour, this system is under the influence of modulating opioid-based physiology. Stereotypy performance was also associated with significantly increased mu receptor density within the VTA and SN. GABA efferents project onto VTA neurons and inhibit DA transmission [43]. The hyperpolarising effect that mu receptor activation exerts upon these GABA neurons results in disinhibition of dopaminergic neurons along the meso-accumbens pathway. For example, social defeat stress in rats, which causes a significant elevation in mu receptor number, causes increased DA transmission into the NA from the VTA [44]. Thus, the level of opioid modulation on altered dopaminergic systems associated with the oral stereotypy phenotype appears to extend to mid-brain (as well as striatal) structures. Interestingly, we have also found a significant increase in binding affinity of mu opioid receptors in the caudate but not the nucleus accumbens of stereotypy horses. This may reflect the different overall functional role of mu opioid receptors acting within the striatum versus the mid-brain whereby the former acts directly to depress activation of GABAergic MSNs [16] whilst the latter is acting indirectly to facilitate dopaminergic input from the VTA into the striatum [43].

The original coping hypothesis of stereotypy suggested that release of opioid neurotransmitters within the CNS produced a hedonic effect that would allow the animal to 'cope' with a sub-optimal environment [12]. Although the hedonic consequences of CNS opioid administration are well documented, it still remains difficult to interpret differences in CNS opioid physiology in the stereotypy phenotype from a functional perspective. This is due to two reasons: 1) changes in opioid physiology as brought about by stress may simply reflect alterations in the CNS that is causally bringing about stereotypy without having any functional significance; 2) even if stereotypy does bring about activation of opioid ligands, it is still difficult to identify markers of this activity in post-mortem material from a functional perspective. Stereotypic behaviour has previously been described as a 'compulsive' behavioural disorder that may bring about over-activation of the CNS opioid system [28] in a way that is similar to the neurophysiological consequences of chronic exposure of natural rewards. Studies have shown that natural rewards (food, sex, social behaviour) operate predominantly through the activation of opioid receptors associated with the mesoaccumbens pathway [45,46]. Although the majority of this work has focused on the general behavioural effects of administrating opioid agonists or antagonists on

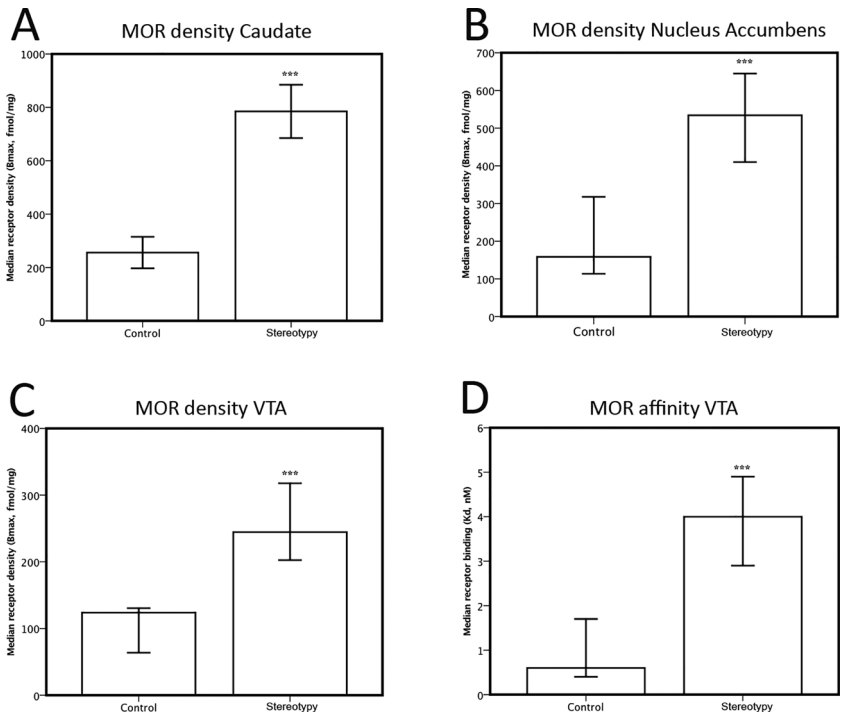

Fig. 2. Statistically significant differences (median ± 95% CI for the median) between opioid receptor number/affinity with oral stereotypy performance; A) Mu opioid receptor (MOR) density Caudate; B) MOR density Nucleus Accumbens; C) MOR density VTA; D) MOR affinity Caudate.

reward-type behaviours, some studies have looked at the effect of natural rewards in changing CNS opioid physiology. For example, intermittent sugar or sweet/fat diet increases mu opioid receptor binding in the nucleus accumbens, cingulate cortex, hippocampus and locus coeruleus [29]. Interestingly, work by Pecina and Berridge [47] has demonstrated that mu opioid receptor activation of the nucleus accumbens (via microinjection of the mu agonist DAMGO) enhanced hedonic 'liking' reactions to a sweet sucrose solution. Within the nucleus accumbens, mu opioid stimulation was found to triple the number of positive orofacial 'liking' reactions elicited by sweetness, in addition to dramatically stimulating intake of palatable food. There are numerous other examples of administration of exogenous opioids increasing the motivation to perform rewarding behaviours, such as eating and sexual activity [48–52], and also enhancing the reward value associated with their performance (see [53] for review). What is interesting about oral stereotypies across a range of species, is that they are performed predominantly as a post-prandial behaviour where intensity of performance is determined by the palatability of the ingested substrate [54]. Given that palatability is heavily mediated via endogenous opioid release, and there is substantial evidence of this type of stimulation leading to a) increased performance of reward type behaviours and b) enhanced hedonic experience of those reward type behaviours, this suggests that some stereotypies (e.g. oral) may have hedonic characteristics.

Appetitive behaviours are considered to be the starting point for stereotypy development as a result of restrictive environments preventing consummatory goals from being attained [33,55]. It has also been demonstrated that this set of behaviours has intrinsic low to moderate levels of reward characteristics [56]. This poses the question as to whether changes in opioid physiology could confer additional reward characteristics to appetitive behaviours, and by doing so increase their functional capacity by a) allowing them to become a form of self-stimulation, b) replacing the consummatory behaviour and hedonic experience that cannot be attained (due to a restricted environment) and c) combatting the physiological consequences of stress associated with a sub-optimal environment. Interestingly, stress-induced neural sensitization is known to reduce reward-threshold (i.e. enhance reward elicited through the same amount of brain stimulation) (e.g [57].) and also increases the reward value of psychostimulants [58] through a process of allostasis [59,60]. Although the latter is considered to be predominantly mediated via dopaminergic systems, it is possible that alterations in opioid physiology also have a strong modulating effect during this process. Further research into the role of the CNS opioid systems in mediating positive feed-back mechanisms/ hedonic experience during appetitive behaviour performance, and how this is altered through allostatic processes, may finally give insight into the putative function of different types of stereotypic behaviour.

5. Conclusion

The aim of the present study was to re-assess opioid physiology in the stereotypy phenotype to help gain additional understanding about the putative role of opioids in orchestrating oral stereotypy from both a functional and a causal perspective. The data reported here indicated increased inhibition (via mu opioid receptors) of the indirect (dorsal striatopallidal) pathway in stereotypy animals ultimately leading to increased disengagement of the 'stop' mechanism of the basal ganglia in its control of behavioural output [33]. The data also demonstrated upregulation of mu opioid receptors at both the proximal and terminal ends of the mesoaccumbens pathway. Given what is known about the effect of opioid receptor activation presynaptically on dopamine neurons along the mesoaccumbens pathway, this would have an overall effect of increased dopaminergic activity within the ventral striatum. One of the roles of the ventral striatum is invigoration of behavioural sequences [61]. Thus, the opioid receptor data presented here gives additional support to the idea that oral stereotypy may be associated with a hyper-motivated endophenotype. Mu opioid receptor activation in certain aspects of the nucleus accumbens has previously been associated with 'hedonic hotspots' [62]. The differences in mu opioid physiology reported here in the oral stereotypy phenotype, although difficult to fully interpret from a functional perspective, may be indicative of allostatic processes whereby appetitive behaviours (as the starting point for stereotypies) develop additional hedonic qualities. This would support the idea that these behaviours, through a process of self-stimulation, could counter the effects of stress, boredom and other attributes of a sub-optimal environment, in a way that would provide further evidence for the 'coping hypothesis' of stereotypic behaviour. Further research directed towards real-time changes in neurophysiolgical and emotional state during different types of stereotypy performance, will help elucidate the putative function of this class of repetitive behaviours.

Author contributions

SDM and AH designed the study. AH and SDM collected samples and performed the laboratory work. CH also performed the laboratory work. MP carried out the statistical analysis of the data and prepared the manuscript. SDM and AH prepared the manuscript. SDM, AH and MP read and commented on the manuscript.

Competing interests

The authors declare no competing financial interests.

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