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Research Report

Olfactory abnormalities in Huntington's disease: Decreased plasticity in the primary olfactory cortex of R6/1 transgenic mice and reduced olfactory discrimination in patients

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ABSTRACT

Reduced neuronal plasticity in the striatum, hippocampus, and neocortex is a common feature of transgenic mouse models of Huntington's disease (HD). Doublecortin (DCX) and polysialylated neural cell adhesion molecule (PSA-NCAM) are associated with structural plasticity in the adult mammalian brain, are markers of newly formed neurons in the dentate gyrus of the adult hippocampus, and are highly expressed in primary olfactory (piriform) cortex. Animal studies have demonstrated that a reduction in plasticity in the piriform cortex is associated with a selective impairment in odour discrimination. Therefore, the number of DCX and PSA-NCAM immunoreactive cells in the piriform cortex were quantified as measures of plasticity in early stage (fifteen week old) R6/1 transgenic HD mice. The transgenic mice had a large reduction in the number of DCX and PSA-NCAM immunoreactive cells in the piriform cortex, similar to that previously reported in the R6/2 mice. We also tested whether odour discrimination, as well as identification and detection, were impaired in HD patients and found that patients (at a similar disease stage as the mice) had an impairment in odour discrimination and identification, but not odour detection. These results suggest that olfactory impairments observed in HD patients may be the result of reduced plasticity in the primary olfactory cortex.

1. Introduction

Huntington's disease is a progressive neurological condition that presents with a combination of motor, psychiatric, and cognitive deficits and is caused by an expansion of CAG repeats in the first exon of the *huntingtin* gene. While the caudate and putamen are traditionally thought of as the most affected structures, early on in the disease there is

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Abbreviations: DCX, Doublecortin; PSA-NCAM, Polysialylated neural cell adhesion molecule

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widespread atrophy in other regions, including the hippocampus, amygdala, and cerebral cortex (Rosas et al., 2003). This extension of pathology outside striatum may also explain the range of symptoms reported in early HD, such as changes in the hypothalamic–pituitary–adrenal axis (Bjorkqvist et al., 2006) and olfactory impairments (Moberg et al., 1987; Nordin et al., 1995; Moberg and Doty, 1997; Hamilton et al., 1999).

Reduced neuronal plasticity is a robust characteristic of the R6 transgenic mouse models of Huntington's disease. Examples include reduced long-term potentiation (LTP) at hippocampal synapses (Lione et al., 1999; Murphy et al., 2000; Gibson et al., 2005; Milnerwood et al., 2006), reduced paired-pulse facilitation in striatal medium spiny neurons (Klapstein et al., 2001), and reduced experience-dependent plasticity in the neocortex, as determined by 2-deoxyglucose brain mapping (Cybulska-Klosowicz et al., 2004; Mazarakis et al., 2005). These studies also correlated reduction in neuronal plasticity with functional deficits on hippocampus-dependent or barrel cortex-dependent behavioural tasks. The transgenic YAC mouse model of HD also has changes in synaptic plasticity, with an impairment of induction and maintenance of LTP at Schaeffer collateral-CA1 synapses, and reduced post-tentanic stimulation (Hodgson et al., 1999). More recently, it has been demonstrated that adult R6 mice have reduced hippocampal neurogenesis (Lazic et al., 2004; Gil et al., 2004, 2005; Phillips et al., 2005; Grote et al., 2005; Lazic et al., 2006), which might also contribute to the hippocampal deficits observed in these mice.

Neurogenesis in the adult dentate gyrus and olfactory bulb/subventricular zone system (OB/SVZ) is becoming increasingly referred to as an example of "structural plasticity" (see Bonfanti, 2006), which includes cell proliferation, migration, differentiation, axonal/dendritic growth and remodelling, and integration of these cells into the neural circuitry, typically as an adaptive response to stimuli. This can be contrasted with classical plasticity, whereby there is a change in the strength of existing synapses, but little in the way of morphological changes in either the pre- or post-synaptic cell. DCX and PSA-NCAM are two markers that have been used for the quantification of structural plasticity (Nacher et al., 2004; Bonfanti, 2006). DCX is a microtubule-associated protein expressed in migrating neuroblasts (Hannan et al., 1999; Gleeson et al., 1999) and has been validated as a marker of hippocampal neurogenesis (Brown et al., 2003; Rao and Shetty, 2004; Couillard-Despres et al., 2005), while NCAM is a member of the immunoglobulin superfamily of adhesion molecules and PSA is a carbohydrate molecule attached to the extracellular part of NCAM. Both DCX and PSA-NCAM are highly expressed in the primary olfactory (piriform) cortex (Nacher et al., 2001).

Since plasticity in the piriform cortex is critically involved in the discrimination of odours (Wilson, 2001; Wilson and Stevenson, 2003), we therefore examined olfactory performance in human HD patients and controls to determine whether specific deficits could be found in odour discrimination, as well as identification and detection. In addition, the number of DCX^+ and $PSA\text{-}NCAM^+$ cells in the piriform cortex were quantified to determine whether these markers of plasticity are altered in this structure.

2. Results

2.1. *Olfactory impairments in HD patients*

Early stage Huntington's disease patients were unable to discriminate between odours as well as controls. In the odd-one-out task, in which they were presented with three odours and had to identify which one was different from the other two, the patients were, on average, correct only 9.5 times out of sixteen trials, while the controls correctly discriminated on 13.9 trials ($t_{(17)}=4.2$, $p<0.001$; Fig. 1A). Patients were also significantly impaired on the olfactory identification task, identifying, on average, three fewer odours out of sixteen than controls ($t_{(17)}=2.6$, $p=0.019$; Fig. 1B). Patients also had a higher detection threshold for butanol, but this difference was not significant ($t_{(17)}=1.1$, $p=0.281$; Fig. 1C). A composite score combining the results of the three tests was calculated (Fig. 1D). All the controls had scores in the normal range (white

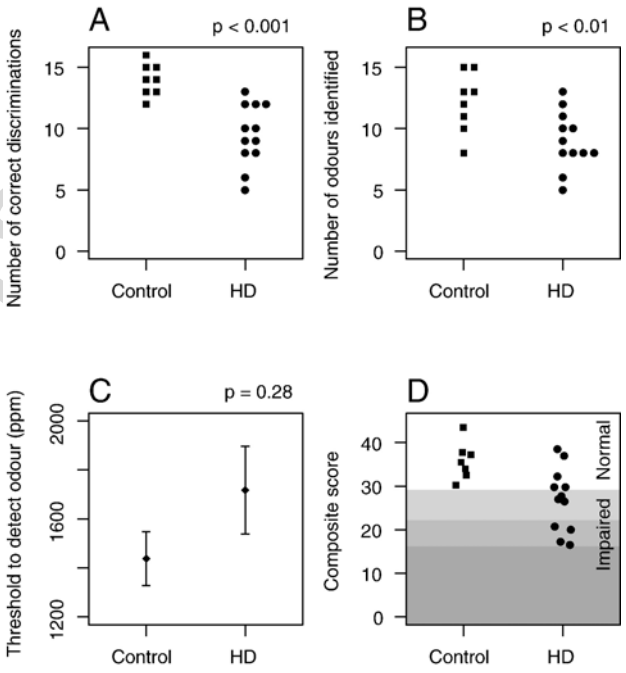

Fig. 1 – Human olfactory data. HD patients were significantly impaired on the olfactory discrimination task, in which they were presented with three odours and had to choose which one was different from the other two (A). Patients were also significantly worse on the olfactory identification task, identifying, on average, three fewer odours out of sixteen than did the controls (B). HD patients had a slightly higher detection threshold for the presence of butanol than did controls, but the difference was not significant (C). A composite score for all three tests was calculated (D) and all the controls had scores in the normal range (white), while some HD patients had scores in the mild hyposmia range (light grey), or severe hyposmia range (medium grey), with some scoring close to the anosmia range (dark grey). Population norms based on Mackay-Sim et al. (2004). Note: values on *y*-axis do not start at zero for (C) ppm = parts per million.

Table 1 – Body weight, brain weight and brain volume of transgenic R6/1 and wild-type mice

	R6/1	Wild type	*p*-value
Body weight (g)	22.3 (2.8)	26.1 (4.7)	0.098
Brain weight (mg)	366 (13)	414 (23)	<0.001
Striatal volume (μm^3)	13.0 (0.6)	14.6 (0.9)	<0.001
Cortical volume (μm^3)	29.2 (1.3)	31.5 (1.4)	0.004

Values represent means (±SD).

background), as did some of the HD patients. The majority of patients however had scores in either the mild hyposmia range (light grey) or severe hyposmia range (medium grey). Although no patients were in the anosmia range (dark grey), some had scores close to the cut-off. Population norms are based on Mackay-Sim et al. (2004).

2.2. *Physical characteristics of transgenic and wild-type mice*

R6/1 mice typically display morphological and neuropathological abnormalities by fifteen weeks of age, and this was confirmed in the sample used in this experiment by measuring the body weight, brain weight, and striatal and cortical volumes of mice (Table 1). The R6/1 mice had brains that were 11.5% lighter than those of controls ($t_{(21)}=4.41$; $p<0.001$). In addition, R6/1 mice had 11.2% smaller striatal volume ($t_{(21)}=3.82$; $p<0.001$) and 7.2% smaller cortical volume ($t_{(21)}=3.26$; $p<0.004$), compared to the wild-type mice. The body weight of the R6/1 mice was 14.8% less than the controls, but this difference was not significant ($t_{(21)}=1.73$; $p=0.098$). The transgenic mice at this age also had neuronal intranuclear inclusions of the transgenic huntingtin protein (data not shown).

2.3. *DCX$^+$ cells are greatly decreased in the piriform cortex of R6/1 mice*

DCX$^+$ cells were found in the granular cell layer and subgranular zone in the dentate gyrus, as well as in layers II and III of the piriform cortex (Figs. 2A and B); the vast majority of labelled cells were in layer II. The number of DCX$^+$ cells was greatly reduced in the dentate gyrus of the R6/1 transgenic mice (Wilcoxon test: $p<0.001$; Fig. 3A), as we have shown previously (Lazic et al., 2006). In addition, there was a large decrease in the number of DCX$^+$ cells in the piriform cortex of the R6/1 mice (Wilcoxon test: $p<0.001$; Fig. 3B), similar to that observed in the R6/2 mice (Phillips et al., 2006). Furthermore, there was also a significant correlation between the number of DCX$^+$ cells in the piriform cortex and the dentate gyrus ($r=0.818$; $p<0.001$; $CI_{95\%}=0.612, 0.920$).

2.4. *PSA-NCAM$^+$ cells are greatly decreased in the piriform cortex of R6/1 mice*

Large numbers of PSA-NCAM$^+$ cells were found in the granular cell layer and subgranular zone of the dentate gyrus, as well as in layers II and III of the piriform cortex, with the vast majority being in layer II (Figs. 2D and E). The number of PSA-NCAM$^+$ cells was greatly reduced in the dentate gyrus of HD mice (Wilcoxon test: $p<0.001$; Fig. 3C), as well as in the piriform cortex (Wilcoxon test: $p<0.001$; Fig. 3D). There was also a strong correlation between the number of PSA-NCAM$^+$ cells in the dentate gyrus and piriform cortex ($r=0.862$; $p<0.001$; $CI_{95\%}=0.699, 0.940$).

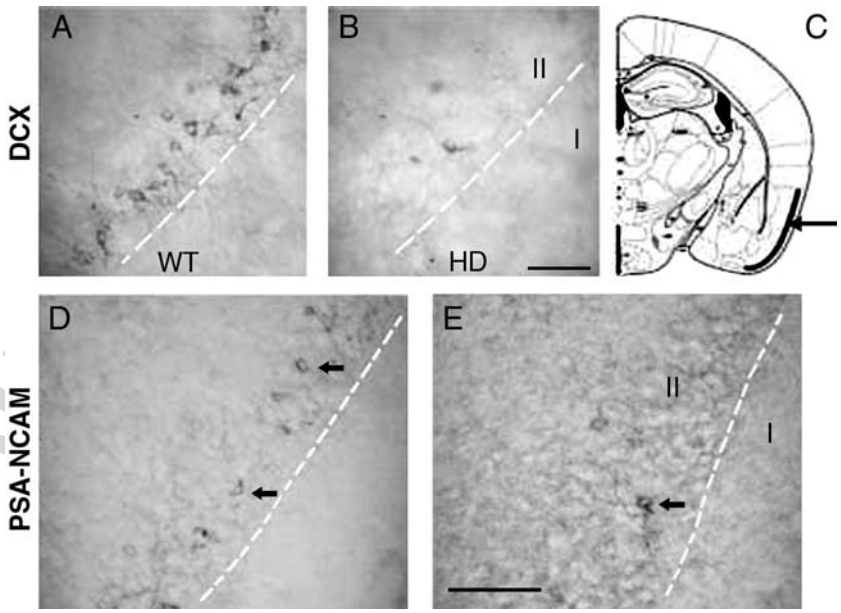

Fig. 2 – DCX$^+$ and PSA-NCAM$^+$ cells in the piriform cortex. The number of DCX$^+$ cells in the piriform cortex of WT mice (A) is greater than in R6/1 HD mice (B). Schematic diagram with the location of the piriform cortex indicated by the arrow (C; adapted from Paxinos and Franklin, 2001). Similarly, the number of PSA-NCAM$^+$ cells is greater in the wild-type (D) compared to the R6/1 mice (E). I = Layer I of piriform cortex; II = Layer II of piriform cortex. Scale bars, A–B = 50 μm; D–E = 200 μm.

3. Discussion

We have previously shown that the number of DCX$^+$ cells in the dentate gyrus is decreased in R6/1 and R6/2 mice, and that there is also a decrease in the number of DCX$^+$ in the piriform cortex of R6/2 mice (Phillips et al., 2005; Lazic et al., 2006; Phillips et al., 2006). We now extend these findings to show a dramatic reduction in the number of DCX$^+$ and PSA-NCAM$^+$ cells in the piriform cortex of R6/1 mice, and that patients with mild to moderate HD have an impairment in olfactory discrimination and identification. This suggests that in HD, the alterations in olfaction may be due to changes in the piriform cortex.

DCX has been validated as a marker of neurogenesis in the dentate gyrus (Brown et al., 2003; Rao and Shetty, 2004; Couillard-Despres et al., 2005), but it is not known if this holds in the piriform cortex, although a recent study has demonstrated neurogenesis in this structure in rats (Pekcec et al., 2006). There is also some evidence in both primates and rabbits that neuroblasts can migrate from the SVZ into the piriform cortex (Bernier et al., 2002; Luzzati et al., 2003), but it is not clear whether the decreased number of DCX$^+$ and PSA-NCAM$^+$ cells in the present study reflects a decrease in cells migrating to the piriform cortex, a decrease in neurogenesis *in situ*, changes in plasticity, or some combination of these. Further examination of the piriform cortex, particularly with retroviral labelling of dividing cells, will provide answers to the above questions.

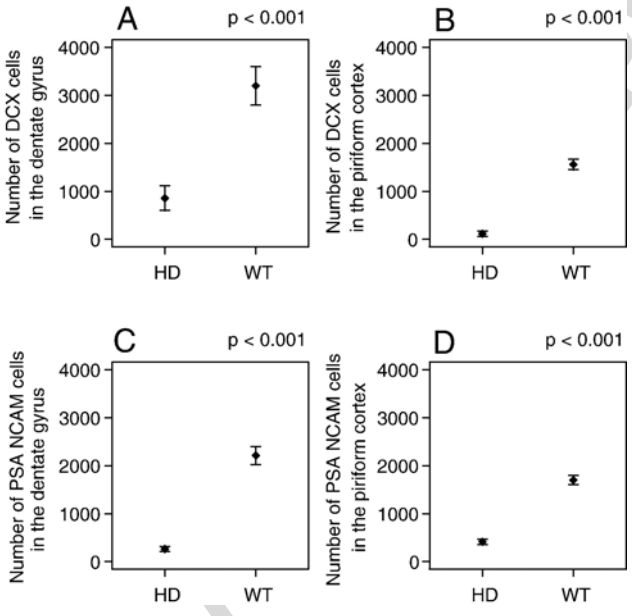

Fig. 3 – Number of DCX$^+$ and PSA-NCAM$^+$ cells in the dentate gyrus and piriform cortex. The number of DCX$^+$ cells is greatly reduced in the dentate gyrus of HD mice (A), as well as in the piriform cortex (B). In addition, R6/1 mice had a large decrease in the number of PSA-NCAM$^+$ cells in the dentate gyrus (C) and in the piriform cortex (D), compared to wild-type controls. Note: the variability was low in the HD group, and therefore the error bars are very small.

DCX might be involved with structural plasticity (Nacher et al., 2001), as it is involved with microtubule reorganisation (Francis et al., 1999), and as such, may play a role in neurite formation, which involves cytoskeletal reorganisation (Jockusch et al., 2004; Bianchi et al., 2005). In addition, the majority of DCX$^+$ cells also express PSA-NCAM (Nacher et al., 2001), which is involved with promoting changes in cell–cell interactions and in facilitating certain forms of plasticity (reviewed in Rutishauser and Landmesser, 1996; Durbec and Cremer, 2001; Bonfanti, 2006). It is therefore not surprising that there was a concomitant decrease in the number of PSA-NCAM$^+$ cells, in both the hippocampus and the piriform cortex of the HD mice. This reduction might be indicative of a general decrease in neuronal plasticity in these mice, which is consistent with previous studies on transgenic HD mice (Lione et al., 1999; Hodgson et al., 1999; Murphy et al., 2000; Klapstein et al., 2001; Cybulska-Klosowicz et al., 2004; Gibson et al., 2005; Mazarakis et al., 2005; Milnerwood et al., 2006).

Since the R6/1 mice have reduced neurogenesis/plasticity in the olfactory cortex, they might be expected to show deficits in olfactory learning or discrimination. Although this has not been investigated in these HD mice, olfactory impairment is a consistent finding in patients with HD (Moberg et al., 1987; Nordin et al., 1995; Moberg and Doty, 1997; Hamilton et al., 1999), and the present study has confirmed and quantified this deficit. Our study suggests that the ability to discriminate between odours is more impaired than the ability to recognise odours, while detection threshold is the least affected, at least in the early stages of the disease. SVZ/OB neurogenesis appears to be specifically involved in olfactory discrimination in mice, but not in threshold detection or olfactory memory (Gheusi et al., 2000). This latter study used NCAM-deficient mice, which have decreased migration of cells from the SVZ into the olfactory bulb; but in addition, these mice may have alterations of PSA-NCAM expression in the piriform cortex which could explain this deficit. Further studies with aged mice and with leukaemia inhibitory factor receptor mutant mice (both of which have reduced OB/SVZ neurogenesis) have shown decreased olfactory discrimination (Enwere et al., 2004). Plasticity in the piriform cortex is critically involved in the discrimination of odours (Wilson, 2001; Wilson and Stevenson, 2003), and therefore the observed discrimination deficit in HD patients is consistent with reduced plasticity in this structure.

There was a strong correlation (r=0.818) between the number of DCX$^+$ cells in the piriform cortex and dentate gyrus, as well as between the number of PSA-NCAM$^+$ cells (r=0.862), possibly indicating that a similar mechanism is responsible for the reduction in number of labelled cells in these two structures in the HD mice. For example, brain-derived neurotrophic factor (BDNF)—a factor known to increase neurogenesis (Lee et al., 2002)—is reduced in the hippocampus, striatum, and cortex of HD mice (Zuccato et al., 2001, 2005; Spires et al., 2004), and while its levels have not been examined in the piriform cortex of these mice, BDNF is known to play a role in modifying synapses in this structure (Nanobashvili et al., 2005). Thus, decreased levels might lead to cellular dysfunction in both the piriform cortex and hippocampus. An alternative aetiological agent may be stress hormones, as they decrease both neurogenesis (Wong and Herbert, 2005, 2006), and synaptic plasticity (reviewed in Kim

et al., 2006). Recently, it has been shown that R6/2 mice have increased plasma and urine corticosterone levels (Bjorkqvist et al., 2006), which are known to decrease the number of DCX^+ and PSA-$NCAM^+$ cells in the piriform cortex (Nacher et al., 2004).

In conclusion, the R6/1 transgenic mouse model of Huntington's disease has a substantial reduction in the number DCX^+ and PSA-$NCAM^+$ cells in the primary olfactory (piriform) cortex compared to wild-type controls, and the number of these cells in the piriform cortex correlates with that in the dentate gyrus, suggesting a common underlying mechanism (e.g. BDNF or glucocorticoid levels). However, it remains to be determined whether this represents decreased neurogenesis or cellular plasticity in the piriform cortex, and whether this affects olfactory learning or discrimination in the transgenic mice. Reduced neurogenesis/plasticity might underlie the olfactory impairments we observed in human HD patients, and such tests of olfactory function may prove useful in monitoring the early changes of patients as well as following the course of the disease.

4. Experimental procedures

4.1. *Human subjects*

Patients ($n=12$) and controls ($n=8$) were recruited from the HD clinic at the Centre for Brain Repair, University of Cambridge. The mean age of patients was 52.9 (SD=7.5) and 53.9 (SD=6.2) for controls. Table 2 displays the demographic characteristics of the subjects. Patients were specifically in the early stages of the disease with a mean total Unified Huntington's Disease Rating Scale (UHDRS) motor score of 15.9 (range=7 to 43) so as to match the stage of disease in the mice. For inclusion into the study, patients must have had (1) official documentation of the presence of the HD mutation by genetic testing, (2) a modified Mini Mental State Examination score >24, (3) a UHDRS chorea score <10, (4) a Total Functional Capacity score >8, (5) an Independence Score >80, (6) non-smoking or light-smoking status (able to abstain for >24 h prior to testing), (7) no history of head trauma, (8) no history of nasal problems, and (9) no concurrent major psychiatric illness.

Controls were, where possible, close family members of a similar age and sex; however, some were recruited from the general public. For inclusion into the study controls had to (1) be in general good health, (2) have non-smoking or light-smoking status, (3) have no history of head trauma, (4) have no history of nasal problems, and (5) be similar to one of the HD subjects by age, sex, and race/ethnicity. Consent for this study was granted by the Cambridge Local Research Ethics Committee and written informed consent was obtained from all subjects after a detailed explanation of the procedures.

Table 2 – Demographic characteristics of patients and controls

	Patients	Controls
Age	52.9 (7.5)	53.9 (6.2)
Sex		
Males	$n=6$	$n=3$
Females	$n=6$	$n=5$
Smokers	$n=3$	$n=1$
UHDRS motor score	15.9 (9.4)	N/A
UHDRS chorea score	5.6 (4.1)	N/A

Values represent means (SD).

4.2. *Human olfactory tests*

Olfactory stimuli were presented via Sniffin' Sticks (Heinrich Burghart, Wedel, Germany) according to the manufacturer's instructions. To determine subjects' threshold for detection of the odour butanol, a three-alternative (odourant and two blanks), blind, forced-choice test was used. A series of 16 dilution steps was used, ranging from 349 ppm to 3055 ppm, according to a staircase procedure. Each successive dilution step was one-third the concentration of the preceding dilution. The order of presentation of the odourant and blanks was randomised in each trial. All subjects began at the weakest concentration and progressed toward the higher concentrations to avoid adaptation. On every trial, the subject was asked to indicate which of the three stimuli presented (odourant or blanks) contained the butanol. An incorrect choice on any trial (selection of a blank), led to a two-step increase in concentration on the next trial. A correct choice led to a repeat presentation of the same concentration. The "starting point" was identified as the first double-correct guess. Once a correct choice had been made twice, a one-step decrease in concentration was made on the next trial. If this was then correctly identified twice in a row, the next lower concentration was then presented. This method was continued until the patient missed one concentration. A one-step higher concentration was then presented. If this concentration was then missed again, the next higher concentration was then given until a dilution step was correctly identified. Once this occurred, a lower concentration was then presented. Testing was concluded when seven reversals occurred. The threshold was calculated as the mean of concentration of the last four reversals.

To assess olfactory discrimination, we gave 16 trials with a three-alternative (two identical odourants and one different), blind, forced-choice test. Subjects were instructed to identify the stick that contained the odourant that differed from the other two. Each stick within a triplet was presented at 5-s intervals, with a 30-s interval between triplets. Each stick was presented only once.

A forced-choice, single presentation test was employed to assess subjects' ability to identify odours. A series of 16 sticks was used, all containing different commonly recognised smells (e.g. cinnamon, bananas, fish). Subjects were presented with one stick at a time and asked to choose from a list of four different smells which one best described the presented smell.

4.3. *Animals*

Wild-type CBA ($n=18$) and R6/1 transgenic mice ($n=5$) from a CBA background were housed in the Department of Physiology, Anatomy and Genetics, Oxford University. Work on animals conformed to the UK Animals (Scientific Procedures)

Act 1986 and was performed under appropriate Home Office project and personal licenses. Mice were bred from a colony that originated from crossing male R6/1 mice (Mangiarini et al., 1996, The Jackson Laboratory, Bar Harbor, ME, USA) with female CBA mice. All mice were kept on a 12/12-h light/dark cycle, with food pellets and water freely available. Both male and female mice were used and they were housed in same-sex groups of four to six per cage (dimensions: 28 cm×44 cm×12 cm). At 4 weeks of age, tail tissue was taken from the mice for PCR genotyping, and a microchip (Labtrac, Uckfield, UK) for identification was inserted subcutaneously under general anaesthesia induced with hypnorm (fentanyl citrate; Janssen Pharmaceutica, Berse, Belgium) and hypnovel (midazolam; Roche) in distilled water (1:1:2; 2.7 mL/kg).

4.4. *Histology*

Mice were killed with a 0.5-mL intraperitoneal injection of Euthatal (pentobarbitone sodium, 200 mg/mL; Merial, UK), which was followed by dislocation of the neck once the mice were unresponsive to stimuli. Mice were perfused transcardially with approximately 50 mL phosphate-buffered saline (PBS) solution followed by another 50 mL of 4% paraformaldehyde fixative. The brains were removed and placed in 4% paraformaldehyde overnight and then transferred to a 30% sucrose solution until they sank. Brains were sectioned at 40-μm intervals in the coronal plane using a freezing stage microtome. Sections were then placed into 96-well plates containing a Tris-buffered saline (TBS) plus 0.05% sodium azide solution and were stored at 4 °C.

Acetylcholinesterase (AChE) staining was used to demarcate the striatum for volumetric measurement using the modified Koelle (1955) method. Briefly, sections were mounted on gelatinized slides and allowed to dry overnight. Sections were then placed in an incubation medium (781 mg copper sulphate, 750 mg glycine, 2.88 g sodium acetate in 1 L water; pH 5.0) for 3 h at 37 °C. Sections were then washed 4 times for 3 min with distilled water and developed in a sulphide solution (2 g sodium sulphide in 200 mL water; pH=7.5) until golden brown. Finally, sections were dehydrated using a series of ascending ethanol solutions (70%, 95%, and 100%) and xylene, and then coverslipped using DPX.

4.5. *Immunohistochemistry*

Staining was performed on free-floating sections. Incubation and washing solutions contained 0.2% Triton X-100 (Sigma) in TBS (Tx-TBS) unless otherwise noted. For DCX staining, sections were quenched for 10 min using 10% H_2O_2 /10% methanol in distilled water, followed by a 3×10 min wash with Tx-TBS. A 1-h block with 3% normal donkey serum (NDS) was followed by incubation with a goat anti-DCX primary antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a 1% NDS blocking solution at room temperature and left overnight. After washing (3×10 min with Tx-TBS), incubation with a donkey anti-goat biotin secondary antibody (1:200; Sigma; plus 1% block) in Tx-TBS for 2 h was followed by another wash (3×10 min with Tx-TBS). Next, a streptavidin–biotin-conjugated complex (1:200; ABC Elite kit; Vector Laboratories, Burlingame, CA, USA) in a Tx-TBS solution was applied for a further 2 h. After another 3×10 min wash with Tx-TBS, three drops (per 5 mL of Tx-TBS solution) of the Vector SG chromogen (Vector Laboratories) and three drops of H_2O_2 (per 5 mL of Tx-TBS solution) were used to visualise the antigen and left on for approximately 2–3 min. Sections were then washed three times with TNS for 10 min each. Sections were mounted on gelatinized glass slides and dehydrated and coverslipped as above. Staining for PSA-NCAM used a similar protocol with a mouse anti-PSA-NCAM primary antibody (1:400; Chemicon, Temecula, CA) and an anti-mouse biotin-conjugated secondary (1:200; Serotec, Oxford, UK).

4.6. *Stereology and quantification*

All quantification was done with the experimenter blind to the genotype of the mice. The Olympus CAST-Grid system (version 1.09; Denmark) was used to quantify the number of DCX^+ and PSA-$NCAM^+$ cells using the $N_v \times V_{ref}$ method (Gundersen et al., 1988). Sections through the dentate gyrus were sampled in a systematic random manner using a 1:12 series. On each section, the dentate gyrus was outlined unilaterally under a 4× objective and the enclosed area was calculated by the CAST-Grid software. Sections within the highlighted area were then sampled at random and cell counts under the 20× objective were used to determine the total number of immunoreactive cells within the sampled region (N_v), using the optical dissector method. This was then repeated on the other side of the brain and on the other sections. The volume (V_{ref}) was calculated by summing the values of all of the area measurements, multiplying by the thickness of the sections (40 μm) and then multiplying by 12 to account for the frequency of sections. For analysis of DCX and PSA-NCAM staining in the dentate gyrus, the size of the counting window was 4844 μm^2 and approximately 50 samples from three sections were examined. There were relatively few immunoreactive cells in the piriform cortex and therefore all the cells on a section were counted (i.e. without any within section sampling), and values are expressed as the total number of cells in the relevant structure or region. Striatal and cortical volumes were calculated as described previously (Lazic et al., 2004).

4.7. *Statistical analysis*

Analysis was conducted with the open-source statistical program R (version 2.3.1, Ihaka and Gentleman, 1996; R Development Core Team, 2006), available at www.r-project.org. To test for differences between genotypes, data were analysed with an independent samples *t*-test, unless assumptions of this test were not met, in which case the nonparametric Wilcoxon rank-sum test was used. A Pearson correlation was used to test for linear correlations among relevant variables. Some dependent variables (e.g. number of labelled cells in the DG and piriform cortex) had positively skewed distributions, and therefore these data were $\log_{10}$ transformed prior to testing for significant correlations. All graphs display the original untransformed data showing means±the standard error of the mean for each group. For all tests, the type I error rate (α) was set at 0.05.

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