Grainyhead-like 3 (Grhl3) deficiency in brain leads to altered locomotor activity and decreased anxiety-like behaviours in aged mice.

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ABSTRACT:

The highly conserved *Grainyhead-like* (*Grhl*) family of transcription factors, comprising three members in vertebrates (*Grhl1-3*), play critical regulatory roles during embryonic development, cellular proliferation and apoptosis. Although loss of *Grhl* function leads to multiple neural abnormalities in numerous animal models, a comprehensive analysis of *Grhl* expression and function in the mammalian brain has not been reported. Here we show that only *Grhl3* expression is detectable in the embryonic mouse brain; particularly within the habenula, an organ known to modulate repressive behaviours. Using both *Grhl3*-knockout mice (*Grhl3*^{-/-}), and brain-specific conditional deletion of *Grhl3* in adult mice (*Nestin-Cre/Grhl3*^{flox/flox}), we performed histological expression analyses and behavioural tests to assess long-term effects of *Grhl3* loss on motor co-ordination, spatial memory, anxiety and stress. We found that complete deletion of *Grhl3* did not lead to noticeable structural or cell-intrinsic defects in the embryonic brain, however aged *Grhl3* conditional knockout (*cKO*) mice showed enlarged lateral ventricles and displayed marked changes in motor function and behaviours suggestive of decreased fear and anxiety. We conclude that loss of *Grhl3* in the brain leads to significant alterations in locomotor activity and decreased self-inhibition, and as such, these mice may serve as a novel model of human conditions of impulsive behaviour or hyperactivity.

INTRODUCTION:

The highly conserved Grainyhead-like (*Grhl*) transcription factors, a family of three vertebrate orthologues (*Grhl1-3*) of the antecedent *Drosophila* gene *grainyhead* (*grh*), are critically important regulators of embryonic development, and have also been implicated in the aetiology and progression of disease. Studies of *grh/Grhl* genes in both *Drosophila* and vertebrates (*Xenopus*, zebrafish and mice) have demonstrated numerous highly conserved functions in dorsal hole/neural tube closure (Ting et al., 2003; Werth et al., 2010; Brouns et al., 2011; Pyrgaki et al., 2011), the planar cell polarity pathway and wound repair (Caddy et al., 2010), skin barrier formation and maintenance (Ting et al., 2003; Tao et al., 2005; Chalmers et al., 2006; Janicke et al., 2010; de la Garza et al., 2012) and craniofacial development (Dworkin et al., 2014), generally relating to the regulation of proliferation, apoptosis and cellular migration. Furthermore, these genes have also been implicated in numerous disease processes, such as age-related hearing impairment and deafness (Peters et al., 2002; Van Laer et al., 2008), tumour suppression (Darido et al., 2011; Georgy et al., 2015), oncogenesis (Chen et al., 2010; Quan et al., 2015), facial dysmorphisms and cognitive delay (Kuechler et al., 2011) and regulation of the epithelial-mesenchymal transition (Cieply et al., 2012; Werner et al., 2013)) in the context of both cancer and neural tube closure (Ray and Niswander, 2016). Importantly, almost all developmental functions ascribed to *Drosophila grh* have subsequently been characterised in higher vertebrates, save for the role of *grh* in the regulation of neuroblast production and function (Almeida and Bray, 2005; Cenci and Gould, 2005).

Two recent studies, however, have implicated a role for this family within the vertebrate brain. Work from our group showed that a zebrafish orthologue of the *Grhl* family, *grhl2b*, is expressed within the brain, and specific antisense oligonucleotide (morpholino)-mediated knockdown of *grhl2b* led to significant defects in the morphology of the midbrain-hindbrain boundary, with concomitant increases in neural apoptosis (Dworkin et al., 2012). This phenotype was also largely recapitulated in a murine ENU-mutagenesis model lacking *Grhl2* (Menke et al., 2015), although this apoptosis was suggested to be a secondary consequence of *Grhl2* loss within the surface (non-neural) ectoderm. These data suggest that the *Grhl* family may play an important role in both the development, and potentially also post-natal cognitive and motor functions in the brain. However, to date, a systematic analysis of the expression

and putative cell-autonomous functional roles of the *Grhl* family in brain has yet to be reported.

Here, we report that the only *Grhl* family member to be expressed in the developing mouse brain is not *Grhl2*, but rather *Grhl3*; furthermore, conditional deletion of *Grhl3* within the brain alters locomotor activity, and potentially also impacts on anxiety and hyperactivity-related behaviours in adult aged mice.

METHODS:

Mouse models – *Grhl3*^{-/-} and generation of *Nestin-Cre* x *Grhl3*^{flox/flox} mice

All experiments were pre-approved by the Alfred Monash Research and Education Precinct (AMREP) Animal Ethics Committee. The generation and genotyping of *Grhl3*^{+/-} and *Grhl3*^{flox/flox} mice (Ting et al., 2003; Darido et al., 2011) has been described previously.

Grhl3^{+/-} mice were crossed with *Nestin-Cre* transgenic mice (Tronche et al., 1999) to generate *Grhl3*^{+/-}/*Nestin*^{Cre+/-} mice. The resultant animals were crossed with *Grhl3*^{flox/flox} mice to provide the *Grhl3*^{Δ/-}/*Nestin*^{Cre+} conditional knockout (cKO) experimental animals (where Δ is the deleted floxed allele; hereafter termed *Grhl3cKO*). Both *Grhl3cKO* mice (n=10) and *Grhl3*^{flox/+}/*Nestin*^{Cre-} wild-type littermate controls (n=7) were aged for a period of 20 months, prior to behavioural testing. The *Grhl3cKO* group consisted of 7 males and 3 females, and wild-type controls consisted of 5 males and 2 females. A single olfactory bulb from aged mice was dissected, and used for DNA extraction and genotyping to determine the level of genomic recombination (Fig. 5H), as described previously (Darido et al., 2011).

Histology and immunostaining

At the completion of the behavioural tests, brains from each mouse were extracted and processed according to standard histological techniques. The antibodies and immunohistochemical methods used to detect cell proliferation/apoptosis (Ki67 and activated caspase-3 respectively) (Darido et al., 2011) and Tyrosine Hydroxylase (Ettrup et al., 2010) immunohistochemistry were utilised and followed as previously reported. The methods for neural stem cell extraction and neurosphere culture were performed as described previously (Dworkin et al., 2009).

Vertical Rod Descent Test to assess balance

Mice were placed on top of an 80 cm dowel rod, maintained at an angle of approximately 65°, and allowed to descend to the bench. The time taken for the mice to descend from the top of the rod until the first paw touched the benchtop, was recorded.

DigiGait test to assess locomotor activity

The DigiGait system was used to assess various indices of gait in all four limbs of experimental mice. Mice were placed on a transparent treadmill set to a speed of 1025cm/sec, and the gait of mice was recorded by video capture of all four paws. Paw prints were digitised, and automated software analysis was used to quantify up to 36 indices of spatial and temporal gait for each limb, including the stance and swing components of stride as well as stride length, duration, and frequency, as described previously (Sashindranath et al., 2015).

Sucrose Preference Test to assess reward preference and pleasure-seeking behaviour

Prior to beginning testing, all mice were habituated to the presence of two drinking bottles (one containing 2% sucrose and the other water) for a period of 3 days in their home cage. Following this acclimatisation, mice had the free choice of either drinking the 2% sucrose solution or plain water for a period of 4 days. Water and sucrose solution intake is measured daily, and the positions of two bottles was switched daily to reduce any confound produced by a side bias. Sucrose preference was calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake and averaged over the 4 days of testing.

Elevated Plus Maze (EPM) Test to assess anxiety

The EPM is comprised of two open arms and two closed arms that extend from a common central platform elevated to a height of 40cm above the floor. The closed arms provide protection via a 15cm high wall with a passageway 4.5cm wide. The open arms have no walls and are the same width. Mice were placed on the centre square, facing an open arm and allowed to freely explore the apparatus for a 10-minute period. A greater relative occupation in the closed arms compared to controls, was considered indicative of anxiety-like behaviour.

Porsolt Swim Test to assess symptoms of despair and depression

Mice were placed in a beaker filled with water, from which there is no escape, for a period of 6 minutes, videoed, and subsequently scored on the time spend mobile and immobile. Depression Scan software was used to quantify mouse movements (incidence and duration of floating, struggling or swimming).

Y-Maze Test to assess spatial memory and inquisitive behaviour

The Y-maze (SD instruments) has 3 identical arms, 30cm in length, which are symmetrical to each other. The walls of the apparatus are 14cm high. For the first trial, mice were placed at the end of the home arm of the Y-maze, facing away from the centre and allowed to explore 2 of the 3 Y-maze arms for a 10 min period. A partition blocking off the novel arm of the maze was in place during this initial trial. Each of the three arms was marked by a unique cue attached to the end to differentiate it from the others. The cues used in the present study were 2-dimensional pictures of black and white symbols including a circle, stripes and triangles. Two hours later, the test was repeated with the partition removed so that all arms, including the novel arm, were available to explore (Trial 2). The time spent in each of the 3 accessible arms was recorded during a 5-minute test via video, and data, including arm entries (with an entry classified as an instance where all four limbs of the mouse were inside the arm) and time spent in each arm, were electronically recorded using TopScan rodent tracking software.

Behavioural testing timeline

Mice were transported to the Howard Florey Institute, and habituated for a period of 1 week. Next, the mice were habituated for 24 hours in the testing room before the first experiment, which was the Y-Maze Test. At the completion of this test, the mice were returned to their housing trolley, and remained in the testing room. The next day, the mice underwent the Elevated Plus Maze Test, returned to the housing trolley, and the entire trolley was returned to the housing room. The mice were again habituated to this new room for 24 hours, and then were subject to the sucrose preference test (in the housing room). Next, the housing trolley was again moved to the testing room, mice were allowed to habituate for 24 hours, and then undertook the Porsolt Swim Test. They were returned to the housing room, and were euthanased within 48 hours (once we ensured that all data from the Porsolt Swim Test had been reliably collected and collated). It should be noted that these tests are routinely performed at the Howard Florey Institute, and the intervals between them are accepted by the Florey Animal Ethics Committee.

RESULTS

***Grhl3* mRNA, but not *Grhl1* or *Grhl2* mRNA, is detected in the embryonic brain**

In order to determine the expression of *Grhl1*, *Grhl2* and *Grhl3* in the brain, we performed in-situ hybridisation (ISH) at 4 separate embryonic developmental timepoints, embryonic (E) days E9.5, E12, E15.5 and E17 days post fertilisation (dpf), in order to comprehensively analyse the spatiotemporal expression profile of these three transcripts throughout embryonic brain formation. The specificity and sensitivity of our probes had been previously characterised and validated (Auden et al., 2006). Although radiolabelled ISH is the most sensitive in vitro assay to detect mRNA in histological sections, we could not detect expression of either *Grhl1* or *Grhl2* in any region of the developing neuroectoderm preceding neural tube closure, nor in any regions of the established brain, despite robust expression being detected in other cranial regions, such as the olfactory and palatal epithelia and overlying surface ectoderm (Auden et al., 2006 and data not shown). Conversely, we were able to detect robust expression of *Grhl3* at E15.5, but not at earlier timepoints, localised specifically to the habenula, striatum, and the ventral-most region of the lateral ventricles (Fig. 1A-C'; higher-magnification images in Fig. S1). *Grhl3* expression in the habenula also persisted until at least E17 (data not shown), although it was significantly reduced within the other two areas at this stage. These results suggest that *Grhl3*, but not *Grhl1* or *Grhl2*, may play a cell-autonomous role within the murine brain.

Loss of *Grhl3* does not impact on proliferation, apoptosis, or morphogenesis in the embryonic brain.

In order to determine whether *Grhl3* regulated any specific aspects of regional brain development, we performed histological examination of the brains of E15.5-E18.5 wild-type and *Grhl3*^{-/-} embryos (Ting et al., 2003; Ting et al., 2005), paying particular attention to regions where *Grhl3* expression was detected by ISH, namely the lateral ventricles, striatum and habenula. Brains of *Grhl3*^{-/-} mice were significantly smaller and lighter at E18.5 (Fig. S2), consistent with an overall reduced embryo size (Ting et al., 2003; Ting et al., 2005). They did not display any obvious defects in morphology (Fig. S2), both by gross examination and Haematoxylin and Eosin (H&E) stained coronal sections of E15.5 embryonic brain [data not shown], nor did they display any qualitative differences in cellularity, cell size or distribution,

save for an apparent weakened adhesion between the brain and overlying dura mater, as we had reported previously (Goldie SJ, 2016).

As *Grhl3* is a known regulator of cellular proliferation and survival, we examined the expression of Ki-67 and activated caspase 3 as markers of proliferation and apoptosis respectively. Again, no differences in cellular production in the striatum, habenula or lateral ventricles were detected (Fig. 1D-G and Fig. S3). Lastly, as cellular proliferation within the murine brain at E14.5 is still very high, to determine whether *Grhl3* loss played a role in neural stem cell (NSC) proliferation and survival, neural stem cells from E14.5 brains of WT and *Grhl3*^{-/-} mice were extracted and cultured as clonal aggregates, termed neurospheres (Dworkin et al., 2009). This assay allows an examination of a putative role for *Grhl3* in the cell-autonomous regulation of neural stem cell proliferation and survival, independent of the in vivo niche. Q-RT-PCR analysis confirmed *Grhl3* expression in neurospheres following 7 days in culture (Fig. S2), however we found that loss of *Grhl3* did not significantly impact on cellular production, growth, regeneration or survival at up to 3 weeks (21 days) of culture (Fig. 1H-I).

Taken together, these data indicate that *Grhl3* is not required for proliferation and survival during neural development, and does not have a major role in the patterning and formation of the embryonic brain.

Loss of *Grhl3* in the brain leads to motor-function alteration in adult mice.

To determine whether *Grhl3* deletion impacts upon neural function in aged adult mice, we examined the consequences of *Grhl3* deletion in the adult brain. Previous studies in patients with hypomorphic mutations in *Grhl2* showed that loss of *Grhl*-factors led to age-related functional impairments, including age-related hearing loss (Van Laer et al., 2008). As constitutive loss of *Grhl3* leads to early post-natal death, *Grhl3* was conditionally deleted in the brain by crossing *Grhl3*^{lox/lox} mice (Darido et al., 2011) with mice expressing *Cre*-recombinase under control of the rat *Nestin* promoter (*Nestin*^{Cre}), which drives conditional, *loxP*-mediated recombination in all neural cells and their progeny (Tronche et al., 1999). Both these *Grhl3cKO* (see methods) and control groups were aged, and routine monitoring did not detect any significant neurological or behavioural phenotypes. At 20 months of age, we formally assessed their locomotor activity and cognitive and behavioural functions, using a number of well-validated movement and behavioural tests (see methods). As the

predominant sites of *Grhl3* expression was in both the medial and lateral habenular nuclei (from which efferent neurons project to the substantia nigra, ventral tegmental area and interpeduncular nucleus) and striatum, we selected behavioural tests which would assess cognitive and motor behaviours known to be regulated by neurons within these regions. These tests included the vertical pole descent test (to assess balance and sensorimotor control; Fig. 2), automated gait analysis using the DigiGait system (to assess motor function; Fig. 2), the Elevated Plus Maze (to assess anxiety; Fig. 3), the Y-Maze spontaneous alternation test (to assess spatial memory, inquisitiveness and willingness to explore new environments; Fig. 3), the sucrose preference test (to assess whether the neural reward pathway was disrupted; Fig. 4) and the Porsolt Forced Swim Test (to determine symptoms of behavioural despair, depression and helplessness; Fig. 4). The results of these tests are presented below; in all cases, significance (p-values) was determined using Student's T-test.

Vertical Rod Descent

This test was used to assess both balance and to examine any potential defects in grip. We found that all mice from both groups were able to grip the rod (data not shown), and there was no significant difference in time taken for each group to make the descent (WT $10.43 \text{ sec} \pm 1.46 \text{ sec}$; *Grhl3cKO* $9.27 \text{ sec} \pm 2.49 \text{ sec}$; $p=0.46$; Fig. 2A). The time taken was not influenced by animal weight, which was not significantly different between the two groups, or between genders (WT $42.8 \text{ g} \pm 9.95 \text{ g}$; *Grhl3cKO* $41.6 \text{ g} \pm 8.1 \text{ g}$; $p=0.65$; Fig. 2B). These data indicate that loss of *Grhl3* in the brain does not lead to impairment in balance during descent.

DigiGait analysis

To determine whether *Grhl3cKO* animals displayed any motor function or walking defects, we used the DigiGait system, which allows for quantitative analysis of the animals' gait characteristics. We found that mice lacking *Grhl3* in the brain displayed a significantly decreased stride length (WT $5.6 \text{ cm} \pm 0.16 \text{ cm}$; *Grhl3cKO* $5.2 \text{ cm} \pm 0.11 \text{ cm}$; $p=0.02$; Fig. 2C-C') coupled with concomitant increase in stride frequency (WT $2.64 \pm 0.04 \text{ steps/second}$; *Grhl3cKO* $2.95 \pm 0.07 \text{ steps/second}$; $p=0.01$; Fig. 2D-D') and total number of steps taken within the 5 second testing period (WT $11.75 \pm 0.17 \text{ steps}$; *Grhl3cKO* $12.55 \pm 0.23 \text{ steps}$;

$p=0.03$; Fig. 2E-E'). These data indicate that *Grhl3* may function in the regulation of locomotor activity during walking.

Elevated Plus Maze (EPM) analysis

The Elevated Plus Maze was used to quantitatively measure relative fear and anxiety (Walf and Frye, 2007), and was predicated by the animals' general aversion for open spaces, unconditioned fear of heights, and a preference to remain close to walls and/or in darkened spaces. We assessed the number of times each mouse entered either the open or closed arm, time spent in each arm, and the total distance travelled in each arm. We found that *Grhl3cKO* mice displayed significantly fewer total entries into the closed arm (WT 32.5 ± 1.7 entries; *Grhl3cKO* 25.5 ± 1.8 entries; $p=0.02$; Fig. 3A), with a concomitant significantly increased incidence of entering the open arm (WT 6.7 ± 1.1 entries; *Grhl3cKO* 13.8 ± 2.4 entries; $p=0.046$; Fig. 3A). This was accompanied by a strong, albeit non-significant, trend towards increased total distance travelled (WT $632\text{mm} \pm 143\text{mm}$; *Grhl3cKO* $2125\text{mm} \pm 673\text{mm}$; $p=0.115$; Fig. 3B) and time spent (WT 30.02 ± 6.21 sec; *Grhl3cKO* $63.17 \text{ sec} \pm 14.60$ sec; $p=0.116$; Fig. 3C) in exploring the open arm, and a similar strong, but non-significant trend to decreased distance travelled (WT $13082\text{mm} \pm 968\text{mm}$; *Grhl3cKO* $10570\text{mm} \pm 1166\text{mm}$; $p=0.166$; Fig. 3B) and time spent (WT $405.27 \text{ sec} \pm 11.97$ sec; *Grhl3cKO* $371.02 \text{ sec} \pm 17.58$ sec; $p=0.192$; Fig. 3C) exploring the closed arm. These data indicate that *Grhl3cKO* preferentially explore the open arm, and are less inclined to explore the closed arm of the maze, suggesting that mice lacking *Grhl3* in the brain display less fear and more inquisitiveness than their WT littermates.

Y-Maze analysis

The Y-Maze test was used to determine whether the *Grhl3cKO* mice showed any inhibition in their willingness to explore new environments, and spatial learning/memory performance. Mice typically prefer to investigate the novel arm in the maze rather than returning to one that has been previously explored. In follow-up sessions, wild-type mice will normally spend a greater proportion of time exploring the previously unexplored zone in preference to the previously visited arms. The number of times the mice entered alternate arms, total arm entries, and the percentage of arm alternation as quantified. We found that *Grhl3cKO* mice

displayed non-significant trends towards increased total arm entries (WT 18.9 ± 2.8 entries; *Grhl3cKO* 24.4 ± 2.9 entries; $p=0.21$; Fig. 3D), increased number of arm alternations (WT 8.00 ± 1.9 alternations; *Grhl3cKO* 11.6 ± 1.2 entries; $p=0.12$; Fig. 3E) and the alternation percentage (WT $42.42\% \pm 9.42\%$; *Grhl3cKO* $54.44\% \pm 4.74\%$; $p=0.23$; Fig. 3F). These data suggest that mice lacking *Grhl3* in the brain do not present with defects in spatial learning/memory, and in fact may actually be more inquisitive and impulsive than their WT littermates.

Porsolt (Forced) Swim Test (PST) analysis

The Porsolt forced swim test (Porsolt et al., 1977) is a widely used model of behavioural despair, based on the notion that an animal suffering behavioural despair will spend a longer time immobile and cease trying to escape when compared to an established baseline set by the control animals. The number of times each mouse floated, struggled, or swam during the 6-minute testing period was measured, as was the total time spent by the mice on each of these activities. We found no significant differences between the two groups in total incidences of floating (WT 18.0 ± 3.1 times; *Grhl3cKO* 18.9 ± 1.9 times; $p=0.48$; Fig. 4A), struggling (WT 31.9 ± 1.7 times; *Grhl3cKO* 30.4 ± 2.1 times; $p=0.21$; Fig. 4A) or swimming (WT 46.7 ± 2.4 times; *Grhl3cKO* 43.3 ± 2.9 times; $p=0.27$; Fig. 4A), and similarly, no significant differences were detected in the total time spent floating (WT $103.5 \text{ sec} \pm 16.8 \text{ sec}$; *Grhl3cKO* $114.5 \text{ sec} \pm 11.3 \text{ sec}$; $p=0.58$; Fig. 4B), struggling (WT $128.7 \text{ sec} \pm 20.7 \text{ sec}$; *Grhl3cKO* $143.1 \text{ sec} \pm 10.5 \text{ sec}$; $p=0.51$; Fig. 4B) or swimming (WT $127.8 \text{ sec} \pm 21.7 \text{ sec}$; *Grhl3cKO* $102.3 \text{ sec} \pm 14.7 \text{ sec}$; $p=0.33$; Fig. 4B). These data indicate that *Grhl3* is unlikely to be a mediator of despair-related behaviour in mice.

Sucrose Preference Test analysis

The sucrose preference test was implemented to analyse each of the two groups' capacities to receive and respond to reward. As all rodents are born with an inherent preference for sweet foodstuffs (Thompson and Grant, 1971), a decreased intake of sucrose-containing water indicates a diminished capacity for reward-seeking and is suggestive of a state of anhedonia (inability to experience pleasure) or depression. The total volume of either plain water, or sucrose-containing water, which the mice drank over a period of 7 days was analysed. We

found no significant differences in the amount of water alone (WT $1.9\text{ml} \pm 0.3\text{ml}$; *Grhl3cKO* $2.1\text{ml} \pm 0.2\text{ml}$; $p=0.73$; Fig. 4C) or sucrose-containing water each group consumed (WT $10.2\text{ml} \pm 0.1\text{ml}$; *Grhl3cKO* $9.4\text{ml} \pm 1.3\text{ml}$; $p=0.77$; Fig. 4D). We did observe a non-significant trend towards a lower ratio of sucrose:water consumption in the *Grhl3cKO* group (WT $6.1 \pm 0.8\text{ml}$; *Grhl3cKO* $4.9\text{ml} \pm 0.6\text{ml}$; $p=0.28$; Fig. 4E). These data do not suggest a significant inhibition in the reward pathway of the brains of mice lacking neural *Grhl3*, and furthermore, do not indicate that loss of *Grhl3* contributes to anhedonia.

Analysis of adult brain structure, proliferation, apoptosis and Tyrosine Hydroxylase production.

At the completion of the tests, the adult brains of both WT and *Grhl3cKO* mice were extracted and analysed for gross abnormalities, size, weight, cell proliferation and apoptosis. Other than subtle (yet consistent) enlargement of the lateral ventricles in *Grhl3cKO* mice ($n=3$ WT and $n=3$ *Grhl3cKO*; Fig. 5A-D), we found no significant abnormalities, no differences in brain size or weight, nor apoptosis or proliferation [not shown]. As the habenula harbors efferent neurons whose terminal projections synapse on neurons within the substantia nigra (SN), and the SN is a critical region involved in the regulation of motor coordination, particularly in the aetiology and onset of Parkinson's Disease, we examined this region in the brains of adult WT and *Grhl3cKO* mice by immunostaining for the dopaminergic neurotransmitter, tyrosine hydroxylase (TH). We were able to detect robust TH expression within the SN (as well as the caudate putamen and ventral striatum; Fig. 5A-D) of both the WT and *Grhl3cKO* mice; quantitation of TH⁺ nuclei within the SN did not show any significant differences in TH expression (Fig. 5E-G). Lastly, we confirmed *Nestin*^{Cre}-mediated deletion by genotyping PCR (Fig. 5H) on DNA extracted from one olfactory bulb per brain. These data confirmed extensive deletion of the *Grhl3* allele within the neural tissue; as expected, complete deletion was not seen, as the olfactory bulb contains numerous cells of non-neural origin (particularly microglia), where *Nestin*^{Cre} does not drive deletion.

DISCUSSION

The *Drosophila grainyhead* (*grh*) gene is evolutionary conserved, and was initially referred to as *neuronal transcription factor 1* (NTF-1), with tissue specific isoforms (Dynlacht et al., 1989; Attardi and Tjian, 1993). When the *Drosophila* neuroblast specific GRH O isoform was

functionally disrupted, the majority of flies died during larval-pupal stage, however a small percentage survived through to adulthood with a lethal phenotype of unco-ordinate movements, manifesting as shaking appendages (Uv et al., 1997). As most *Drosophila grh* functions have parallels in higher vertebrates, we hypothesised that deletion of the relevant mammalian *grh* orthologue in the brain might also impact on neuronal motor and/or behavioural function.

The mammalian orthologues of *grh*, the *Grainyhead-like* family, comprise three family members (*Grhl1-3*) in mice. *Grhl1* had not been previously implicated in mediating neural function, and in concordance with this, we did not detect any expression of *Grhl1* within the murine brain in the present study. Previous studies had shown that disruption of *Grhl2* led to increased apoptosis of neural progenitors (Menke et al., 2015), although *Grhl2* expression within the brain proper was not examined. Our previous work in zebrafish also showed that the fish orthologue of mouse *Grhl2*, *grhl2b* was expressed within the vertebrate brain, and that transient *grhl2b* inhibition led to significantly elevated apoptosis at the midbrain-hindbrain boundary (Dworkin et al., 2012). These data suggested that *Grhl2* may function within the mammalian brain to regulate cell survival. However, despite careful examination of numerous brain sections at multiple embryonic timepoints, we could not detect any *Grhl2* expression within the developing mouse brain. These results indicate both of evolutionary divergence from the zebrafish, and suggest that the previously-reported apoptosis of neural progenitors in murine models is due to a secondary effect, perhaps through defects in the overlying surface ectoderm (Menke et al., 2015), where *Grhl2* is robustly expressed (Auden et al., 2006).

Our study characterises the expression pattern, and gives insights into the function of, the highly-conserved transcription factor *Grhl3* in the mouse brain. Our in-situ hybridisation analyses of multiple stages of embryonic brain development detected expression of *Grhl3* within the posterior lateral ventricles, the striatum, and the habenula. The lateral ventricles serve to allow passage of cerebro-spinal fluid, which not only aids in cushioning the brain from impact trauma, but also to aid in circulation of nutrients and waste removal. We did not detect any differences in lateral ventricle size or cellular composition in *Grhl3*^{-/-} embryonic brains, however conditional deletion of *Grhl3* in the adult brain led to consistent enlargement of the lateral ventricles in all animals examined. Whilst we have noted defective reabsorption of fluids from cavities in previous conditional deletion experiments (e.g. hydronephrosis following conditional deletion of *Grhl3* using *Keratin14-Cre*, data not shown), enlarged brain

ventricles are consistent with neural stem cell (NSC) defects and impaired adult neurogenesis (Malaterre et al., 2008). Adjacent to the ependymal cells, which line the ventricles, lies a region termed the sub-ventricular zone (SVZ) which contains a repository of NSCs which persist into adulthood (Morshead et al., 1994; Doetsch et al., 1999). Both in vivo proliferation/apoptosis analysis, and in vitro NSC (neurosphere) assays from embryonic WT and *Grhl3*^{-/-} mice did not indicate any cell intrinsic defects in NSC formation during embryogenesis, however it is possible that NSC defects may become apparent in *Grhl3cKO* mice as a consequence of aging.

Although *Grhl3* expression was visible in the lateral ventricles and striatum, the strongest and most extensive expression of *Grhl3* was within the habenula. This expression was of particular interest, given the relative paucity of known habenula-restricted markers. Even the gene product classically used as a habenular marker, *Gpr151* (Broms et al., 2015), is expressed in other regions of the brain, including the anterior paraventricular, the rhomboid, the central lateral, and the parafascicular thalamic nuclei (Wagner et al., 2014), suggesting that *Grhl3* may be a novel marker of the murine habenula, particularly at E17 (where *Grhl3* expression in the striatum and lateral ventricles had largely disappeared). Our future work will be aimed at determining the downstream transcriptional targets of *Grhl3* within the habenula, as well as identifying and characterising elements within the *Grhl3* upstream regulatory region which may direct habenular *Grhl3* expression. Previous work has shown that the expression of two genes within the habenula, *tissue-type plasminogen activator (t-PA)* and *insulin*, is driven by a functional binding site (within the promoter) for the transcription factor *nuclear factor of activated T-cells (NFAT; AGGGAAA)* (Yu et al., 2001). Our *in silico* analyses of the mouse *Grhl3* promoter indicates a candidate AGGGAAA site is present, ~4kb upstream of the transcriptional start site (TSS). Future work will determine whether this site is functional within the *Grhl3* promoter, and whether this element, when fused to a LacZ reporter (Yu et al., 2001; Attanasio et al., 2013), is capable of driving expression within the habenula.

Whereas the lateral ventricles contain cells which primarily perform structural or regenerative roles, the striatum and habenula are regions which are known to regulate risk-reward function (striatum), and numerous locomotor and cognitive functions, including action planning and decision making, fear and aversion, mechanisms of avoidance, regulation of risk/reward and memory (habenula) (Hikosaka, 2010). Therefore, we performed several behavioural tests in

order to broadly determine whether loss of *Grhl3* in these regions led to any changes in behaviour pertaining to memory, fear, anxiety, spatial learning, depression, inquisitiveness, and hedonism. One caveat to our experiments is that all our behavioural tests were performed on mice housed either in isolation or in pairs. As the striatum has also been implicated in reward outcomes in social situations (Baez-Mendoza and Schultz, 2013), our future work will focus on the behaviour of mice in groups, in order to determine whether loss of *Grhl3* leads to any changes in the social behaviour of mice.

Loss of *Grhl3* within the brain, either via constitutive knockout in embryos, or via conditional (*Nestin-Cre*) deletion in adults, did not lead to significant structural, proliferative or apoptotic differences when compared to WT littermates, nor did *Grhl3* deletion lead to defective tyrosine hydroxylase (TH) production in dopaminergic neurons of the substantia nigra. Our behavioural tests showed two clearly significant results. Firstly, aged *Grhl3cKO* experimental animals displayed significantly less fear and anxiety-like symptoms in the Elevated Plus Maze test, spending a greater amount of time exploring the open arm of the maze relative to WT controls. Secondly, our data shows that aged *Grhl3cKO* experimental animals showed significant defects in locomotor activity as assessed by the DigiGait test, taking shorter, quicker and more frequent steps, which is an indicator that the animals may be experiencing hyperactivity. These data are particularly suggestive of a habenular defect, as the habenula is a region of the brain which operates as a “handbrake” to prevent over-stimulation of both the serotonergic and dopaminergic systems (Hikosaka, 2010). Animals with habenular lesions display hyperactivity, restlessness and distraction, make premature motor movements in response tasks (Lee and Huang, 1988; Lecourtier and Kelly, 2005) and show decreased reward response (Hikosaka, 2010). Although not examined within our behavioural testing paradigms, animals with habenular defects may be particularly susceptible to outside stressors, and perform more poorly on behavioural tasks (Heldt and Ressler, 2006). Future studies, assessing how the *Grhl3cKO* mice perform under conditions of stress (e.g. physical effort, isolation, food deprivation etc.) (Heldt and Ressler, 2006) may be necessary to extend our data.

Our study, for the first time, describes a putative role for *Grhl3* in the mammalian brain, suggesting a novel link between *Grhl3* loss and potential disruption of neural pathways which regulate both motor-coordination and inhibition of anxiety. We aim to extend the behavioural analyses reported here to extensively characterise the role of *Grhl3* in impulsive behaviour,

across both social settings and situations of stress. Our goal will be to determine whether mice with conditional brain-specific deletion of *Grhl3* may be used as an effective model of human neural conditions, such as increased risk-taking behaviours, cognitive decline and attention deficit hyperactivity disorders, and ultimately, to determine whether loss or mutation of *Grhl3* may underpin any known human neural behavioural pathologies.

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REFERENCES:

- Almeida MS, Bray SJ. 2005. Regulation of post-embryonic neuroblasts by *Drosophila* Grainyhead. *Mech Dev* 122:1282-1293.
- Attanasio C, Nord AS, Zhu Y, Blow MJ, Li Z, Liberton DK, Morrison H, Plajzer-Frick I, Holt A, Hosseini R, Phouanavong S, Akiyama JA, Shoukry M, Afzal V, Rubin EM, FitzPatrick DR, Ren B, Hallgrimsson B, Pennacchio LA, Visel A. 2013. Fine tuning of craniofacial morphology by distant-acting enhancers. *Science* 342:1241006.
- Attardi LD, Tjian R. 1993. *Drosophila* tissue-specific transcription factor NTF-1 contains a novel isoleucine-rich activation motif. *Genes Dev* 7:1341-1353.
- Auden A, Caddy J, Wilanowski T, Ting SB, Cunningham JM, Jane SM. 2006. Spatial and temporal expression of the Grainyhead-like transcription factor family during murine development. *Gene Expr Patterns* 6:964-970.
- Baez-Mendoza R, Schultz W. 2013. The role of the striatum in social behavior. *Front Neurosci* 7:233.
- Broms J, Antolin-Fontes B, Tingstrom A, Ibanez-Tallon I. 2015. Conserved expression of the GPR151 receptor in habenular axonal projections of vertebrates. *J Comp Neurol* 523:359-380.
- Brouns MR, De Castro SC, Terwindt-Rouwenhorst EA, Massa V, Hekking JW, Hirst CS, Savery D, Munts C, Partridge D, Lamers W, Kohler E, van Straaten HW, Copp AJ, Greene ND. 2011. Over-expression of *Grl2* causes spina bifida in the Axial defects mutant mouse. *Hum Mol Genet* 20:1536-1546.
- Caddy J, Wilanowski T, Darido C, Dworkin S, Ting SB, Zhao Q, Rank G, Auden A, Srivastava S, Papenfuss TA, Murdoch JN, Humbert PO, Parekh V, Boulos N, Weber T, Zuo J, Cunningham JM, Jane SM. 2010. Epidermal wound repair is regulated by the planar cell polarity signaling pathway. *Dev Cell* 19:138-147.
- Cenci C, Gould AP. 2005. *Drosophila* Grainyhead specifies late programmes of neural proliferation by regulating the mitotic activity and Hox-dependent apoptosis of neuroblasts. *Development* 132:3835-3845.
- Chalmers AD, Lachani K, Shin Y, Sherwood V, Cho KW, Papalopulu N. 2006. Grainyhead-like 3, a transcription factor identified in a microarray screen, promotes the specification of the superficial layer of the embryonic epidermis. *Mech Dev* 123:702-718.
- Chen W, Dong Q, Shin KH, Kim RH, Oh JE, Park NH, Kang MK. 2010. Grainyhead-like 2 enhances the human telomerase reverse transcriptase gene expression by inhibiting DNA methylation at the 5'-CpG island in normal human keratinocytes. *The Journal of biological chemistry* 285:40852-40863.
- Cieply B, Riley Pt, Pifer PM, Widmeyer J, Addison JB, Ivanov AV, Denvir J, Frisch SM. 2012. Suppression of the Epithelial-Mesenchymal Transition by Grainyhead-Like-2. *Cancer research*.
- Darido C, Georgy SR, Wilanowski T, Dworkin S, Auden A, Zhao Q, Rank G, Srivastava S, Finlay MJ, Papenfuss AT, Pandolfi PP, Pearson RB, Jane SM. 2011. Targeting of the tumor suppressor GRHL3 by a miR-21-dependent proto-oncogenic network results in PTEN loss and tumorigenesis. *Cancer cell* 20:635-648.
- de la Garza G, Schleiffarth JR, Dunnwald M, Mankad A, Weirather JL, Bonde G, Butcher S, Mansour TA, Kousa YA, Fukazawa CF, Houston DW, Manak JR, Schutte BC, Wagner DS, Cornell RA. 2012. Interferon Regulatory Factor 6 Promotes Differentiation of the Periderm by Activating Expression of Grainyhead-Like 3. *The Journal of investigative dermatology*.
- Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97:703-716.

- Dworkin S, Darido C, Georgy SR, Wilanowski T, Srivastava S, Ellett F, Pase L, Han Y, Meng A, Heath JK, Lieschke GJ, Jane SM. 2012. Midbrain-hindbrain boundary patterning and morphogenesis are regulated by diverse grainy head-like 2-dependent pathways. *Development* 139:525-536.
- Dworkin S, Malaterre J, Hollande F, Darcy PK, Ramsay RG, Mantamadiotis T. 2009. cAMP response element binding protein is required for mouse neural progenitor cell survival and expansion. *Stem Cells* 27:1347-1357.
- Dworkin S, Simkin J, Darido C, Partridge DD, Georgy SR, Caddy J, Wilanowski T, Lieschke GJ, Doggett K, Heath JK, Jane SM. 2014. Grainyhead-like 3 regulation of endothelin-1 in the pharyngeal endoderm is critical for growth and development of the craniofacial skeleton. *Mech Dev*.
- Dynlacht BD, Attardi LD, Admon A, Freeman M, Tjian R. 1989. Functional analysis of NTF-1, a developmentally regulated Drosophila transcription factor that binds neuronal cis elements. *Genes Dev* 3:1677-1688.
- Ettrup KS, Sorensen JC, Bjarkam CR. 2010. The anatomy of the Gottingen minipig hypothalamus. *J Chem Neuroanat* 39:151-165.
- Georgy SR, Cangkrama M, Srivastava S, Partridge D, Auden A, Dworkin S, McLean CA, Jane SM, Darido C. 2015. Identification of a Novel Proto-oncogenic Network in Head and Neck Squamous Cell Carcinoma. *J Natl Cancer Inst* 107.
- Goldie SJ AB, Anderson P, Auden A, Partridge DD, Jane SM, Dworkin S. 2016. Mice lacking the conserved transcription factor Grainyhead-like 3 (Grhl3) display increased apposition of the frontal and parietal bones during embryonic development. *BMC Developmental Biology* In Press.
- Heldt SA, Ressler KJ. 2006. Lesions of the habenula produce stress- and dopamine-dependent alterations in prepulse inhibition and locomotion. *Brain Res* 1073-1074:229-239.
- Hikosaka O. 2010. The habenula: from stress evasion to value-based decision-making. *Nat Rev Neurosci* 11:503-513.
- Janicke M, Renisch B, Hammerschmidt M. 2010. Zebrafish grainyhead-like1 is a common marker of different non-keratinocyte epidermal cell lineages, which segregate from each other in a Foxi3-dependent manner. *Int J Dev Biol* 54:837-850.
- Kuechler A, Buysse K, Clayton-Smith J, Le Caignec C, David A, Engels H, Kohlhase J, Mari F, Mortier G, Renieri A, Wieczorek D. 2011. Five patients with novel overlapping interstitial deletions in 8q22.2q22.3. *Am J Med Genet A* 155A:1857-1864.
- Lecourtier L, Kelly PH. 2005. Bilateral lesions of the habenula induce attentional disturbances in rats. *Neuropsychopharmacology* 30:484-496.
- Lee EH, Huang SL. 1988. Role of lateral habenula in the regulation of exploratory behavior and its relationship to stress in rats. *Behav Brain Res* 30:265-271.
- Malaterre J, Mantamadiotis T, Dworkin S, Lightowler S, Yang Q, Ransome MI, Turnley AM, Nichols NR, Emambokus NR, Frampton J, Ramsay RG. 2008. c-Myb is required for neural progenitor cell proliferation and maintenance of the neural stem cell niche in adult brain. *Stem Cells* 26:173-181.
- Menke C, Cionni M, Siggers T, Bulyk ML, Beier DR, Stottmann RW. 2015. Grhl2 is required in nonneural tissues for neural progenitor survival and forebrain development. *Genesis*.
- Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, Weiss S, van der Kooy D. 1994. Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron* 13:1071-1082.
- Peters LM, Anderson DW, Griffith AJ, Grundfast KM, San Agustin TB, Madeo AC, Friedman TB, Morell RJ. 2002. Mutation of a transcription factor, TFCP2L3, causes progressive autosomal dominant hearing loss, DFNA28. *Hum Mol Genet* 11:2877-2885.
- Porsolt RD, Bertin A, Jalfre M. 1977. Behavioral despair in mice: a primary screening test for antidepressants. *Arch Int Pharmacodyn Ther* 229:327-336.
- Pyrgaki C, Liu A, Niswander L. 2011. Grainyhead-like 2 regulates neural tube closure and adhesion molecule expression during neural fold fusion. *Dev Biol* 353:38-49.

- Quan Y, Xu M, Cui P, Ye M, Zhuang B, Min Z. 2015. Grainyhead-like 2 Promotes Tumor Growth and is Associated with Poor Prognosis in Colorectal Cancer. *J Cancer* 6:342-350.
- Ray HJ, Niswander LA. 2016. Grainyhead-like 2 downstream targets act to suppress epithelial-to-mesenchymal transition during neural tube closure. *Development* 143:1192-1204.
- Sashindranath M, Daglas M, Medcalf RL. 2015. Evaluation of gait impairment in mice subjected to craniotomy and traumatic brain injury. *Behav Brain Res* 286:33-38.
- Tao J, Kuliyeve E, Wang X, Li X, Wilanowski T, Jane SM, Mead PE, Cunningham JM. 2005. BMP4-dependent expression of *Xenopus* Grainyhead-like 1 is essential for epidermal differentiation. *Development* 132:1021-1034.
- Thompson RD, Grant CV. 1971. Automated preference testing apparatus for rating palatability of foods. *J Exp Anal Behav* 15:215-220.
- Ting SB, Caddy J, Wilanowski T, Auden A, Cunningham JM, Elias PM, Holleran WM, Jane SM. 2005. The epidermis of *grhl3*-null mice displays altered lipid processing and cellular hyperproliferation. *Organogenesis* 2:33-35.
- Ting SB, Wilanowski T, Auden A, Hall M, Voss AK, Thomas T, Parekh V, Cunningham JM, Jane SM. 2003. Inositol- and folate-resistant neural tube defects in mice lacking the epithelial-specific factor *Grhl-3*. *Nat Med* 9:1513-1519.
- Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schutz G. 1999. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet* 23:99-103.
- Uv AE, Harrison EJ, Bray SJ. 1997. Tissue-specific splicing and functions of the *Drosophila* transcription factor Grainyhead. *Molecular and cellular biology* 17:6727-6735.
- Van Laer L, Van Eyken E, Fransen E, Huyghe JR, Topsakal V, Hendrickx JJ, Hannula S, Maki-Torkko E, Jensen M, Demeester K, Baur M, Bonaconsa A, Mazzoli M, Espeso A, Verbruggen K, Huyghe J, Huygen P, Kunst S, Manninen M, Konings A, Diaz-Lacava AN, Steffens M, Wienker TF, Pyykko I, Cremers CW, Kremer H, Dhooge I, Stephens D, Orzan E, Pfister M, Bille M, Parving A, Sorri M, Van de Heyning PH, Van Camp G. 2008. The grainyhead like 2 gene (*GRHL2*), alias *TFCP2L3*, is associated with age-related hearing impairment. *Human molecular genetics* 17:159-169.
- Wagner F, French L, Veh RW. 2014. Transcriptomic-anatomic analysis of the mouse habenula uncovers a high molecular heterogeneity among neurons in the lateral complex, while gene expression in the medial complex largely obeys subnuclear boundaries. *Brain Struct Funct*.
- Walf AA, Frye CA. 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc* 2:322-328.
- Werner S, Frey S, Riethdorf S, Schulze C, Alawi M, Kling L, Vafaizadeh V, Sauter G, Terracciano L, Schumacher U, Pantel K, Assmann V. 2013. Dual roles of the transcription factor grainyhead-like 2 (*GRHL2*) in breast cancer. *J Biol Chem* 288:22993-23008.
- Werth M, Walentin K, Aue A, Schonheit J, Wuebken A, Pode-Shakked N, Vilianovitch L, Erdmann B, Dekel B, Bader M, Barasch J, Rosenbauer F, Luft FC, Schmidt-Ott KM. 2010. The transcription factor grainyhead-like 2 regulates the molecular composition of the epithelial apical junctional complex. *Development* 137:3835-3845.
- Yu H, Schleuning WD, Michl M, Liberatore G, Tan SS, Medcalf RL. 2001. Control elements between -9.5 and -3.0 kb in the human tissue-type plasminogen activator gene promoter direct spatial and inducible expression to the murine brain. *Eur J Neurosci* 14:799-808.

Figure Legends

Fig. 1 *Grhl3* expression and characterisation of *Grhl3*^{-/-} embryonic brains

The expression of *Grhl3* was examined in sequential coronal (A-A') or transverse (B-C') sections of E15.5 wild-type embryonic mouse brain (A-C) using radiolabelled ISH (A'-C'). Expression was detected within the habenula (Ha; A'), striatum (St; B') and the ventral-most region of the lateral ventricle (LV; C'). No differences were seen in *Grhl3*^{-/-} brains compared to wild-type brains with respect to cellular proliferation (Ki67; D-E) or apoptosis (activated caspase-3; F-G). Similarly, no cell-intrinsic differences were seen following in vitro neurosphere culture from *Grhl3*^{-/-} brains compared to wild-type brains with respect to proliferation over 7 days (H) or 21 days (I). All images are shown at x20 magnification.

Fig. 2 Analysis of locomotor activity via vertical rod-descent and DigiGait analyses

The average time taken to descend from the top of an 80cm vertical rod was not significantly different between WT and *Grhl3cKO* animals (A); this was not due to any differences in size or weight between the two groups (B). When assessed using DigiGait analysis, the stride length of the left (C) and right (C') hind limbs was significantly shorter in the *Grhl3cKO* animals, with a commensurate increase in stride frequency (D-D') and therefore total number of steps (E-E') within the 5-second testing period. (*p < 0.05).

Fig. 3 Analysis of anxiety, memory and inquisitive behaviour using the Elevated Plus Maze (EPM) and the Y-Maze

The average number of entries into either the open or closed arm of the EPM (A), total distance travelled in either arm (B) and total time spent in either arm (C) was quantified between WT and *Grhl3cKO* animals. A trend towards significance was seen when measuring total arm-entries into each arm of the Y-Maze (D), the total number of alternate entries into each arm (E) and the percentage of arm alternation (F) between WT and *Grhl3cKO* animals. (*p < 0.05).

Fig. 4 Analysis of depressive behaviour using the Porsolt (forced) Swim Test (PST) and analysis of preference for reward behaviours using the Sucrose Preference Test.

An analysis of both the number of occasions (**A**) and duration (**B**) which mice spent floating, struggling or swimming in the PST was not significantly different between WT and *Grhl3cKO* animals. Furthermore, the preference for sucrose-containing water, as analysed by water consumption per day (**C**), ratio of sucrose:water consumed (**D**) and total sucrose consumption per day (**E**) was not decreased in *Grhl3cKO* animals.

Fig. 5 Analysis of the brains of adult WT and *Grhl3^{Δc}/Nestin^{Cre+}* (cKO) mice shows no significant differences in tyrosine hydroxylase expression

Grhl3 cKO mice displayed a slight qualitative increase in lateral ventricle size (LV; **A-D**). Robust Tyrosine Hydroxylase (TH) expression (TH⁺ neurons) was detected in the caudate putamen (CP), ventral striatum (VS) and substantia nigra (SN) of both WT and *Grhl3cKO* mice (**A-B**; coronal sections; **C-D**; sagittal sections; boxed regions show the SN). The number of TH⁺ neurons within the SN (**E-F**) was quantified, showing no significant differences in cell number between WT and *Grhl3cKO* (n=3 per group) animals (**G**). Genomic deletion PCR (**H**) was performed on olfactory bulb DNA extracted from WT and *Grhl3cKO* aged mice, showing robust deletion in the *Grhl3cKO* mice.

Supplementary Figure Legends

Fig. S1 Analysis of *Grhl3* expression within the surface ectoderm and brain at gestational timepoints E14.5-E16.5 by autoradiographic in situ hybridisation.

(A-B’); sagittal sections of whole mouse embryos at E14.5 and E16.5 showing expression of *Grhl3* in the surface ectoderm (red arrows). (C-F’) Coronal sections of embryonic brain at E15.5 again showing expression of *Grhl3* in surface ectoderm, and absence of *Grhl3* expression within the brain, save for the habenula (“h” in E’). (G – G’) Magnified view of boxed region in E – E’, highlighting strong expression in the surface ectoderm, but no expression visible in the dermis, calvaria, cerebral cortex or dura mater.

Fig. S2 Characterisation of WT and *Grhl3*^{-/-} E18.5 embryonic mouse brains.

(A-B) Dissecting-microscope images of WT and *Grhl3*^{-/-} embryonic brains (E18.5) show a decrease in size, but no obvious changes in morphology, of *Grhl3*^{-/-} brains. (C-D) Quantitation of brain weights of WT and *Grhl3*^{-/-} brains (presented as overall pooled data [C] and individual weights [D]) shows that *Grhl3*^{-/-} brains are significantly smaller, consistent with overall decreased embryo size. (E) Quantitative Real-Time Polymerase Chain Reaction (Q-RT-PCR) analysis of mRNA extracted from neural stem/progenitor cells (NSPCs) cultured for 7 days as neurospheres, confirming that the expression of *Grhl3* seen in control neurospheres is lost in *Grhl3*^{-/-} NSPCs.

Fig. S3 Characterisation of cellularity, proliferation and apoptosis in WT and *Grhl3*^{-/-} embryonic mouse brains.

(A-D) Higher-magnification images (x20 and x100) of proliferation within the habenula (red arrows) and lateral ventricles (LV) of WT and *Grhl3*^{-/-} brains at E15.5. (E-H) Proliferation (shown by immunohistochemical analysis of proliferating cell nuclear antigen [PCNA] expression) within WT and *Grhl3*^{-/-} brains at E18.5. Although no qualitative differences were observed, we noted separation of brain tissue from the overlying dura mater (red arrow) in *Grhl3*^{-/-} brains. (I-J) Apoptosis (shown by immunohistochemical analysis of caspase 3 expression) within WT and *Grhl3*^{-/-} brains at E18.5, showing no specific areas of cell death.

FIG.1

Fig. 1 Grhl3 expression and characterisation of Grhl3^{-/-} embryonic brains
The expression of Grhl3 was examined in sequential coronal (A-A') or transverse (B-C') sections of E15.5 wild-type embryonic mouse brain (A-C) using radiolabelled ISH (A'-C'). Expression was detected within the habenula (Ha; A'), striatum (St; B') and the ventral-most region of the lateral ventricle (LV; C'). No differences were seen in Grhl3^{-/-} brains compared to wild-type brains with respect to cellular proliferation (Ki67; D-E) or apoptosis (activated caspase-3; F-G). Similarly, no cell-intrinsic differences were seen following in vitro neurosphere culture from Grhl3^{-/-} brains compared to wild-type brains with respect to proliferation over 7 days (H) or 21 days (I). All images are shown at x20 magnification.

190x275mm (96 x 96 DPI)

FIG.2

Fig. 2 Analysis of locomotor activity via vertical rod-descent and DigiGait analyses. The average time taken to descend from the top of an 80cm vertical rod was not significantly different between WT and Grhl3cKO animals (A); this was not due to any differences in size or weight between the two groups (B). When assessed using DigiGait analysis, the stride length of the left (C) and right (C') hind limbs was significantly shorter in the Grhl3cKO animals, with a commensurate increase in stride frequency (D-D') and therefore total number of steps (E-E') within the 5-second testing period. (* $p < 0.05$).

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FIG.5

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