RESCUE OF AN AGGRESSIVE FEMALE SEXUAL COURTSHIP IN MICE BY CRISPR/Cas9 SECONDARY MUTATION IN VIVO.

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We had previously reported [1] a mouse line carrying the Atypical female courtship (HoxD^{Afc}) allele, where an ectopic accumulation of Hoxd10 transcripts was observed in a sparse population of cells in the adult isocortex, as a result of a partial deletion of the HoxD gene cluster (Figure 1A). Female mice carrying this allele displayed an exacerbated paracopulatory behavior, culminating in a severe mutilation of the studs’ external genitals. To unequivocally demonstrate that this intriguing phenotype was indeed caused by an illegitimate function of the HOXD10 protein, we use CRISPR/Cas9 technology to induced a microdeletion into the homeobox of the Hoxd10 gene in cis with the HoxD^{Afc} allele [2]. Females carrying this novel HoxD^{Del(1-9)d10hd} allele no longer mutilate males. We conclude that a brain malfunction leading to a severe pathological behavior can be caused by the mere binding to DNA of a transcription factor expressed ectopically. We also show that in HoxD^{Afc} mice, Hoxd10 was expressed in cells containing Gad1 and Cck transcripts, corroborating our proposal that a small fraction of GABAergic neurons in adult hippocampus may participate to some aspects of female courtship.

Although the heterozygous HoxD^{Afc} genotype proved semi-lethal in both sexes, only sexually mature females displayed an aberrant courtship behavior. When placed with a male for mating, and regardless of the male genotype (i.e. HoxD^{Afc} heterozygous or wildtype), females repeatedly bit and injured the male penises, often up to their complete ablation. In such adult HoxD^{Afc} heterozygous mice, ectopic Hoxd10 transcript accumulation was found in numerous scattered cells in the hippocampus [1], while Hox genes are never expressed rostral to the hindbrain and its derivatives [3]. To confirm the causal role of Hoxd10 ectopic expression in this unusual behavior, we induced a deletion in the homeobox of the Hoxd10 gene in cis with the HoxD^{Afc} allele (Figure 1A). Non-homologous end joining of genomic DNA after exposure to a single guide RNA and the Cas9 endonuclease in fertilized eggs resulted in a 10 base-pair large deficiency in the Hoxd10 homeobox, giving rise to the HoxD^{Del(1-9)d10hd} allele. This mutant allele had lost the third alpha-helix of the homeodomain necessary for the binding of this transcription factor to its DNA target sites (Figure 1B), due to a protein truncation from the 40th position of the homeodomain onwards, replacing 34 residues by a 10 residues frameshifted sequence (Figure 1C).

We crossed this allele out through three consecutive generations and observed twelve adult females caged with males. Studs were followed for the appearance of injuries at their external genitals. Heterozygous HoxD^{Del(1-9)d10hd} females bred successfully, without any indications of atypical female courtship (0 out of 12). This was in marked contrast with
the observation of 12 out of 18 $HoxD^{Afc}$ females carrying the intact $Hoxd10$ homeobox sequence and showing genital biting [1]. Other abnormal phenotypic traits associated with the $HoxD^{Afc}$ allele, like malocclusion and slow postnatal weight gain were also rescued [2]. These results provide strong genetic evidence of the direct role of the $HOXD10$ transcription factor in bringing about the courtship aberration observed in $HoxD^{Afc}$ mice.

This courtship anomaly occurred in animals with a low abundance of $Hoxd10$ positive cells in adult forebrain, which display molecular and neuroanatomical characteristics reminiscent of a small subpopulation of GABAergic interneurons [1,4], as characterized by the simultaneous detection of both the $Gad1$ and $Cck$ markers. Double labeling FISH analyses with $Hoxd10$-dig and $Gad1$-fluor pair of probes indeed showed $Hoxd10$ positive cells localized selectively in the hippocampus, distributed in any of the layers of CA fields where it co-localized with $Gad1$ (Figure 1). Furthermore, by using $Cck$-fluor and $Hoxd10$-dig probes, we scored the $Hoxd10$ specific red signal in cell accumulating $Cck$ transcripts (Figure 1). As all $Cck$ positive non-principal cells seemed included in the $Gad1$ labeled pool, and since all $Hoxd10$ positive cells were part of the $Cck$ positive non-principal pool, we concluded that ectopic $Hoxd10$ transcripts accumulated in a very sparse subpopulation of $Cck$ positive GABAergic cells. Of note, $Hoxd10$ like other $Hox$ genes is not expressed in any cells of a normal adult forebrain [5].

The $HoxD^{Afc}$ phenotype followed a gender-specific pattern of expressivity, despite the fact that ectopic expression of $Hoxd10$ was similar in both sexes. The ectopic presence of this HOX product in CCK positive GABAergic neurons in adult hippocampus may thus interfere with the implementation of a particular genetic program in a sexually dimorphic manner, perhaps through the property of such proteins to exert a dominant negative effect in various contexts [6]. CCK signaling was previously associated with a sex-dependent control of behavior and its level seems to be modulated during the estrus cycle [7]. Also, the inactivation of the $Cck2$ receptor, which presumably mediates some effects of CCK neuropeptides in postsynaptic neurons, elicits behavioral alterations distinct in females as compared to males [8]. Altogether, this is consistent with a gender-specific role of CCK positive GABAergic cells in the modulation of behavior [9]. A persistent ectopic expression of $HOXD10$ in CCK positive hippocampal GABAergic cells may thus interfere with the function of these cells in controlling the dynamic physiological status of females during the estrous cycle [10].

**Material and Methods**

Experiments were conducted according to the Swiss law on animal protection (LPA) under licenses #GE/81/14 and #GE/29/26. The CRISPR/Cas9 induced allele was described in
Freshly dissected brains were mounted in OCT and stored at -80°C. In most experiments, pairs of hemi-brains of HoxD^{Alt} and HoxD^{Del4-9} heterozygous or wild type control adult females were mounted in the same block, cut, collected on the same slides and processed together to allow for direct comparison of the Hoxd10 signals under identical conditions. Usually four parallel sub-series of 14 µm thick coronal cryo-sections were collected, air-dried and stored at -80°C. One of the sub-series was stained with Cresyl violet and the position of the sections along the Coronal Allen Brain Atlas was determined. On the day of hybridization, slides were thawed, air-dried and fixed in 4% paraformaldehyde in PBS. In situ hybridizations were carried out at 63.5°C overnight, followed by stringency washes at 61°C. The binding of the antisense probe was revealed either by the NBT/BCIP alkaline phosphatase substrate (e.g. Allen Brain Institute http://mouse.brain-map.org/gene), or with the FASTRED alkaline phosphatase substrate to detect DIG labeled probes and the Tyramide amplification procedure (PerkinElmer SAT700001EA), followed by Streptavidin Alexa Fluor 488 conjugate (Invitrogen S32354) to detect Fluorescein labeled probes. Gad1 and Cck antisense riboprobes were synthesized using cDNA plasmid clones as templates (http://www.imagenes-bio.de). Briefly, mouse Gad1 cDNA clone IRAKp961I2154Q was linearized with Kpn1 and transcribed by T7 polymerase (Promega, #P2075). Mouse Cck cDNA clone IRAVp968E034D was linearized with EcoRI and transcribed with T3 polymerase (Promega, #P2083). Mouse Hoxd10 cDNA clone [1] was digested with EcoRI and transcribed by T7 polymerase. Labeled nucleotides were incorporated using DIG RNA Labeling Mix (Roche 1122707390), or Fluorescein RNA Labeling Mix (Roche 11685619910). We successfully detected Hoxd10 with DIG, yet not when a fluorescein labeled antisense cRNA probe was used. This may reflect a higher sensitivity of the alkaline phosphatase enzymatic reaction, which was also supported by the easier detection of the Gad1 and Cck signals with DIG/FAST RED, as compared to the fluorescein/Tyramide enhancement. In double FISH experiments Hoxd10 specific red signal was scored at probe concentrations, when red stained cellular profiles were detected only in the HoxD^{Alt}, and not in either control samples, indicating that conditions were appropriate for specific detection of Hoxd10 transcripts. The double FISH procedure was carried out as in [11]. Pictures were taken with HBO 100 illumination using the appropriate filter sets to visualize red, green and blue fluorescence signals (set 43, 10 and 49 respectively), on a Zeiss Axioplan 2 microscope (Figure 1 C-E). Hoxd10 red hybridization signals were accepted as positive if the signal could be seen with a 5x/0.25 n.a. 0.17 Zeiss FLUAR objective using filter set 43. Upon higher magnification, a clear cytoplasm signal zone included a negative zone corresponding to the position of a cell nucleus (perikaryon). Images were taken with a Leica DFC300 FX digital color camera. Brightness and contrast
were adjusted in Photoshop CS3. Red and blue or green and blue double color images were generated using the HDR2 plug-in.

Acknowledgements

We thank N. Flores-Ramirez for help with in situ hybridizations and I. Rodriguez for discussions. This work was supported by funds from the University of Geneva, the Ecole Polytechnique Fédérale (Lausanne), the Swiss National Research Fund (No. 310030B_138662) and the European Research Council grants SystemHox (No 232790) and RegulHox (No 588029).

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Figure 1

(A) Comparison of wild type HoxD and the HoxD^{Afc}, alias Del(1-9) and HoxD^{Del(1-9)d10hd} mutant alleles. Discontinuity of the horizontal line indicates the absence of the genomic segment and the red X indicates the position of the CRISPR/Cas9 hit in the Hoxd10 homeobox, leading to the generation of the HoxD^{Del(1-9)d10hd} allele. (B) Amino-acid sequence of the HOXD10 homeodomain in both the wild type and the HoxD^{Afc} alleles. The three alpha helical subdomains are underlined. (C) Amino-acid sequence of the homeodomain in the truncated HOXD10hd protein product. The sequence of the remaining two alpha helical subdomains are underlined and an asterisk indicates an out of frame stop codon. (D, E). Details of representative coronal sections of heterozygous HoxD^{Afc} female brains. The contours of the hippocampal formation are indicated by red dots and three landmark cytoarchitectonic layers are annotated (sr, sp, so, for strata radiatum, pyramidale and oriens, respectively). (D). Gad1 specific antisense probe reveals positive cells distributed in all layers of CA. (E) A Cck specific antisense
probe shows few strongly stained cells in all layers of CA, and a relatively weaker signal in the rest of the cells located in sp. (F, G, H). Simultaneous fluorescent in situ hybridization. Nuclei are shown in blue. (F) Expression of Cck in green (top) is detected in one of four Gad1 positive cells shown below in red (bottom) in CA3 sr. (G). Expression of Hoxd10 (red, top) in one of four Gad1 positive cells (green, bottom) in CA3 so. (H). Expression of Hoxd10 in (red, top) in one of the three Cck positive cells (green, bottom) in CA1 so.
