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Report

# A Genetic Basis for Altered Sexual Behavior in Mutant Female Mice

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## Summary

**Although neural substrates of mammalian female mating behavior have been described [1, 2], the association between complex courtship activity and specific underlying mechanisms remains elusive [3]. We have isolated a mouse line that unexpectedly shows altered female social behavior with increased investigation of males and increased genital biting. We investigated adult individuals by behavioral observation and genetic and molecular neuroanatomy methods. We report exacerbated inverse pursuits and incapacitating bites directed at the genitals of stud males. This extreme deviation from wild-type female courtship segregates with a deletion of the *Hoxd1* to *Hoxd9* genomic region. This dominant *Atypical female courtship* allele ($HoxD^{Afc}$) induces ectopic *Hoxd10* gene expression in several regions in newborn forebrain transitorily and stably in a sparse subpopulation of cells in the *cornu ammonis* fields of adult hippocampus, which may thus lead to an abnormal modulation in the sexual behavior of mutant females. The resulting compulsive sexual solicitation behavior displayed by the most affected individuals suggests new avenues to study the genetic and molecular bases of normal and pathological mammalian affect and raises the potential involvement of the hippocampus in the control of female courtship behavior. The potential relevance to human 2q.31.1 microdeletion syndrome [4, 5] is discussed.**

## Results

During the production of a stock of mice carrying a targeted microdeletion within the *HoxD* gene cluster, including from the *Hoxd1* gene to *Hoxd9* (*Del1–9*), we observed that heterozygous females displayed aberrant courtship behavior, transmitted to their offspring for at least ten generations. Progenies were rarely observed in harems with more than one such female. Instead, the male external genitals became selectively and severely wounded (see Figure S1A available online), such that mating had to be stopped. We analyzed full-night video recordings and witnessed females chasing males assiduously (see Movies S1, S2, and S3). In the course of such inverse pursuits, they often bit the genitals, very specifically, while the hind paws of the male were off the floor (Figure 1A). Inverse pursuits involving such bites were directed at males exclusively, never at bystander females. Such apparent aggressive behavior was surprising, because in cages of group-housed males or females of this line, we did not observe fighting or mutilations over a period of several years. Furthermore, the overall pattern of behavior did not fit descriptions either of species specific aggressive postures characteristic of mice [6] or of the pathological aggressiveness described for male and female *fierce* mouse mutant [7]. We carefully observed when females physically contacted the ano-genital regions of males, by video recording groups of two to four females caged with a male using visual tracking, which allowed quantitative individual scoring. We found that females often approached the males from behind and sniffed their tail and the ano-genital region while following them along. This was seen with wild-type (WT) females and was more frequent during the night of sexual receptivity. Therefore, we scored for the timing and length of all ano-genital investigation episodes in time-lapse recordings of WT, *Del4–9*, *Del1–10*, and *Del1–9* females when caged with adult males. In cages of two harems of four females housed together with a male during several days, we found that in case of controls, ano-genital investigations were rare and the total time spent in this occupation rarely added up to more than a few dozens of seconds per night, when the female was not receptive (Figure S1B). On the night of sexual receptivity, the number of instances and the total time spent in ano-genital investigations increased to around 100 s in case of both control females and a minority of *Del1–9* females. We considered this increase in male seeking behavior as part of the normal courtship of female laboratory mice. The incidence and total span of ano-genital investigation increased dramatically on the night of receptivity, in case of the majority of *Del1–9* females, taking the form of inverse pursuits alternating with sexual intercourses and suggesting that in these mutant females the courtship activity was severely exacerbated. Chased males could be of any genotype, including WT and, surprisingly, they did not display any aggressive behavior, even after their genital region was mutilated.

We quantified selected aspects of the female courtship behavior on the night of receptivity in order to numerically compare this abnormal strain with control animals (Table 1). Twelve out of 18 (see Table 1) *Del1–9* mutant mice showed excessive physical contacts, which we scored as lift and/or bite. In contrast, a single such frame among the 19 mating control females was found depicting a WT female engaging in such a lift and/or bite behavior. This difference was statistically significant and thus confirmed the causal role of the *Del1–9* mutant females in male mutilations. Due to the short duration of the lifts, the count of such episodes, which were embedded in sequences of ano-genital investigations, was underestimated at least 4-fold, when recorded in time-lapse mode (see Figure S1C). *Del1–9* females initiated a higher number of ano-genital investigations, and the total time spent in this activity was also significantly longer, on average (Table 1). A minority of *Del1–9* females were scored normal by all behavioral observation criteria (see also Figures S1B and S1D). This could indicate the effect of presently noninvestigated genetic modifiers or physiological variation in the expressivity of the phenotype. Surprisingly, a number of *Del1–9* females were involved in a significantly higher number of actual copulations, as signaled by the higher count of lordosis episodes. In

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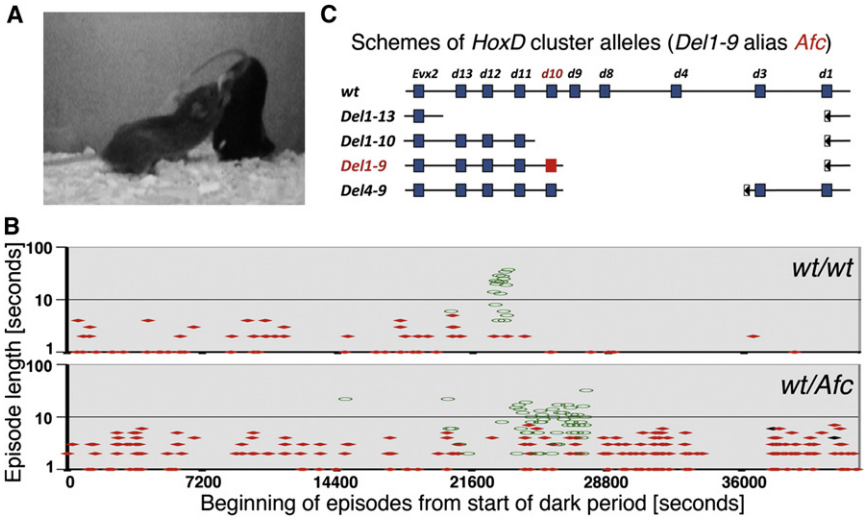


Figure 1. Heritable Atypical Female Courtship in Mice

(A) A female in the course of ano-genital contact with a stud male (black coat color), with his hind legs lifted up.

(B) Plots of timing and length of ano-genital investigations and copulations (red diamonds and green circles, respectively), comparing a WT female (top) and a *HoxD*$^{Afc}$ mutant female (bottom) during an entire night, based on time-lapse video recordings. Two dark diamonds at the bottom indicate lift and/or bite episodes similar to that depicted in (A). Note the enhanced frequency of investigations by the mutant female, in particular after copulations had occurred (see also Movies S1, S2, and S3).

(C) Schematic representation of the alleles used in this study, with the WT gene cluster on the top and the various deletions below: *Del1–13* [8], *Del1–10* [9], *Del1–9* (alias *HoxD*$^{Afc}$), and *Del4–9* [28]. All stocks are maintained by serial backcrossing of heterozygous males to (Bl6/CBA)F1 females to keep comparable heterogeneity in genetic backgrounds over generations. Only *Del1–9* shows ectopic expression of *Hoxd10* (in red) in forebrain.

Detailed description of digital video recordings, allele structure, and derivation are provided in Supplemental Experimental Procedures. The *Del1–9* (alias *HoxD*$^{Afc}$) novel allele was produced using the TAMERE breeding protocol [29] using *Del1–13* and *10–9flox* [30] allele. See also Figure S1.

course of these extended copulation sequences, it was the female that approached the male immediately preceding the mounts. Overall, the ano-genital investigation episodes primarily occurred prior to copulations in case of controls, whereas most *Del1–9* mutants continued inverse pursuits after copulation bouts as well (Figure 1B, Figure S1D). We concluded to a case of altered social behavior with increased investigation of males and increased genital biting, aspects of female courtship that amounted to sexual harassment. We refer to this allele as *Atypical female courtship* (*HoxD*$^{Afc}$).

Because this phenotype involved complex cognitive processes and nonadaptive goal directed behaviors, we expected an anomaly in either the structure or the physiology of the forebrain. Because the *HoxD*$^{Afc}$ condition is dominant and because *Hox* genes are normally not functional and poorly expressed there, if at all, we suspected a gain of function to cause this phenotype. Also, mice carrying the related *Del1–13* and *Del1–10* deletions (Figure 1C) did not display this anomaly, after more than ten years of nonstop breeding [8, 9]. Because the only difference between *Del1–9* and *Del1–10* is the presence of *Hoxd10* at the former locus, we predicted that ectopic expression of *Hoxd10* in forebrain was involved. We first looked for *Hoxd10* transcripts accumulation by in situ hybridization in *HoxD*$^{Afc}$ newborns by using the related yet phenotypically silent *Del4–9* allele as control. Both *Del1–9* and *Del4–9* deletions induced similar ectopic expression of *Hoxd10* in the hindbrain (data not shown). In midbrain and forebrain, however, ectopic *Hoxd10* transcripts were only found in *Del1–9* (*HoxD*$^{Afc}$) (Figure 2A) mice and neither in *Del4–9*, nor in WT control animals (Figure 2B). Several regions of ectopic expression were observed and appeared of special interest in the context of the above-described phenotype, such as, for example, nuclei in habenula and the amygdala. This suggested that the *Afc* phenotype was associated with ectopic *Hoxd10* transcription either in forebrain or in midbrain.

Because the phenotypic anomaly is selectively expressed during adulthood in females, and in order to better discriminate between involvement of these various regions of ectopic expression, we investigated the adult brain. First, areas expressing robust levels of ectopic *Hoxd10* during development appeared grossly normal in adults, suggesting that brains of mutant animals were largely unaffected at a gross structural level, consistent with the apparently normal behavior of *HoxD*$^{Afc}$ females beside this severe mating anomaly. Second, although a few labeled cells were seen in the amygdala, ectopic expression of *Hoxd10* was no longer detected in the rest of forebrain regions positive in newborns. Instead the vast majority of cells ectopically expressing *Hoxd10* was in the hippocampus of *HoxD*$^{Afc}$ mutant forebrains (Figure 2D). There again, *Hoxd10* positive cells were not scored in control forebrains carrying the *Del4–9* allele, neither in the amygdala nor in the hippocampus. This confirmed our prediction based on the genomic structure of our allelic series and further supported the correlation showing abnormal gene regulation in adult forebrain when the behavioral phenotype is manifest. In the adult *Afc* brain, most *Hoxd10*-positive cells were selectively located in the *cornu ammonis* (CA) fields, with, in CA1, about 15% of the cells localized within the principal pyramidal cell layer, whereas the remaining positive cells were in the neighbor layers. Because this distribution resembled the localization of cells expressing glutamic acid decarboxylase *(Gad1*; Figure 2E), it raised the possibility that *Hoxd10* was ectopically expressed in CA nonprincipal GABAergic neurons [10].

## Discussion

In contrast to the vast majority of heritable pathological behaviors described in the laboratory mouse, the *HoxD*$^{Afc}$ phenotype we report here follows a gender-specific inheritance pattern. This suggests that the affected mechanism underlies a female-specific behavior. Control females of these stocks expectedly approach males more frequently when they are in proestrous, rather than when in other phases of the estrous cycle. These approaches include intimate ano-genital investigations. The courtship displayed by *HoxD*$^{Afc}$ mice also follows this temporal dynamics, yet it seems largely exaggerated and uncontrolled. These females indeed recognize the male and display proceptive behaviors [11], but they appear

Table 1. Results of Overnight Time-Lapse Observations of Female Sexual Behavior[a]

| | WT,$^{+/+}$ (n = 9) | *Del4–9*/+ (n = 5) | *Del1–10*/+ (n = 5) | *Del1–9*/+ (n = 18) |
|---|---|---|---|---|
| Lift and/or bite episodes[b] | 1/9 | 0/5 | 0/5 | 12/18[e] |
| Count, average[c] | 1* | 0 | 0 | 3.8 ± 1 |
| Ano-genital investigations[b] | 9/9 | 5/5 | 5/5 | 18/18 |
| Count, maximum | 93 | 62 | 32 | 345 |
| Count, minimum | 1 | 5 | 6 | 34 |
| Count, average[c] | 46.2 ± 12 | 35.6 ± 11 | 16.4 ± 5 | 119.2 ± 21[f] |
| Total span, average[d] | 95.6 ± 19 | 111.2 ± 29 | 43.8 ± 10 | 311.2 ± 60[g] |
| Lordosis response[b] | 9/9 | 5/5 | 5/5 | 18/18 |
| Count, maximum | 41 | 36 | 27 | 107 |
| Count, minimum | 3 | 2 | 5 | 2 |
| Count, average[c] | 17.5 ± 5 | 10.8 ± 6 | 17.8 ± 5 | 31.0 ± 7[h] |
| Total span, average[d] | 348.4 ± 87 | 171.6 ± 61 | 240.4 ± 67 | 306.4± 37[i] |
| Plugs[b] | 5/9 | 2/5 | 5/5 | 13/18 |
| Rejected mounts[c] | 12.3 ± 5 | 7.6 ± 5 | 6.8 ± 2 | 13.6 ± 3[j] |

[a]Thirty-seven females recorded on the night of sexual receptivity, grouped by *HoxD* genotype.
[b]Number of mice, which showed that particular behavior over total.
[c]Number of episodes by mice, which showed that particular behavior, mean ± SEM. * represents a single case.
[d]Sum of length of all episodes in s, mean ± SEM.
[e]Chi-square test: Yates, with Bonferroni correction (n = 15): $p < 0.006$ *Del1–9* vs. all other groups combined.
[f]ANOVA after log transformation to stabilize the variance, $p < 0.0009$. Post hoc t tests assuming equal variances with Bonferroni correction (n = 15): $p<0.001$ for *Del1–9* vs. all other groups combined. The rest of the pairwise comparisons were not statistically significant ($p >> 0.05$).
[g]ANOVA after log transformation to stabilize the variance, $p < 0.0006$. Posthoc t tests assuming equal variances, with Bonferroni correction (n = 15): $p<0.001$ for *Del1–9* vs. all other groups combined; $p < 0.04$ for *Del1–9*, vs. WT. The rest of the pairwise comparisons were not statistically significant ($p >> 0.05$).
[h]ANOVA after log transformation to stabilize the variance, $p = 0.08$.
[i]ANOVA after log transformation to stabilize the variance, $p = 0.2$.
[j]ANOVA after log transformation to stabilize the variance, $p = 0.6$.

hyperreactive, suggesting defective behavioral inhibition, as illustrated by the frequency of both inverse pursuits and tactile stimulation directed at the external genitals.

Although mouse females' proceptive behavior is not well documented, some reports mention that rodent females actively contribute to mating [12]. A common element among various species is a form of approach behavior, where the female establishes physical proximity to the male [13, 14]. There is also experimental evidence indicating that female mice actively control the pace of sexual intercourses [15]. Furthermore, tactile stimulation was recognized to be part of the courtship routine in diverse female mammals [14, 16]. In the courtship behavior of *HoxD$^{Afc}$* females, both alternating approaches and withdrawals and physical contact responses show compulsive exaggeration in the most affected individuals.

*Hox* genes are believed to have no function in neuroectoderm derived cells in forebrain [17, 18]. They encode transcription factors and are selectively repressed throughout the midbrain and forebrain of all vertebrates. Remarkably, we found ectopic expression of the *Hoxd10* gene in diverse regions of the developing forebrain in *Del1–9* (*HoxD$^{Afc}$*) newborns. Later, in adult, expression was maintained only in a few cells in the basomedial amygdala, and a new domain of ectopic expression appeared in the hippocampus. Consequently, the phenotype we describe could be related to two distinct deleterious effects of *Hoxd10* in the brain. In the first scenario, an early gain of function of *Hoxd10* in the brain would either modify the structure or the differentiation of some nuclei, inducing a stable functional modification leading to behavioral alteration 2 months later. In the second scenario, the *Afc* phenotype is triggered by ectopic expression of *Hoxd10* in hippocampus in adult brain. Because any ectopic expression domain persistent in adult brain would be more likely to interfere with the very dynamic physiological status of mutant females we favored the second hypothesis and thus concentrated on ectopic *Hoxd10* expression in adult brain. There, *Hoxd10* was observed in rare cells in CA, reminiscent of the cytoarchitectonic distribution of nonprincipal GABAergic neurons. Therefore, in the case of *HoxD$^{Afc}$*, we hypothesize that the ectopic presence of this HOX protein in forebrain, i.e., cells that normally never experience it, may trigger important changes in the implementation of their genetic program. It is indeed well documented that proteins like HOXD10 can exert a dominant-negative effect in many contexts [19]. In this particular case, it may impinge upon the capacity of these cells to modulate behavioral responses, thereby leading to the observed exacerbation of an innate behavior.

Although the mechanisms underlying both the specific gain of function of *Hoxd10* in this cell type and the rational for its deleterious effect remain elusive, some elements suggest avenues to further investigate. First, the *Afc* phenotype selectively affects *Del1–9* females, even though heterozygous adult males also display ectopic expression in CA nonprincipal cells. This may relate to the fact that the altered behavior is already gender-specific under normal circumstances. In fact, the acute phase of this behavioral anomaly occurs during proestrous, a phase known to induce complex modifications in the female rodent hippocampus [20], including global changes in transcription [21]. The presence of ectopic HOXD10 may interfere with these complex hormonal regulations. During female courtship, some cells in CA may contribute to the modulation of courtship behavior, restricting both its amplitude and its occurrence to the preovulatory period. In this view, the *Afc* condition may illustrate the role of the hippocampus in "states of emotion, especially disappointment and frustration," that was suggested nearly half a century ago [22, 23].

Finally, this correlation between the ectopic presence of a transcription factor, as a consequence of copy number variation, and a behavioral anomaly further illustrates that genomic rearrangements can lead to pathologically significant gain of function, rather than to mere haplo insufficiencies [24]. It is conceivable that microdeletions or altered epigenetic mechanisms [25] at this or other loci may induce brain malfunction, including pathological behaviors due to ectopic expression of normally silent genes. The possibility also exists that the genomic region, which lies between the centromeric breakpoint of *Del1–9* and that of *Del1–10* contains regulatory elements that, in the mutant condition, would misregulate the expression of other, yet-to-be-identified locus, besides *Hoxd10*. In this view, the *Del1–9* allele would generate a gain of function of an unknown gene unrelated to the *Hox* gene family. However, we do not favor this hypothesis, which would involve the presence of an alternative target gene located either in *trans* or in *cis*, but far away. In humans, the 2q31.1 syntenic *HoxD*-containing locus is a target of multiple deletions, duplications or inversions. Patients display a range of anomalies

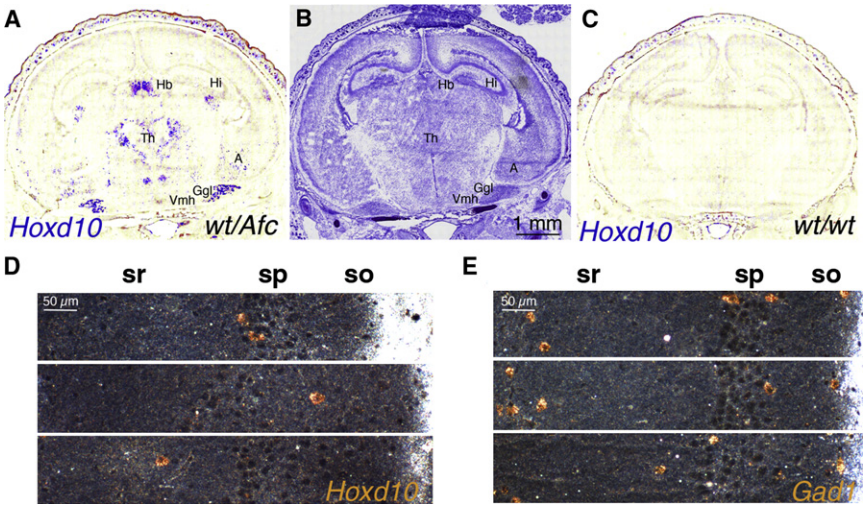


Figure 2. Ectopic Expression of *Hoxd10* in Newborn and Adult Brain

(A) A coronal section of a heterozygous *HoxD*$^{Afc}$ newborn female head, hybridized with *Hoxd10*-specific probe. Note that many cells in the Gasser ganglion at the base of the skull and broad domains in forebrain, including parts of the cortex and the diencephalon, show blue staining due to ectopic accumulation of *Hoxd10* transcripts.

(B) A neighbor section of the same heterozygous *HoxD*$^{Afc}$ newborn female head, stained with Cresyl violet. A few landmark structures are annotated both in (A) and (B). (A, amygdala; Ggl, Gasser ganglion; Hb, habenula; Hi, hippocampus; Th, thalamus; Vmh, ventromedial hypothalamic nucleus.)

(C) In situ hybridization showing a comparable coronal section of a WT newborn female head. Blue staining was absent both in Gasser ganglion and in brain.

The blue signal in the neck region seen in both cases was due to endogenous alkaline phosphatase activity that was also detected in the absence of antisense riboprobe. Both sections were processed simultaneously in the same hybridization experiment, scanned at the same settings, and mounted and modified together in Photoshop to set curves, levels, brightness, and contrast simultaneously, to ensure comparable sensitivity of visualization.

(D) Details of in situ hybridization showing the CA1 fields of a heterozygous adult *HoxD*$^{Afc}$ female brain, hybridized with the *Hoxd10* antisense riboprobe.

(E) Details of in situ hybridization showing the CA1 fields of an adult heterozygous *HoxD*$^{Afc}$ female brain, hybridized with the *Gad1* antisense riboprobe. Rare positive cells are detected by blue chromogene NBT/BCIP (appearing light-brown due to dark-field illumination; sr, sp, so, *strata radiatum*, *pyramidale*, and *oriens*, respectively).

Detailed description of brain sections, probes and hybridization methods are given in Supplemental Experimental Procedures. See also Figure S2.

related to those scored in this mutant *Afc* stock, including craniofacial and limb malformations, neural symptoms, and growth retardation [4, 5]. Pathological sexual behavior, however, has not yet been reported in 2q.31.1 microdeletion patients. In mice, however, none of the many engineered deletions at this locus [26], but the *Del1–9*, was ever reported to show this abnormal behavior, which emphasizes the importance of this very breakpoint, not yet found in a human condition. As it stands, the *Afc* phenotype we report in this paper may be closer to other human conditions like complex psychomotor temporal lobe epilepsy or Kluver Bucy syndrome [27].

### Experimental Procedures

Detailed description of behavioral observations, graphical representations, in situ hybridization analyzes are provided in Supplemental Information. Experimental procedures involving animals were carried under proper authorization and according to Swiss law on animal protection.

### Supplemental Information

Supplemental Information includes two figures, Supplemental Experimental Procedures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.06.067.

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