# Sex chromosome genes directly affect brain sexual differentiation

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Sex differences in the brain are caused by differences in gonadal secretions: higher levels of testosterone during fetal and neonatal life cause the male brain to develop differently than the female brain[1]. In contrast, genes encoded on the sex chromosomes are not thought to contribute directly to sex differences in brain development, even though male (XY) cells express Y-chromosome genes that are not present in female (XX) cells, and XX cells may have a higher dose of some X-chromosome genes. Using mice in which the genetic sex of the brain (XX versus XY) was independent of gonadal phenotype (testes versus ovaries), we found that XY and XX brain cells differed in phenotype, indicating that a brain cell's complement of sex chromosomes may contribute to its sexual differentiation.

An exception has been reported[2,3] to the well-supported theory that sex differences in the brain are caused by gonadal secretions. Dissociated cell cultures from embryonic day 14 (E14) rodent mesencephalon or diencephalon sexually differentiate *in vitro*—for example, by developing sex differences in the number of dopamine neurons—even though E14 is before the emergence of sex differences in plasma levels of testosterone[4–6]. Therefore, these XX and XY brain cells differ in cell-autonomous programs of development, not because of differential action of gonadal secretions. This conclusion is strengthened by the finding that treating pregnant dams with estrogen or androgen antagonists before harvesting the cells does not prevent the XX–XY differences in cell behavior[7]. The testes may begin to secrete testosterone prior to E14, however, making it impossible to completely exclude a role for gonadal steroids.

We used mice with an unusual Y chromosome ($Y^-$), in which the testis-determining gene *Sry* is deleted so that testis development fails[8]. $XY^-$ mice have ovaries and are thus considered females when gonadal phenotype is used to define the animal's sex. The $Y^-$ chromosome is complemented by an autosomal *Sry* transgene (TgN(*Sry*-129)Ei)[9], so that $XY^-$*Sry* males are fertile. Mating $XY^-$*Sry* males to XX females produces four types of progeny: XX females, $XY^-$ females, XX*Sry* males and $XY^-$*Sry* males. These males are masculinized equivalently by testicular secretions during development, and thus differ phenotypically from both female groups[10]. We prepared dissociated cell cultures from E14.5 mesencephalon of these mice using established methods[6] (see **Supplementary Methods** online for further details). Phenotypic differences in cultures within sex ($XY^-$ females versus XX females; $XY^-$*Sry* males versus XX*Sry* males) were due to differences in the complement of sex chromosomes in the cells. Differences across sex ($XY^-$ females versus $XY^-$*Sry* males; XX females versus XX*Sry* males) were due to gonadal secretions and/or expression of the *Sry* gene in brain cells themselves.

Cells from each embryo were plated individually into four wells. After 6 or 11 days *in vitro* (d.i.v.), we immunostained the cells in three of the wells to label dopaminergic neurons (by tyrosine hydroxylase immunoreactivity, THir), and in the remaining well, we labeled all neurons (by microtubule-associated protein 2 immunoreactivity, MAP2ir). We compared the four genotypes using a two-way ANOVA with main factors of sex (male versus female) and sex chromosome complement ($XY^-$ versus XX). At both times, there was a highly significant main effect of sex chromosome complement on the number of THir neurons ($P < 0.000001$), with $XY^-$ cultures having more THir neurons than XX cultures regardless of gonadal phenotype (**Fig. 1**). There was no main effect of sex. The interaction of the two factors was significant at 6 d.i.v. ($P = 0.002$) but not at 11 d.i.v. ($P = 0.056$). Similar group differences were found in the ratio of THir to MAP2ir cells (TH/MAP2): a main effect of sex chromosome complement ($P < 0.000001$ at both d.i.v.), no main effect of sex, and a significant interaction at 6 d.i.v. only ($P = 0.002$). Planned comparisons (**Supplementary Methods**) confirmed the sex-

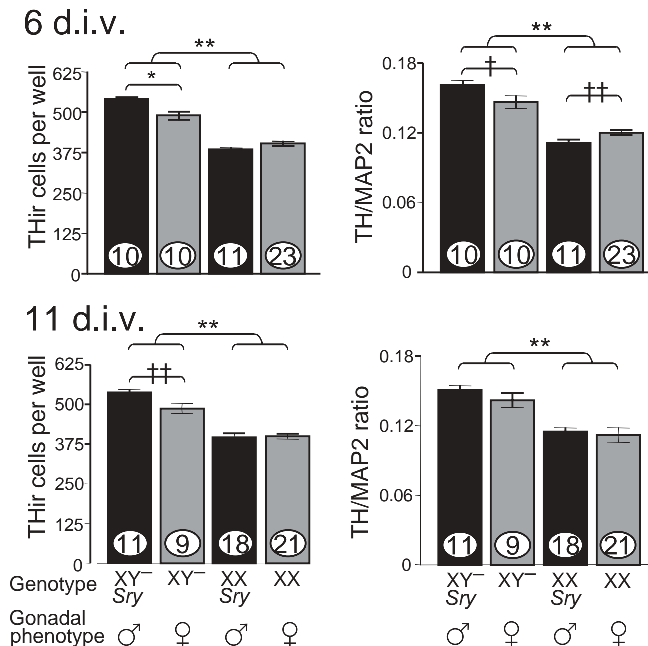


**Fig. 1.** Sex chromosome effect on neuronal phenotype. Left, $XY^-$ cultures develop more THir neurons than XX cultures, irrespective of the gonadal sex of the embryo, at 6 or 11 days *in vitro* (d.i.v.). Right, the fraction of total neurons expressing the THir phenotype (TH/MAP2) was influenced by sex chromosome complement in a similar manner. Smaller effects of sex (male versus female) were also seen. Data are mean ± s.e.m. Black bars are cultures from males (embryos with testes), gray bars are from females (with ovaries). Group sizes (*n* = number of platings, each plating is from a single embryo) are at the bottom of each bar. $^{**}P < 0.000001$, $^{*}P < 0.01$, $^{\dagger\dagger}P < 0.02$, $^{\dagger}P < 0.05$. All animal procedures were approved by the UCLA Chancellor's Animal Research Committee.

chromosome effect: for both dependent variables at both times, XX differed from $XY^-$ and XX*Sry* differed from $XY^-$*Sry* ($P < 0.02$). Planned comparisons also revealed weaker sex effects: at 6 d.i.v., $XY^-$*Sry* cultures had more THir neurons than $XY^-$ cultures (THir, $P < 0.01$, TH/MAP2, $P < 0.05$), but XX cultures had more THir neurons than XX*Sry* cultures (TH/MAP2, $P < 0.02$). At 11 d.i.v., $XY^-$*Sry* cultures had more THir neurons than $XY^-$ cultures ($P < 0.02$), but no significant differences between sexes were found in other groups or in the TH/MAP2 ratio.

The sex chromosome effect was robust, replicated using different embryos at two different times *in vitro*. The XX/$XY^-$ difference cannot be attributed to an effect of the *Sry* gene, which did not vary among the groups compared: *Sry* was absent in comparisons of XX and $XY^-$ but present in comparisons of XX*Sry* and $XY^-$*Sry*. Rather, the sex chromosome effect can be attributed to differences in (i) expression of Y genes that are normally present only in male cells, (ii) differences in expression of X genes, especially those that escape X inactivation and/or (iii) differences in paternal imprinting of X genes, which occurs in some XX but not in XY cells[1,11]. The sex chromosome effect was specific to THir neurons because it was seen in the TH/MAP2 ratio, and groups did not differ in number of MAP2ir neurons. The sex differences (XX versus XX*Sry*; $XY^-$ versus $XY^-$*Sry)* could be due to either a differential effect of gonadal secretions before cell harvesting, or the expression of *Sry* in male but not female brain cells[12]. Most of the sex differences reflect a higher number of dopamine cells when *Sry* was present ($XY^-$ *Sry* > $XY^-$), but in a single case (TH/MAP at 6 d.i.v.), the sex effect was paradoxically in the opposite direction (XX > XX*Sry)*.

The present results draw further attention to mesencephalic dopamine systems as potential sites of direct X or Y gene action during sexual differentiation of the brain, and raise the question of the site and mechanism of action of the sex chromosomes in neuronal development *in vivo*. The nigrostriatal and mesolimbic dopamine systems show pervasive sex differences in rodents and humans[3,13,14]. Thus, we also question whether sex chromosome genes lead to functional differences and influence these neurons' susceptibility to disease. The *in vitro* system described here offers significant advantages for unraveling the cellular and molecular mechanisms of sex-chromosome gene action.

*Note: Supplementary information is available on the Nature Neuroscience website.*

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**Competing interests statement**

*The authors declare that they have no competing financial interests.*

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1. Arnold, A. P. in *Hormones, Brain and Behavior* (eds. Pfaff, D. W. *et al.*) 105–135 (Academic Press, New York, 2002).
2. Reisert, I. & Pilgrim, C. *Trends Neurosci.* **14**, 467–473 (1991).
3. Pilgrim, C. & Reisert, I. *Horm. Metab. Res.* **24**, 353–359 (1992).
4. Weisz, J. & Ward, I. L. *Endocrinology* **106**, 306–316 (1980).
5. Picon, R. *J. Endocrinology* **71**, 231–238 (1976).
6. Sibug, R. *et al*. *Devel. Brain Res.* **93**, 136–142 (1996).
7. Beyer, C., Eusterschulte, B., Pilgrim, C. & Reisert, I. *Cell Tissue Res.* **270**, 547–552 (1992).
8. Lovell-Badge, R. & Robertson, E. *Development* **109**, 635–646 (1990).
9. Mahadevaiah, S. K. *et al*. *Hum. Mol. Genet*. **7**, 715–727 (1998).
10. De Vries, G.J. *et al. J. Neurosci.* (in press).
11. Xu, J., Burgoyne, P. S., & Arnold, A. P. *Hum. Mol. Genet.* **11**, 1409–1419 (2002).
12. Lahr, G. *et al. Mol. Brain Res.* **33**, 179–182 (1995).
13. Becker, J. B. *Pharmacol. Biochem. Behav.* **64**, 803–812 (1999).
14. Dluzen, D. E. & McDermott, J. L. *J. Gend. Specif. Med.* **3**, 36–42 (2000).

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# Auditory midbrain neurons that count

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Many acoustic communication signals, including human speech and music, consist of a precise temporal arrangement of discrete elements, but it is unclear whether this precise temporal patterning is required to activate the sensory neurons that mediate signal recognition. In a variety of systems, neurons respond selectively when two[1–3] or more[4] sound elements are presented in a particular temporal order and the precise relative timing of these elements is particularly important for 'delay-tuned' neurons[2], including 'tracking' types[5], in bats. Here we show that one class of auditory neurons in the midbrain of anurans (frogs and toads) responds only to a series of specific interpulse intervals (IPIs); in the most selective cases, a single interval that is slightly longer or shorter than the requisite interval can reset this interval-counting process.

Many anuran vocalizations consist of a series of pulses, repeated at specific and constant intervals[6] (**Fig. 1a**). This temporal patterning of pulses is required for call recognition[6,7], and female anurans are able to differentiate between calls that differ in pulse number[8]. 'Pulse-integrating' neurons in the midbrain may be the neural substrate for this selectivity, as they show sharp tuning to pulse repetition rate (PRR)[9,10]. We found that they also seem to require a threshold number of pulses to fire (**Fig. 1b**), and respond to nearly every presentation of a stimulus that has just one more than the threshold number of pulses (**Fig. 1c**).

Do these cells require a certain number of correct IPIs (interval-counting hypothesis), or a certain average pulse rate (number of pulses in an integration time window)?

We tested the interval-counting hypothesis by recording extracellularly from 33 pulse-integrating cells while presenting stimuli that differed in their pulse-interval distributions (data from a representative cell in **Fig. 2a–c**). This neuron responded when five or more pulses were presented at a rate of 100 pulses/s; there was a strong, tonic response when 10 pulses were presented with 10-ms IPIs (**Fig. 2b**). Across neurons, responses to the constant-interval stimulus ranged from phasic to tonic. No responses were seen, however, to a mixed-interval stimulus (**Fig. 2c**), even though it had the same average pulse rate as the constant-interval stimulus. That is, it consisted of 10 pulses in 100 ms. Repeated IPIs of 5 ms (the shorter