EFFECTS OF THE SEX RATIO ORGANISM ON *IN VITRO* DIFFERENTIATION OF DROSOPHILA EMBRYONIC CELLS

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ABSTRACT

Effects of Sex Ratio Organism (SRO) on the development of embryonic cells of *Drosophila melanogaster* were studied *in vitro*. The single embryo culture technique and a histochemical staining method were employed to distinguish male embryo cultures from female ones. SRO-infected Zw^n females were crossed to wild-type males, and their embryos were separately dissociated and cultured *in vitro*. Cell growth and differentiation in each culture were examined by phase contrast microscopy and described both qualitatively and quantitatively. Neurons, imaginal disk cells and plasmatocyte-like cells barely differentiated in male (Zw^n/Y) cultures, whereas muscle and fatbody cells were not so severely affected. These symptoms were generally specific to male cultures. Female (Zw^n/+) cultures were relatively normal. These observations are consistent with the results of mosaic analysis. The susceptibility of cells to SRO is discussed in relation to the dose of X chromosome in the cells.

DEVIATIONS from the normal 1:1 ratio of sexes have been known for a long time in several species of Drosophila (GERSHENSON 1928; STURTEVANT and DOBZHANSKY 1936). The cause of the deviation may be a nuclear mutation (e.g., *da*, CLINE 1976; *mle*, FUKUNAGA, TANAKA and OISHI 1975; *msl*, BELOTE and LUCCHESI 1980) or a maternally transmitted microorganism called Sex Ratio Organism (SRO, MALOGOLOWKIN AND POULSON 1957; MALOGOLOWKIN 1958). Transfer of SRO from *D. willistoni* and from *D. nebulosa* to *D. melanogaster* by injection of hemolymph (SAKAGUCHI and POULSON 1963) permitted genetic analysis of the cause of the differential lethality. The conclusion is that embryos with two X chromosomes survive, whereas those with a single X are killed by SRO infection regardless of the presence of a Y chromosome and of the phenotypic sex (SAKAGUCHI and POULSON 1963; MIYAMOTO and OISHI 1975).

TSUCHIYAMA, SAKAGUCHI and OISHI (1978) found that some XX//XO mosaic individuals were produced by SRO-infected mothers. Using the contour method (HOTTA 1978), they showed that the primary target site of SRO mapped to the ventral region of the blastoderm fate map. The region included both primordial

mesoderm and nervous system, but the resolution of the mosaic analysis was not sufficient to determine which of these was the SR target tissue. Histological observation of *D. willistoni* embryos did not give information about the target because of a rapid and almost total breakdown of male embryos (COUNCE and POULSON 1962).

Another approach to the detailed analysis of SR phenomena at the cellular level is to cultivate cells from SRO-infected embryos and to examine their development *in vitro*. For this purpose, cultures must be prepared from single embryos since SRO-infected mothers produce both male and female embryos. In this report, we describe the results of the analysis of SR phenomena *in vitro* by means of the single embryo culture technique; the culture genotype was determined by following glucose-6-phosphate dehydrogenase (G6PD) segregation histochemically.

MATERIALS AND METHODS

Fly strains: Canton-S (CS) of *Drosophila melanogaster* was used as wild-type. Flies were reared at 25°, 70% humidity, on the usual cornmeal-agar medium. DR. B. SAKAGUCHI generously gave us his Oregon-R strain carrying SRO from *D. nebulosa* (designated as ORNSR). We transferred SRO further from ORNSR to Canton-S and *y w* Zw^n by the transferring technique of SAKAGUCHI and POULSON (1963). These infected strains (designated as CSNSR and ZwNSR, respectively) were maintained according to the method of OISHI (1971). They produced hundreds of daughters but no sons (Table 1). Zw^n (KANKEL and HALL 1976) is a null allele of G6PD; for other mutants see LINDSLEY and GRELL (1968).

Preparation of cell cultures: Our method of single embryo culture was based on the techniques of SEECOF *et al.* (1971) and of SHIELDS, DÜBENDORFER and SANG (1975). Dechorionated embryos were observed under a dissecting microscope, and those with ventral furrows, cephalic furrows and posterior invaginations were used. According to POULSON (1950) and also to our own observations, these invaginations could be seen together only for a few minutes at about 3.75 hr after fertilization. The selected embryos were then submerged under the culture medium and held there for 30–60 min before initiation of the culture. This preincubation of the embryos caused a great improvement in the differentiation of muscle and imaginal disc cells. The contents of an embryo were withdrawn with a glass micropipette and expelled into 5 μl of the culture medium. The cell suspension was mounted as a column drop in the vessel originally developed by SHIELDS, DÜBENDORFER and SANG (1975) and incubated at 23° in a humid atmosphere of 5% CO_2 and 95% air. At 1–2 hr of the culture, culture vessels were inverted to separate yolk and debris from the cells. This caused a great improvement in cell viability, especially of single cells. The frequency of unsuccessful cultures in which no cell types differentiated was less than 4% in both SR and control cultures.

The culture medium used was M3(BF) (CROSS and SANG 1978) supplemented with 0.6 mg/ml of glutathione and 10% heat-inactivated fetal bovine serum (M. A. Bioproducts, Walkersville, Maryland). Antibiotics were not used since SRO was found to be sensitive to them. Omission of antibiotics also improved cell viability. The pH of the medium was adjusted to 6.85 with a solution of 1 N NaOH containing 10 U/ml of insulin, just before use. Although the amount of NaOH-insulin solution added to the medium differed from experiment to experiment, the final concentration of insulin was usually in the range of 6–15 mU/ml, which was comparable with the optimal dose for differentiation *in vitro* of Drosophila embryonic cells (SEECOF and DEWHURST 1974). For details of the culture methods, see our previous report (KOANA and MIYAKE 1982).

Cells from non-SR embryos were also cultured in medium containing SRO. To prepare the SRO-containing medium, CSNSR females aged at least 12 days after eclosion were anesthetized, washed twice with 70% ethanol, and injected with as great a volume of normal culture medium as possible. After a few minutes, hemolymph was taken out with a glass micropipette, and 3 μl of it (from five to ten females) were diluted in 2 ml of normal culture medium. The SRO-containing medium was filtered through a 0.45-μm Millipore filter to prevent contamination by hemocytes and bacteria.

TABLE 1

Number of progeny of SRO-infected mothers

Parent			No. of parental females	Progeny	
♀		♂	♀	♀	♂
CSNSR[a]	×	CS	29	453	0
CS	×	CS	20	549	604
ZwNSR[b]	×	CS	34	1014	0
ywZw^n	×	CS	19	561	562

[a] CS females carrying SRO.
[b] *y w Zw*^{n} females carrying SRO.

Genotype identification of cultures: Embryos were taken from the cross of homozygous *Zw*^{n} (= G6PD-null) females and CS (G6PD-positive) males; female embryos will therefore be G6PD-positive and male embryos G6PD-null. After observation and description of cell development in each culture were completed, the G6PD activity of each culture was determined histochemically. The staining procedure has been described in our previous paper (KOANA and MIYAKE 1982). Briefly, the cultured cells were frozen with dry ice and thawed. A reaction mixture containing glucose-6-phosphate and NADP was layered on the cells. After a 15-min incubation at room temperature, another reaction mixture containing nitroblue tetrazolium was added (separation of dyes from substrates made the sensitivity higher). The reaction was terminated by rinsing in hot tap water. When the cells in a culture had G6PD activity, the reduction of tetrazolium gave a deep purple precipitation of diformazan; when they were G6PD-null, no precipitate was made (see Figure 2 of KOANA and MIYAKE 1982). Almost all cell types seemed to be stained in *Zw*$^+$ cultures. However, cells without *de novo* G6PD activity could be stained, since the cell wall was partially destroyed by the freeze-thaw procedure, and G6PD is quite soluble in the reaction mixtures (KANKEL and HALL 1976).

RESULTS

Differentiation in vitro of cells from normal and SRO-infected embryos: In cultures from wild-type embryos, morphologically undifferentiated gastrula cells attached to the substratum by culture for 1 hr. By 5 hr, flat, spindle-shaped cells appeared, and they seemed to turn into macrophage-like cells. Later, several more types of cells differentiated. We have identified syncytial muscle cells, ganglion-like neuron clusters, fat body cells, three types of hemocytes and cellular spheres of imaginal disc cells (KOANA and MIYAKE 1982). Our observations are consistent with the results of several other authors (SHIELDS and SANG 1970; KURODA 1974; SHIELDS, DÜBENDORFER and SANG 1975; CROSS and SANG 1978).

Embryos were also taken from the cross between CSNSR females and CS males. Among 36 single embryo cultures, 18 showed specific morphological abnormalities. Several cell types failed to differentiate normally. The flat, spindle-shaped cells which appear first were observed only in the 18 apparently normal cultures (Figure 1a). In the 18 abnormal cultures, such cells could not be found, and a lot of (cellular) debris was seen (Figure 1b). A remarkable reduction of cell number was observed. At 24 hr of culture, the morphological aberrations in the 18 abnormal cultures became much more pronounced (Figure 1c and d). The most striking symptom in SR cultures was the remarkable paucity of the number and size of neuron clusters. A Canton-S wild-type culture contained,

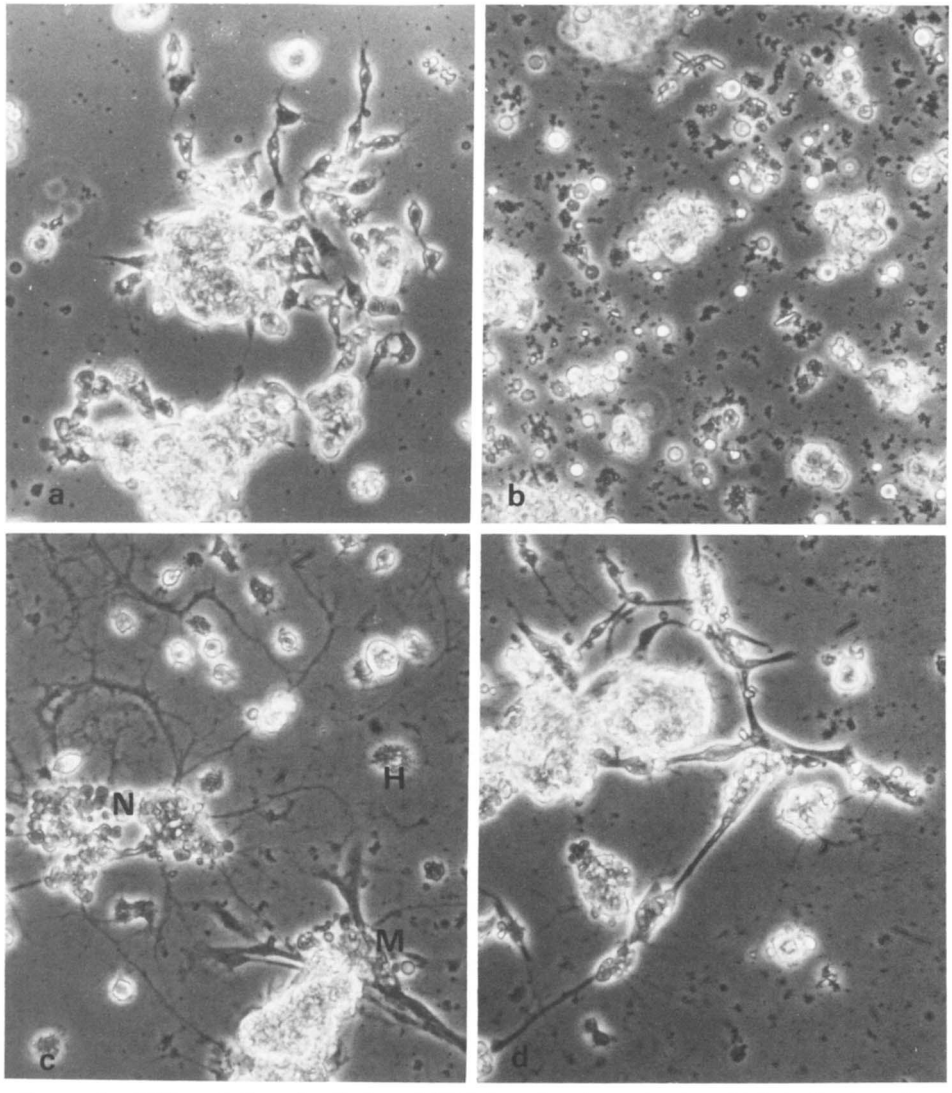

FIGURE 1.—Differentiation of cells from SRO-infected embryos *in vitro*. (a) Spindle-shaped cells in an apparently normal culture at 5 hr after the initiation of the culture. They seemed to turn into hemocytes at a later stage. (b) An abnormal culture at 5 hr. Spindle-shaped cells are rarely found, and a lot of (cellular) debris is observed. (c) A normal culture at 24 hr. Pulsating muscle cells (M), a neuron aggregate (N) with a lot of axon bundles and hemocytes (H) are observed. (d) An abnormal culture at 24 hr. Few axons and hemocytes are found. Muscle cells are functioning normally. Of 36 embryos taken from CSNSR × CS, 18 showed normal development *in vitro*, whereas the other 18 had this specific SR syndrome. Phase contrast ×310.

on an average, more than 50 ganglion-like neuron aggregates. At 48 hr after the initiation of the culture they usually consisted of tens to hundreds of closely packed neurons. A number of axons bunched together linked neighboring aggregates. Among 36 cultures made from SR embryos, the 18 apparently normal cultures had such ganglion-like structures at 48 hr (Figure 2a). The other

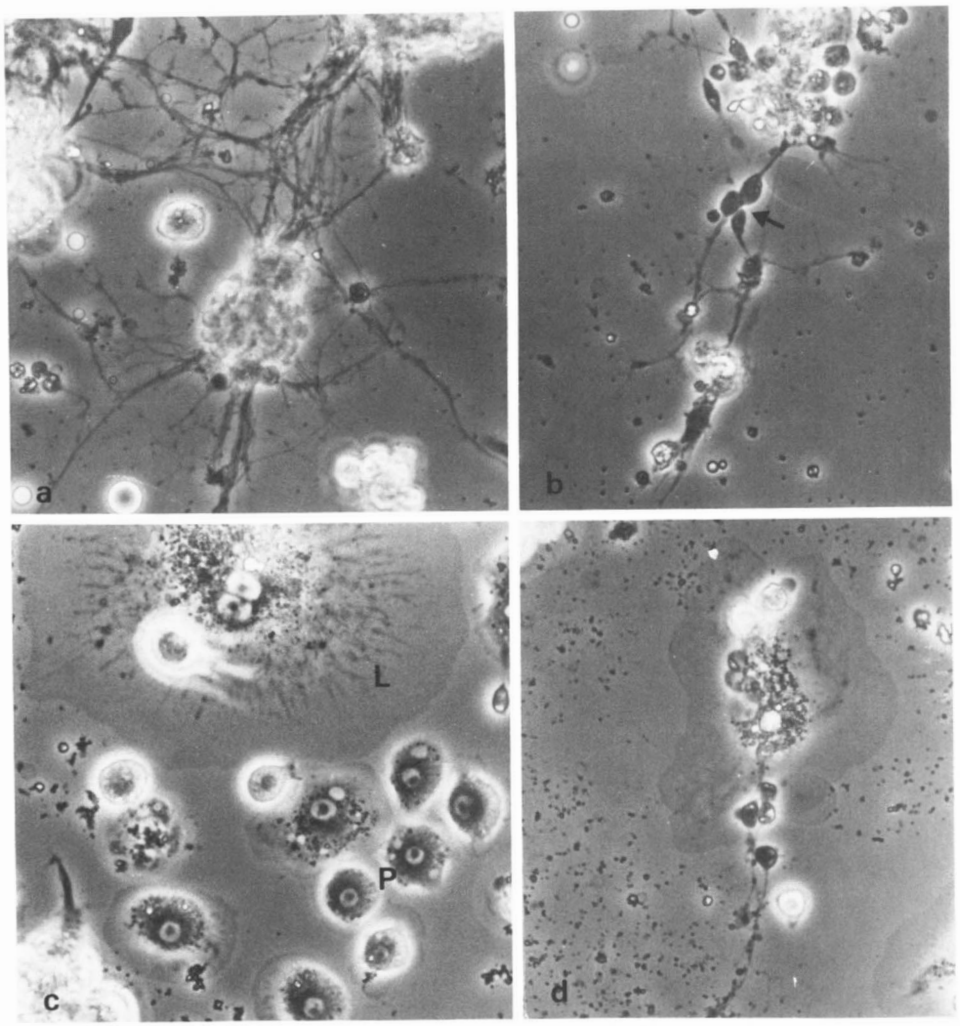

FIGURE 2.—High magnification view of the cultures from SRO-infected embryos. (a) Ganglion-like neuron aggregates in a normal culture at 24 hr. Many extended axon bundles and closely packed neurons are seen. (b) A small neuron cluster (arrow) in an abnormal culture at 24 hr. Neurons are irregularly shaped and loosely packed, and there are few axons. (c) Hemocytes in a normal culture at 7 days. A syncytial lamellocyte-like cell (L) and plasmatocyte-like (P) cells are seen. (d) An irregularly shaped lamellocyte-like cell in an abnormal culture. No plasmatocyte-like cells are seen. Phase contrast ×510.

18 cultures, on the other hand, had no such ganglion-like structures at any time of culture. There were some extremely small neuron clusters that consisted of only several neurons (Figure 2b). Individual neurons were often morphologically irregular and loosely packed. The number of axons per cluster never exceeded ten. The number of neuron clusters per culture was less than one-third that of the large ganglion-like aggregates in wild-type cultures.

In addition, roundish hemocytes (plasmatocytes) were rarely found in the 18 abnormal cultures. At a later stage of the culture, macrophage-like cells (lamel-

locytes) were sometimes observed, but they were irregularly shaped and usually syncytial (Figure 2c and d). The cellular spheres of imaginal disc cells were not found in the abnormal cultures. It is unknown whether disc cells did not differentiate or failed to be organized into the monolayer spheres. Muscle cells and fat body cells seemed not to be affected severely. Myocytes made apparently normal syncytial myotubes which pulsed quite actively (Figure 1d). The numbers of myotubes and pulsations per culture were not very much smaller than those of the 18 apparently normal cultures, although both were reduced compared with Canton-S cultures (Table 2).

Correlation between SR syndrome and sex of cultures: Half of the single embryo cultures (18 of 36) made from the cross between CSNSR females and CS males showed this specific syndrome. It is likely that cultures from male embryos showed the SR syndrome and those from female ones were normal. To test this, embryos were taken from the cross between G6PD-null ZwNSR females and G6PD-positive CS males. After observation and description had been completed, cultures were stained for G6PD activity. Table 3 shows that there is a good correlation between the SR syndrome and the sex of the embryos. Fourteen of the 15 cultures with the SR syndrome were G6PD-null; it is, therefore, inferred that they were made from male embryos. Fourteen of the 15 cultures without the SR syndrome were G6PD-positive; it is, therefore, inferred that they were made from female embryos. Two exceptions (one G6PD-positive culture with mild SR syndrome and one G6PD-null culture showing normal development) might suggest that female embryos can be affected occasionally to the same extent as male ones by SRO, and that male embryos can escape *in vitro*, although they cannot *in vivo* (Table 1). However, the possibility that these rare exceptions were also exceptional in their chromosomes—resulting from X-chromosome nondisjunction—cannot be eliminated.

Differentiation of cells from non-SR embryos in the SRO-containing culture medium: In addition to culturing cells from SRO-infected embryos, we also cultured cells from non-SR embryos in the medium containing SRO. Among 40 cultures made from the cross between non-SR *y w* Zw^n females and CS males, 14 G6PD-null (male) cultures and two G6PD-positive (female) ones showed typical SR syndrome (Table 4). Neurons and cellular spheres of imaginal disc cells were rarely seen. Hemocytes were also affected severely, whereas muscle and fat body cells seemed relatively well differentiated. A large amount of (cellular) debris was again observed.

Since SRO diluted in the culture medium still actively affects male (and some female) embryonic cells, a preliminary characterization of SRO was done. SRO medium lost its activity when it was filtered through a 0.05-μm Millipore filter instead of a 0.45-μm one (*cf.* POULSON and SAKAGUCHI 1961). None among 18 cultures showed the SR syndrome. UV irradiation for 10 sec (by 15-W UV lamp from 30 cm distance) did not completely inactivate SRO (four among 19 cultures showed slight SR syndrome). SRO lost activity completely when UV-irradiated for 10 min (none among 22 cultures showed the SR syndrome).

DISCUSSION

To understand the entire range of the SR phenomenon, analysis at the cellular level is necessary. This is difficult to do *in vivo* because of complex interactions

TABLE 2

SR syndrome in vitro

Type of element	Average no. per culture		
	CSNSR × CS		
	SR syndrome (18 cultures)	Normal (18 cultures)	CS × CS (16 cultures)
Ganglion-like aggregates	17.6[a]	59.8	53.7
Cellular spheres	0	1.0	1.9
Myotubes	16.3	26.0	44.1
Pulsating centers	3.8	10.0	16.9

[a] These were extremely small neuron clusters rather than normal-sized aggregates.

TABLE 3

Correlation between SR syndrome and genotype of the culture

Parents' genotype	G6PD activity[a]	No. of cultures	
		Normal	SR syndrome
ZwNSR[b] × CS	+ (female)	14	1
	− (male)	1	14
y w Zw^n × CS	+	12	0
	−	11	0

[a] G6PD activity present (+) or absent (−).
[b] *y w* Zw^n females carrying SRO.

TABLE 4

Cell differentiation in SRO-containing medium

Parents' genotype	G6PD activity[a]	No. of cultures	
		Normal	SR syndrome
y w Zw^n × CS	+	19	2
(medium containing CSNSR hemolymph)	−	5	14
y w Zw^n × CS	+	5	0
(medium containing CS hemolymph)	−	5	0

[a] G6PD activity present (+) or absent (−).

between host, SRO and the environment (COUNCE and POULSON 1966); in *D. willistoni* death and degeneration usually occur before gastrulation (COUNCE and POULSON 1962) so the possibility of differential cellular effects cannot be addressed. Although in *D. melanogaster*, which is less severely affected by SRO than *D. willistoni*, morphological abnormality in the central nervous system was occasionally found (S. TSUCHIYAMA-OHMURA, personal communication), the lethal phase of male embryos is broad; many embryos die before gastrulation, whereas others survive until just before hatching. It is, therefore, not

possible to determine whether SRO has cell type-specific effects in intact organisms. However, we have found that dissociated gastrula cells from non-SRO infected *D. melanogaster* embryos show considerable differentiation in culture and that affected SRO-infected embryos consistently show a specific array of defects (the SR "syndrome").

COUNCE and POULSON (1962) found that about two-thirds of the embryos from SR mothers had morphological abnormalities; therefore, they suggested that some of the "late-dying" embryos were female. Our results also indicate that female embryos are affected by SRO. One G6PD-positive (presumably female) culture from Table 3 and two from Table 4 had the SR syndrome. Table 2 shows that, even in "normal" cultures from embryos from CSNSR × CS, the average number of myotubes and pulsating centers was reduced relative to cultures from noninfected CS embryos. These observations suggest that, at a cellular level, most of the female embryos are affected by SRO to some extent and that a small fraction of them is affected as severely as male embryos.

Our results also indicate that the sensitivity to SRO is cell type specific. Even in male cultures, there are differences in SRO sensitivity between cell types; neurons are destroyed almost completely, whereas muscle cells and fat body cells are less severely affected. TSUCHIYAMA, SAKAGUCHI and OISHI (1978) showed, by mosaic analysis, that the primary target site of SRO was in the region including the primordial mesoderm and the nervous tissue. The results in this current work suggest that the major target tissue of SRO is most likely the nervous tissue. Muscle cells could be a minor target of SRO or could be affected as a secondary result of other cell death. Imaginal disc cells were also affected, which is consistent with the mosaic data obtained by TSUCHIYAMA, SAKAGUCHI and OISHI (1978). They found that mosaic progeny from SRO-infected mothers usually had much smaller male external body parts than the mosaics from non-SR control mothers. This suggested that SRO also affected imaginal disc cells, at least as a minor target site. We also found that hemocytes were affected rather severely. As the origin of hemocytes is still not fully established (POULSON 1950; FULLILOVE, JACOBSON and TURNER 1978), we cannot discuss whether our observation is consistent or inconsistent with the results of mosaic analysis.

An important problem still unsolved is why male embryos are more sensitive to SRO effects than females and also why cell types differ. At the embryo level, two X chromosomes confer resistance to SR killing, whereas single X individuals are sensitive. At the cellular level, we found that syncytial (myotube) and polyploid (fat body) male cells were more resistant than diploid cells (imaginal disc, neurons, etc.). The parallel between number of X chromosomes per cell and SRO effects at both the embryo and at the cellular level is striking and suggests the hypothesis that SRO sensitivity in *D. melanogaster* may reflect absolute number of X chromosome per cell, rather than X:*A* ratio. The hypothesis can be tested by examining the SRO sensitivity of established cell lines. The hypothesis predicts that cell lines that were originally XY but which have become aneupolid (XXY) or polyploid should be resistant to SRO, whereas lines that were originally XX but which have become aneuploid (*XO*) or haploid

should be sensitive. Moreover, the basic hypothesis predicts that mutants that affect dosage compensation should have no effect on SRO sensitivity.

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