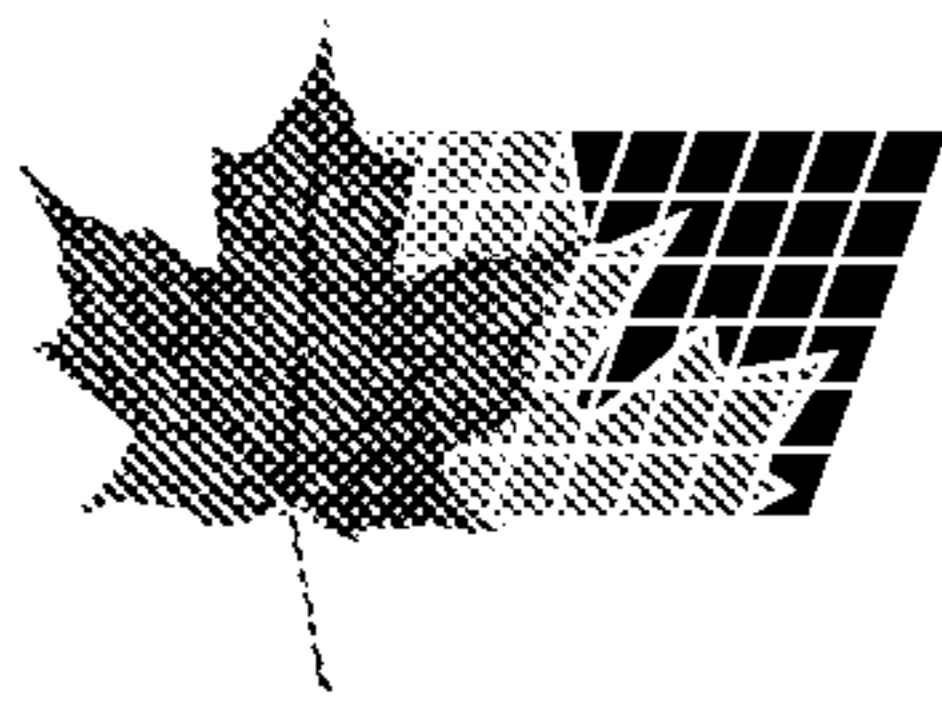




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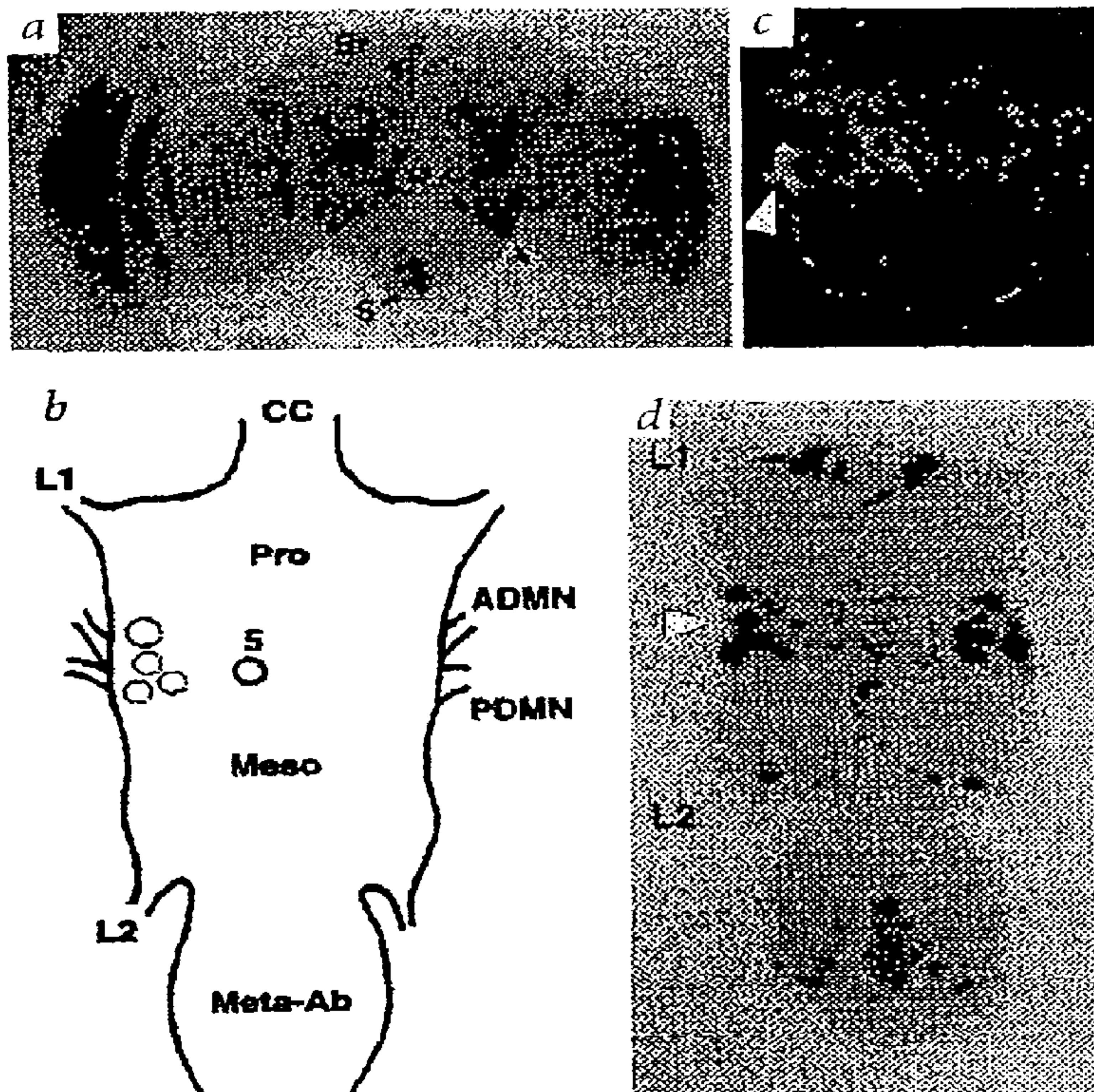
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(54) **PROLONGATION DE LA DUREE DE VIE PAR
SUREXPRESSION D'UN GENE QUI AUGMENTE LE
METABOLISME D'OXYGENE REACTIF**

(54) **EXTENSION OF LIFESPAN BY OVEREXPRESSION OF A GENE
THAT INCREASES REACTIVE OXYGEN METABOLISM**



Title: Extension of Lifespan by Overexpression of a Gene that Increases Reactive Oxygen Metabolism

BACKGROUND OF THE INVENTION

Reactive oxygen (RO) has been identified as an important effector in aging and lifespan determination¹⁻³. However, the specific cell types in which oxidative damage acts to limit lifespan of the whole organism have not been explicitly identified.

SUMMARY OF THE INVENTION

We show that the association between mutations in the oxygen radical metabolizing enzyme CuZn superoxide dismutase (SOD) and loss of motoneurons in the brain and spinal cord that occurs in the life-shortening paralytic disease, Familial Amyotrophic Lateral Sclerosis (FALS)⁴, cause chronic and unrepaired oxidative damage occurring specifically in motoneurons which is a critical causative factor in aging. To prove this hypothesis, we generated transgenic *Drosophila* with expression of human SOD targeted to adult motoneurons. Here we show that overexpression of a single gene, SOD, specifically in a single cell type, the motoneurons, extends normal lifespan by up to 40% and restores the lifespan of a short-lived SOD-null mutant. Elevated resistance to oxidative stress suggests that the lifespan extension observed in these flies is mediated by enhanced RO metabolism. These results show that SOD activity in motoneurons is an important factor in aging and lifespan determination in *Drosophila*.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. GAL4-activated expression of HS in motoneurons. Whole mounts of adult brain and ventral ganglia hybridized *in situ* with a full length dioxygenin-labeled human SOD (HS) cDNA. Tissues were examined from transgenic flies bearing one copy each of HS1 and D42-GAL4 (HS1/+; GAL4/+). (a) Transgenic HS expression was detected primarily in the central brain (Br), lateral margins adjacent to the lobula/lubula plate (arrowheads), and suboesophageal ganglia (S). No expression was detected in the optic lobes (OL) or retina (R). (b) A schematic of the ventral ganglia depicting the location of four ganglionic regions: prothoracic (Pro), mesothoracic (Meso), and combined metathoracic and abdominal ganglia (Meta–Ab). Peripheral nerves which act as landmarks are also shown, (ADMN, PDMN; L1 and L2). Four of the five identifiable flight motoneurons (red circles) are ventrally located, the fifth is located dorsally. (c) The expression of the D42-GAL4 line was determined by immunofluorescence after crossing to flies containing a UAS-GFP transgene. Illustrated is the result of a z-series of confocal images through the ventral ganglia. The location of four of the large flight muscle motoneurons is indicated by an arrowhead. (d) Expression of HS can be detected within flight muscle motoneurons 1-4 (*) as well as other motoneurons distributed at various locations within the ventral ganglia. Scale bar for (a) = 200 microns and for (b) = 100 microns.

Fig. 2. Detection of transgenic HS protein. (A) Immunoblot analysis of adult extracts using an antibody to human SOD. The arrow indicates a 21 kD immunoreactive protein corresponding to human SOD (a) HS1/+; GAL4/+; (b) HS2/+; GAL4/+; (c) HS1/+; +/+; (d) Authentic purified human SOD (0.025 g). The strains in lanes a-c were also homozygous for the *Drosophila* SOD^{+} genes. (B) Assay of SOD activity in nondenaturing polyacrylamide gels (6)). Residual Mn SOD activity is indicated by (*). (a) HS2/HS2; +/+; (b) HS2/HS2; GAL4/GAL4; (c) HS1/HS1; +/+; (d) HS1/HS1; GAL4/GAL4; (e) +/+; GAL4/GAL4; (f) wild type, Oregon R strain. The strains in lanes a-e are homozygous for the SOD^{x39} mutation that precludes formation of indigenous *Drosophila* SOD. SOD specific activity (Units/mg protein) was also determined for each strain (30): (a) HS2/HS2; +/+; 0.1 ± 0.8 ; (b) HS2/HS2; GAL4/GAL4; 6.2 ± 0.8 ; (c) HS1/HS1; +/+; 0; (d) HS1/HS1; GAL4/GAL4; 7.5 ± 2.6 ; (e) +/+; GAL4/GAL4; 0. Values represent the mean $\pm$ SEM of 12 determinations (3 different extracts each assayed 4 times) after correction for residual Mn SOD activity. The "0" values are 1 SEM.

Fig. 3. Extension of normal adult lifespan by selective augmentation of HS in motoneurons.

Adult SOD^{+} males (0-24 hrs old) bearing a single copy of HS1 (A) or HS2 (B) and either one or no copies of D42-GAL4 were maintained at 25°C in shell vials (10 flies per vial) containing standard cornmeal agar medium. The starting population size for each genotype was 250. Flies were transferred to fresh medium and scored for survivorship every two days. The mean (50% mortality) and maximum (90% mortality) lifespan for each genotype is as follows: HS1/+; +/+ (mean = 45.1 ± 3.4 ; max. = 56.3 ± 3.6); HS1/+; D42GAL4/+ (mean = 63.7 ± 4.3 ; max. = 73.2 ± 3.4); HS2/+; +/+ (mean = 52.2 ± 1.8 ; max. = 58.8 ± 1.5); HS2/+; D42GAL4/+ (mean = 60.6 ± 2.2 ; max. = 71.0 ± 2.7). The lifespan of the D42-GAL4/D42-GAL4; +/+ control is very similar to the HS/+; +/+ strains. In addition, expression of HS under the transcriptional control of other GAL4 drivers, including a heatshock-GAL4 driver which drives expression broadly at all stages of development and an elav-GAL4 driver which drives expression at high levels in embryonic and larval neurons, did not extend lifespan (data not shown).

Fig. 4. Restoration of adult lifespan in an SOD-null mutant by auxiliary expression of HS in motoneurons. (A) Adult males (0-48 hrs old) homozygous for the SOD-null mutation SOD^{x39} and also bearing different combinations of HS and D42-GAL4 transgenes were maintained at 25°C in shell vials (10 flies per vial) containing standard cornmeal agar medium. The starting population size for each genotype was 50 flies. Flies were scored daily for survivorship and transferred to fresh vials every two days. (B) Gene dosage effects on restoration of adult

lifespan. Adult males (0-48 hrs old) homozygous for the SOD-null mutation SOD^{x39} and also bearing one or two copies each of HS1 and the D42-GAL4 activator were constructed and lifespan studies were conducted as in (A). The starting population sizes were 180, 335 and 70 for the 0 dose, 1 dose and 2 dose genotypes, respectively. The zero dose control bears 2 copies of HS1 but no D42-GAL4 activator. The data presented are representative of at least two separate experiments.

Fig. 5. Motorneuronal SOD and lifespan in *Drosophila*. The lifespans of the SOD genotypes used in this study in relation to wildtype.

Fig. 6. Resistance to oxidative stress of a SOD-null mutant conferred by expression of HS in motorneurons. (A) Resistance to paraquat. Adult males (0-48 hrs old) homozygous for the SOD-null mutation SOD^{x39} and also bearing different combinations of UAS-HS and D42-GAL4 transgenes were maintained at 25°C in shell vials containing filter pads saturated with aqueous paraquat and scored for survivorship after 24 hours. Each point represents 50 flies (5 vials of 10 flies each). (B) Resistance to ionizing radiation. Adult males (24-48 hrs old) homozygous for the SOD-null mutation SOD^{x39} and also bearing different combinations of UAS-HS and D42-GAL4 transgenes were exposed to 100 kRad γ -radiation (190 min at ~ 520 Rads/min in a cobalt⁶⁰ source) and then maintained at 25°C in shell vials containing standard cornmeal agar medium and scored daily for survivorship. The data are representative of at least two separate experiments.

DETAILED DESCRIPTION OF THE INVENTION

Expression of a human SOD transgene (HS) in *Drosophila* motorneurons was achieved using the yeast GAL4/UAS system⁵⁻⁷. The D42-GAL4 activator used in these experiments is expressed broadly during embryogenesis but becomes restricted to motorneurons and interneurons within the larval nervous system and with the exception of a few unidentified neurons within the central brain, is restricted to motorneurons in the adult nervous system. The HS transgene consists of a human SOD cDNA coupled to a yeast UAS element within a *Drosophila* P transformation vector. Two independent UAS-HS transgenic lines, designated HS1 and HS2, were used in these experiments. Because lifespan is strongly affected by variation in genetic background, a series of genetic schemes was employed to introduce the D42-GAL4 and UAS-HS transgenes into uniform SOD^{+} or SOD^{-} genetic backgrounds and to construct expressing and non-expressing strains that were essentially co-isogenic.

In situ hybridization shows that expression of the HS transgene is limited to adult

motorneurons including a set of five bilaterally symmetrical motorneurons which control flight muscles in *Drosophila* (Fig. 1). Similar results were obtained for whole mount preparations of *Drosophila* larvae (data not shown). To confirm that functional human SOD proteins were expressed in these cells, whole fly extracts were analyzed by immunoblot and SOD activity assays (Fig. 2). Using an antibody to HS that does not cross-react with *Drosophila* SOD, substantial HS protein was detected in flies arising from a cross between the D42-GAL4 and the UAS-HS lines, while no HS protein was detected in the D42-GAL4 line nor in either of the 2 UAS-HS lines. Assay of SOD activity in GAL4-UASHS transgenic flies that are also homozygous for a SOD-null mutation demonstrates that the transgenic HS is enzymatically active.

To determine the consequences of SOD overexpression in motorneurons on normal longevity, we genetically introduced the D42-GAL4 and UAS-HS transgenes into flies with a normal SOD^+ genetic background. Previous studies in which SOD levels had been increased broadly throughout many tissues showed little to no effect on adult lifespan in *Drosophila*⁸⁻¹⁰ unless combined with a similar increase in catalase¹¹. In contrast, we find that if SOD overexpression is targeted selectively to motorneurons it causes a dramatic extension of lifespan (Fig. 3). Transgenic HS1 flies overexpressing SOD in motorneurons exhibit mean and maximum adult lifespans up to 40% longer than non-expressing isogenic controls. The most striking feature of the postponed mortality in these flies is the extension of the premortality plateau phase of the life curve (<5% mortality) from approximately 27 days to 50 days. That is, selectively enhanced expression of SOD in motorneurons nearly doubles the time before the onset of significant mortality. The HS2 strain, which exhibits SOD activities without and with GAL4 activation of 1.3% and 83%, respectively, of the level of activity in activated strain HS1, confirms the relationship between the level of motorneuron-enhanced SOD activity, postponed senescence and lifespan extension. These results demonstrate that enhancing SOD activity in motorneurons can markedly postpone the age-dependent onset of senescent mortality in *Drosophila*. We conclude from these results that RO metabolism specifically in motorneurons is a critical factor in senescence and lifespan determination in *Drosophila*.

One of the characteristics of SOD-null mutants of *Drosophila* is the foreshortening of the adult lifespan by 85-95%. Complete rescue of normal lifespan in SOD-null mutants is afforded by a genomic *Drosophila* SOD transgene that expresses SOD in the normal pattern throughout the body¹². Our results, above, predict that lifespan of a SOD-null mutant would also be substantially restored if SOD were selectively expressed just in motorneurons. To test this prediction, the D42-GAL4 and HS transgenes were introduced into flies with a SOD-null mutant genetic background and lifespans were determined. The SOD mutation used in these experiments, SOD^{x39} is an internal deletion that precludes synthesis of SOD protein¹⁴. As can

been seen (**Fig.4**), selective expression of SOD in motoneurons restores the lifespan of SOD-null mutant flies in a clearly dose-dependent manner. Flies bearing one or two copies each of the D42-GAL4 and UAS-HS transgenes exhibit mean adult lifespans of approximately 10 and 36 days, respectively, compared to the 3 day mean lifespan of the isogenic SOD-null mutant control. This represents a restoration of the lifespan from 5% (exhibited by the SOD-null mutant) to greater than 60% of the isogenic SOD⁺ control. The full range of lifespans produced in this study by controlling the expression of SOD in motoneurons of flies with SOD-null and SOD⁺ genetic backgrounds is summarized in **Figure 5**.

That restoration of lifespan is not complete shows that there is a requirement for SOD and RO metabolism in other tissues. However, we have examined the effects of enhanced SOD expression in several other tissues during development and not observed any significant ability to enhance lifespan. (data not shown). Whether increasing the levels of SOD in motoneurons or providing additional factors such as catalase would further increase the rescue of SOD mutants and the lifespan of wildtype flies remains to be determined. Nonetheless, the impressive rescue achieved by overexpression of SOD in motoneurons implies that motoneuron dysfunction arising from the lack of SOD is the principal cause of the reduced lifespan of SOD-null mutants.

To determine if the mechanism of lifespan extension involves the catalytic activity of SOD and enhanced superoxide metabolism in motoneurons, we oxidatively stressed flies bearing combinations of UAS-HS1 and D42-GAL4 transgenes by exposure to the RO-generating agents paraquat and ionizing radiation (**Figure 6**). Expression of HS in motoneurons provided significant resistance to both challenges. This strengthens the view that the mechanism underlying extended lifespan in these flies involves elevated RO metabolism in motoneurons.

To determine if the observed extension of lifespan in our transgenic HS lines could be attributed to lower metabolic rates we measured respiration rates in flies which expressed HS in motoneurons as compared to controls (**Table 1**). The results clearly demonstrate that activation of the HS transgene in motoneurons does not depress metabolic rate, effectively excluding this as a possible mechanism for the lifespan extension seen in these experiments.

The results of this study show an important refinement of the free radical (oxidative damage) hypothesis of aging^{1, 2}, namely, that lifespan is determined by RO metabolism in a small number of critical cell types that includes motoneurons. The same genetic strategy used in this study can now be applied to identify other factors and cell types that are critical in lifespan determination and to define the aspects of RO metabolism in these cells that impose such lifespan-limiting effects on the whole organism.

The gradual diminution of motor function is one of the hallmarks of aging in animals and has significant ramifications in gerontology. Moreover, the sensitivity of motoneurons to

oxidative impairment is well documented in both vertebrates and invertebrates. Our ability to substantially postpone senescent mortality and to alleviate mutant symptoms arising from impaired RO metabolism by antioxidant intervention in motoneurons in *Drosophila* suggests a possible strategy for reducing the morbidity of normal senescence in other animals, including humans.

This invention relates to a method of reducing reactive oxygen (RO) damage by expressing therapeutic genes or other genes of interest in cells. The cells transformed with the gene that increases RO metabolism are preferably nervous system cells. In a more preferred embodiment, the cells are motoneurons. In the most preferred embodiment, the cells are human motoneurons.

The invention includes nucleotide modifications of the gene sequences disclosed in this application (or fragments thereof) that are capable of producing proteins that increase RO metabolism in cells. The SOD gene from any organism may be used to increase RO metabolism. In a preferred embodiment, the gene is the SOD gene from a mammal. In the most preferred embodiment, the gene is the human SOD gene. The SOD gene sequence (and the sequences of other genes encoding proteins that increase RO metabolism) may be modified using techniques known in the art. Modifications include substitution, insertion or deletion of nucleotides or altering the relative positions or order of nucleotides. The invention includes DNA which has a sequence with sufficient identity to a nucleotide sequence described in this application to hybridize under stringent hybridization conditions (hybridization techniques are well known in the art). The genes that may be used to increase RO metabolism of the invention also include genes (or a fragment thereof) with nucleotide sequences having at least 70% identity, at least 80% identity, at least 90% identity, at least 95% identity, at least 96% identity, at least 97% identity, at least 98% identity or, most preferred, at least 99% identity to the gene sequences of the invention, such as SOD. Other genes may also be used to increase RO metabolism. These include SOD2, SOD3, catalase gene, glutathione peroxidase, genes involved in metabolism in a broader sense by radical scavenging, (for example synthesis of uric acid), genes for synthesis or metabolism of vitamin E, or fragments thereof. Identity refers to the similarity of two nucleotide sequences that are aligned so that the highest order match is obtained. Identity is calculated according to methods known in the art. For example, if a nucleotide sequence (called "Sequence A") has 90% identity to a portion of the SOD gene, then Sequence A will be identical to the referenced portion of the SOD gene except that Sequence A may include up to 10 point mutations (such as deletions or substitutions with other nucleotides) per each 100 nucleotides of the referenced portion of the SOD gene.

The DNA sequences of the invention (regulatory element sequences and therapeutic gene sequences) may be obtained from a cDNA library, for example using expressed sequence tag analysis. The nucleotide molecules can also be obtained from other sources known in the art such as genomic DNA libraries or synthesis.

The genes are preferably expressed by vectors containing DNA regulatory elements that direct the expression of the genes for use in research, protein production and gene therapy in cells and tissues (preferably human cells and tissues). Viral vectors may be used, for example, adenovirus vectors. For example, in addition to the expression vector described in the examples above, the promoter for neuronal enolase gene or the promoter that specifies neurofilaments or VAMP1 (synaptobrevin) may be used to express genes. Other regulatory elements from other mammals, yeast, bacteria, viruses, birds or insects may also be used to express the genes. The specific regulatory elements chosen for a particular vector may vary depending on factors such as the level of activity of the cassette desired or the characteristics of the gene to be expressed. One skilled in the art can modify the sequences of the regulatory elements and the gene to be expressed using techniques disclosed in this application and known in the art.

The gene may be expressed to prevent RO damage *in vivo* or *in vitro*. Cells transformed *in vitro* can be used as a research tool. The gene is also useful for gene therapy by transforming cells *in vivo* to express a therapeutic protein that increases RO metabolism. Gene therapy may be used to treat diseases, disorders and abnormal physical states such Amyotrophic Lateral Sclerosis, Huntington's disease, Parkinson's disease and Alzheimer's disease and any other disease, disorder or abnormal physical state of the motoneurons induced by reactive oxygen.

The SOD protein is one therapeutic protein which may be expressed *in vivo* or *in vitro* to increase RO metabolism. Other proteins may also be used to increase RO metabolism. These include the proteins produced by SOD2, SOD3, catalase gene, glutathione peroxidase, genes involved in metabolism in a broader sense by radical scavenging, (for example synthesis of uric acid), genes for synthesis or metabolism of vitamin E, or fragments thereof. Changes in the nucleotide sequence which result in production of a chemically equivalent (for example, as a result of redundancy of the genetic code) or chemically similar amino acid (for example where sequence similarity is present), may also be used as therapeutic proteins with the expression cassettes of the invention.

Pharmaceutical compositions used to treat patients having diseases, disorders or abnormal physical states include a gene that encodes a protein that increases RO metabolism and an acceptable vehicle or excipient (Remington's Pharmaceutical Sciences 18th ed, (1990, Mack Publishing Company) and subsequent editions). Vehicles include saline and D5W (5% dextrose and water). Excipients include additives such as a buffer, solubilizer, suspending agent, emulsifying agent, viscosity controlling agent, flavor, lactose filler, antioxidant, preservative or dye. There are preferred excipients for stabilizing peptides for parenteral and other administration. The excipients include serum albumin, glutamic or aspartic acid, phospholipids and fatty acids. The protein may be formulated in solid or semisolid form, for example pills, tablets, creams, ointments, powders, emulsions, gelatin capsules, capsules,

suppositories, gels or membranes. Routes of administration include oral, topical, rectal, parenteral (injectable), local, inhalant and epidural administration. The compositions of the invention may also be conjugated to transport molecules to facilitate transport of the molecules. The methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients are known in the art.

The pharmaceutical compositions can be administered to humans or animals. Dosages to be administered depend on individual patient condition, indication of the drug, physical and chemical stability of the drug, toxicity, the desired effect and on the chosen route of administration (Robert Rakel, ed., Conn's Current Therapy (1995, W.B. Saunders Company, USA)). The pharmaceutical compositions are used to treat diseases, disorders and abnormal physical states described above in this application.

The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. All such modifications are intended to be included within the scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Methods

Transgenic strains. The UAS-HS transgene was constructed by inserting an HS cDNA in the polycloning site behind the UAS element of the P expression vector, pUAST, that contains a *miniwhite*⁺ reporter gene. Transformants were made using *white*¹ recipients and standard P transformation methodology¹³. The D42-GAL4 strain carries a 3rd chromosome GAL4 enhancer trap that selectively expresses the GAL4 transcriptional activator in adult motoneurons⁷.

Genetic constructions. To generate flies with D42-GAL4-activated expression of 2nd chromosome P(*w*⁺)UAS-HS transgenes and appropriate non-activated controls in an isogenic *Drosophila* SOD⁺ background, homozygous *w*¹;P(*w*⁺)UAS-HS; + females were mated in parallel to *w*¹ recipient strain males and to homozygous *w*¹;+;P(*w*⁺)D42-GAL4 males. *w*¹;P(*w*⁺)UAS-HS/+;P(*w*⁺)D42-GAL4/+ males, carrying one copy of each of the transgenes and therefore showing D42-activated SOD expression, could then be compared directly to *w*¹;P(*w*⁺)UAS-HS/+;+/+ males lacking expression.

To generate flies homozygous for both a UAS-HS transgene and the D42-GAL4 activator in a SOD-null mutant background, it was necessary to construct a recombinant chromosome carrying both the $P(w^+)$ D42-GAL4 insert and the SOD^{x39} mutation because both of these genetic elements reside on chromosome III. The D42R20 ($D42-GAL4^-$, SOD^{x39}) and D42R40 ($D42-GAL4^+$, SOD^{x39}) third chromosomes were constructed in parallel through a crossing scheme involving recombination between chromosomes carrying the $P(w^+)$ D42-GAL4 insert and the SOD^{x39} mutation, the latter chromosome marked with the recessive eye colour marker, *red*. Based on the relative frequencies of each progeny class, the D42R20 and D42R40 chromosomes could be inferred to differ by at most the ~26 cM segment of chromosome III between the *SOD* and *red* loci, or by as little as the $P(w^+)$ D42-GAL4 insert itself. A crossing scheme employing a w^1 stock carrying both 2nd and 3rd chromosome balancers was devised for construction of GAL4-UAS doubly balanced stocks to minimize variation in genetic background between stocks. Based on this crossing scheme, each of the four resulting GAL4-UAS stocks will carry w^1 chromosomes from identical sources, each of which derive ultimately from the X chromosome of the original w^1 recipient strain. The second chromosomes of each stock, having been balanced throughout, will also directly derive from the w^1 recipient strain, and will differ only in the position of the $P(w^+)$ UAS-HS inserts. Apart from the presence or absence of $P(w^+)$ D42-GAL4, the recombinant third chromosomes of each stock will also be very similar, as described above. Thus, the experimental GAL4-UAS-HS stocks and their controls can be considered as virtually isogenic for most of the genome, with minimal differences in the genetic background between strains. This allows us to attribute phenotypic characteristics specifically to the GAL4-activated HS expression without concern for phenotypic effects arising from other differences in genetic background.

To obtain expression of a single copy of a UAS-HS transgene activated by a single copy of the D42-GAL4 activator in a SOD-null mutant background, doubly balanced stocks carrying a UAS-HS transgene on the second chromosome and the SOD-null allele, SOD^{x39} , on the third chromosome were constructed by standard genetic techniques. Virgin females of the genotype w^1/w^1 ;UAS-HS/UAS-HS; $SOD^{x39}/TM3$ were collected and mated in parallel to w^1 ;D42R40($D42-GAL4$, SOD^{x39})/TM3 males and to w^1 ;D42R20($D42-GAL4^-$, SOD^{x39})/TM3 males. Male progeny of the genotype w ;UAS-HS/+;D42R40/ SOD^{x39} , carrying single copies of both the $P(w^+)$ UAS-HS transgene and the $P(w^+)$ D42-GAL4 activator, were collected for direct comparison to male sibs of the genotype w^1 ;UAS-HS/+;D42R20/ SOD^{x39} , carrying one copy of

the $P(w^+)$ UAS-HS transgene but no $P(w^+)$ D42-GAL4 activator, in a SOD-null mutant genetic background that is co-isogenic for most of the genome. It is important to note here that the SOD^{x39} allele used to make the SOD-null background contains a 365 bp deletion that includes the both the transcription and translation start sites and therefore makes neither a transcript nor a protein product¹⁴. Thus, the only SOD protein in these flies is that specified by the UAS-HS transgene. This prevents any possible interference by indigenous *Drosophila* SOD subunits in the formation of homodimeric HS.

Motorneuron specificity of D42-GAL4. Expression of the D42-GAL4 activator was determined by crossing the D42-GAL4 line to a UAS-GFP (green fluorescent protein) line followed by fluorescence microscopy, or to a UAS-lacZ line followed by immunocytochemistry using an anti- β -galactosidase antibody. The pattern of D42-GAL4 in embryos is described elsewhere⁷. In larvae, D42-GAL4 is expressed in motorneurons, interneurons and some peripheral glial cells. Low levels of expression were also detected in the fat body. In the adult, D42-GAL4 expression is restricted to a small number of cells within the central brain and to motorneurons within the ventral ganglia.

SOD assay. SOD activity was assayed qualitatively following electrophoresis in nondenaturing polyacrylamide gels¹⁵. For quantitative assay of SOD activity, extracts were made by homogenizing 20 adult males (24 hr old and previously frozen at -80°C) in 200 ml of 0.05 M sodium phosphate pH 7.4, 0.1 mM EDTA. After centrifugation at 13,000 g for 5 min at 4°C , the supernatants were partially deproteinized by treatment with chloroform/ethanol¹⁶ and assayed for CuZn SOD activity by the 6-hydroxydopamine autoxidation method¹⁷. Proteins were determined using the Bio-Rad Protein Assay Kit.

Lifespan Determination. For statistical analysis, the mean and maximum (90%) lifespan of each strain was calculated from the time (in days) at which survival reached 50% and 10% of the starting population in each of the 25 cohorts of each strain. The means and variances of these estimates were calculated, and used to establish a 99% confidence interval for mean and maximum lifespan values. The values calculated are as follows:

HS1/+; +/+.....mean 45.1 +/- 3.4.....max 56.3 +/- 3.6

HS1/+;D42/+.....mean 63.7 +/- 4.3.....max 73.2 +/- 3.4

HS2/+; +/+.....mean 52.2 +/- 1.8.....max 58.8 +/- 1.5

HS2/+;D42/+.....mean 60.6 +/- 2.2.....max 71.0 +/- 2.7

Oxidative Stress. Adult flies were challenged with paraquat and ionizing radiation as described

in Parkes et al¹².

Oxygen Consumption. Oxygen consumption was measured with a Gilson Single-Valve Differential Respirometer using standard methods (Umbreit, 1964). Twenty-five flies were weighed, placed in the respirometer and left undisturbed for 2 hours at 25°C. The rate of change of volume was expressed as uL of dry O₂ consumed per mg of wet weight per hour at standard temperature and pressure (STP). Rates were examined for differences with 1-way ANOVAs, and means were compared using the SNK test.

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Key elements of Drosophila work:

Upgrading of reactive oxygen metabolism in motoneurons has major impact on extended lifespan

The extended lifespan is characterized by decreased morbidity; that is, during the period of extended life, the organism is characterized by vigour and activity and robust behaviour typical of youthfulness. The period of senescent decline in these animals is of normal duration, and thus occupies a proportionately smaller percent of the total lifespan than normal.

Predictions from our Drosophila work regarding potential applications in humans:

1. Our work indicates that motoneurons should be the focus of interventions via reactive oxygen metabolism to reduce the morbidity of normal aging and neurodegenerative disease.

Commercial Utility:

- Research tool

- Gene therapy

- further possibilities (projects underway) include targetting other cell types such as other neurons, and skeletal muscle. Also other genes in reactive oxygen metabolism such as catalase, glutathione peroxidase, genes in urate synthesis, glutathione synthesis and vitamin E synthesis and transport.

We claim:

1. A method of increasing reactive oxygen metabolism in a cell, comprising inserting in the cell a gene that encodes a protein that increases reactive oxygen metabolism and expressing the gene.
2. The method of claim 1, wherein increasing reactive oxygen metabolism prolongs the life of the cell.
3. The method of claim 1, wherein the gene comprises SOD.
4. The method of claim 1, wherein the gene comprises a gene selected from the group consisting of a gene having at least 70% sequence identity with SOD and encoding a protein having SOD activity, and a gene encoding a protein having SOD activity.
5. The method of claim 1, wherein the gene comprises a gene selected from the group consisting of SOD2, SOD3, catalase gene, glutathione peroxidase, radical scavenging genes, genes for synthesis or metabolism of vitamin E, and fragments of the aforementioned genes.
6. The method of claim 1, wherein the cell comprises a motor neuron.
7. The method of claim 1, wherein the cell is affected by a disease, disorder or abnormal physical state selected from a group consisting of Amyotrophic Lateral Sclerosis, Huntington's disease, Parkinson's disease and Alzheimer's disease and a disease, disorder or abnormal physical state of the motoneurons induced by reactive oxygen.
8. A method of preventing disease, disorder or abnormal physical state in a patient or prolonging the life of a patient, comprising:
 - administering to the patient an amount of a gene that increases reactive oxygen metabolism so that the expression cassette is inserted in the patient's cells;
 - expressing the gene to produce the protein so that the protein increases reactive oxygen metabolism.
9. A vector for the expression of a gene that increases reactive oxygen metabolism in a cell, comprising
 - regulatory elements for expression of the gene, and
 - the gene operatively associated with the regulatory elements and capable of expression in the cell.
10. The vector of claim 9, wherein the gene comprises a SOD gene.
11. The vector of claim 9, wherein the gene comprises a human SOD gene.

12. The vector of claim 9, wherein the gene comprises a gene selected from the group consisting of a gene having at least 70% sequence identity with SOD and encoding a protein having SOD activity, and a gene encoding a protein having SOD activity.
13. The vector of claim 9, wherein the gene comprises a gene selected from the group consisting of SOD2, SOD3, catalase gene, glutathione peroxidase, radical scavenging genes, genes for synthesis or metabolism of vitamin E, and fragments of the aforementioned genes.
14. The vector of claim 9, wherein the cell comprises a motoneuron.
15. The vector of claim 9, wherein the cell is affected by a disease, disorder or abnormal physical state selected from a group consisting of Amyotrophic Lateral Sclerosis, Huntington's disease, Parkinson's disease and Alzheimer's disease and a disease, disorder or abnormal physical state of the motoneurons induced by reactive oxygen.
16. A pharmaceutical composition comprising a therapeutically effective amount of the SOD gene and a pharmaceutically acceptable carrier.
17. A pharmaceutical composition comprising a therapeutically effective amount of the vector of any of claims 9 to 15 and a pharmaceutically acceptable carrier.

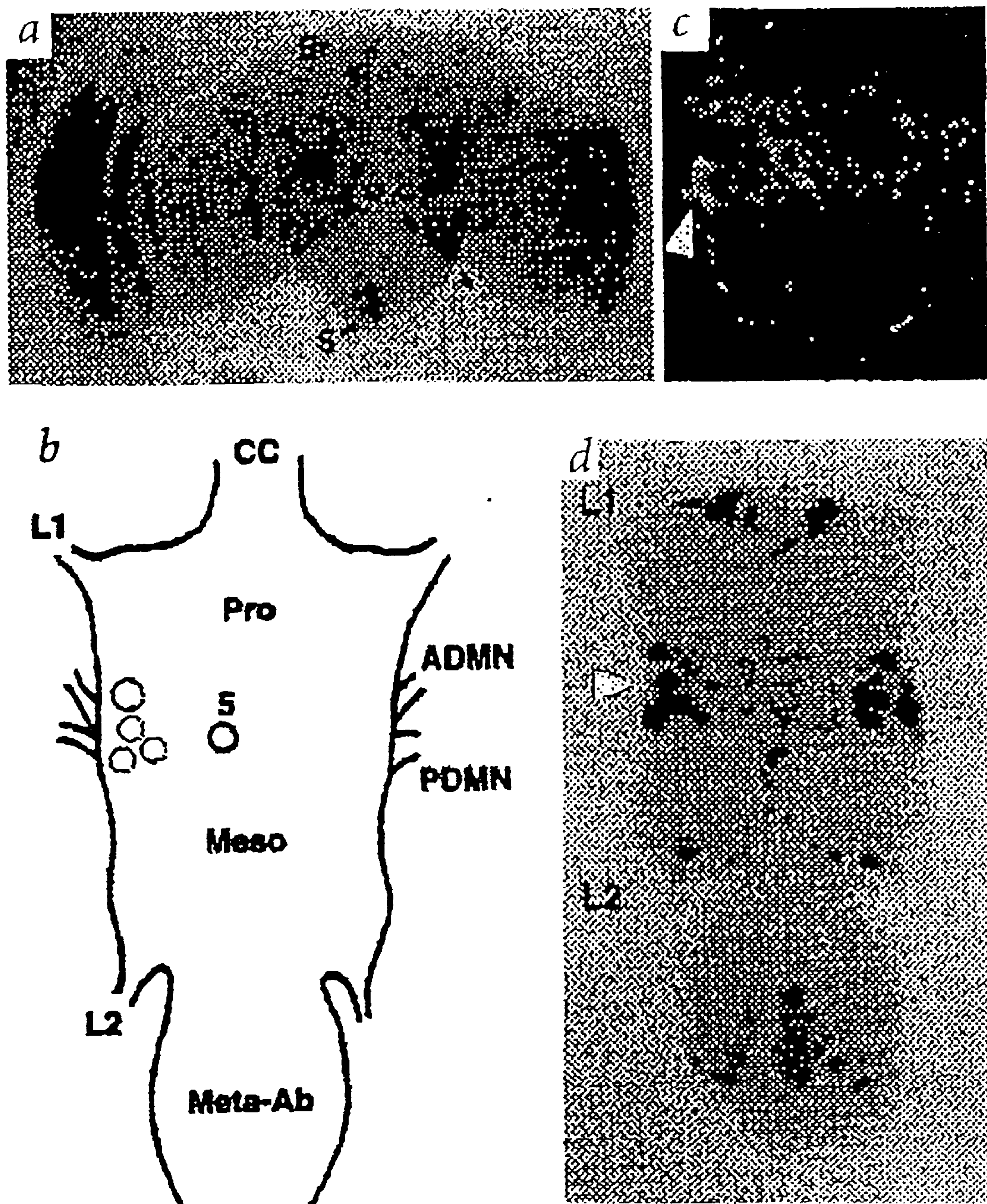


Figure 1

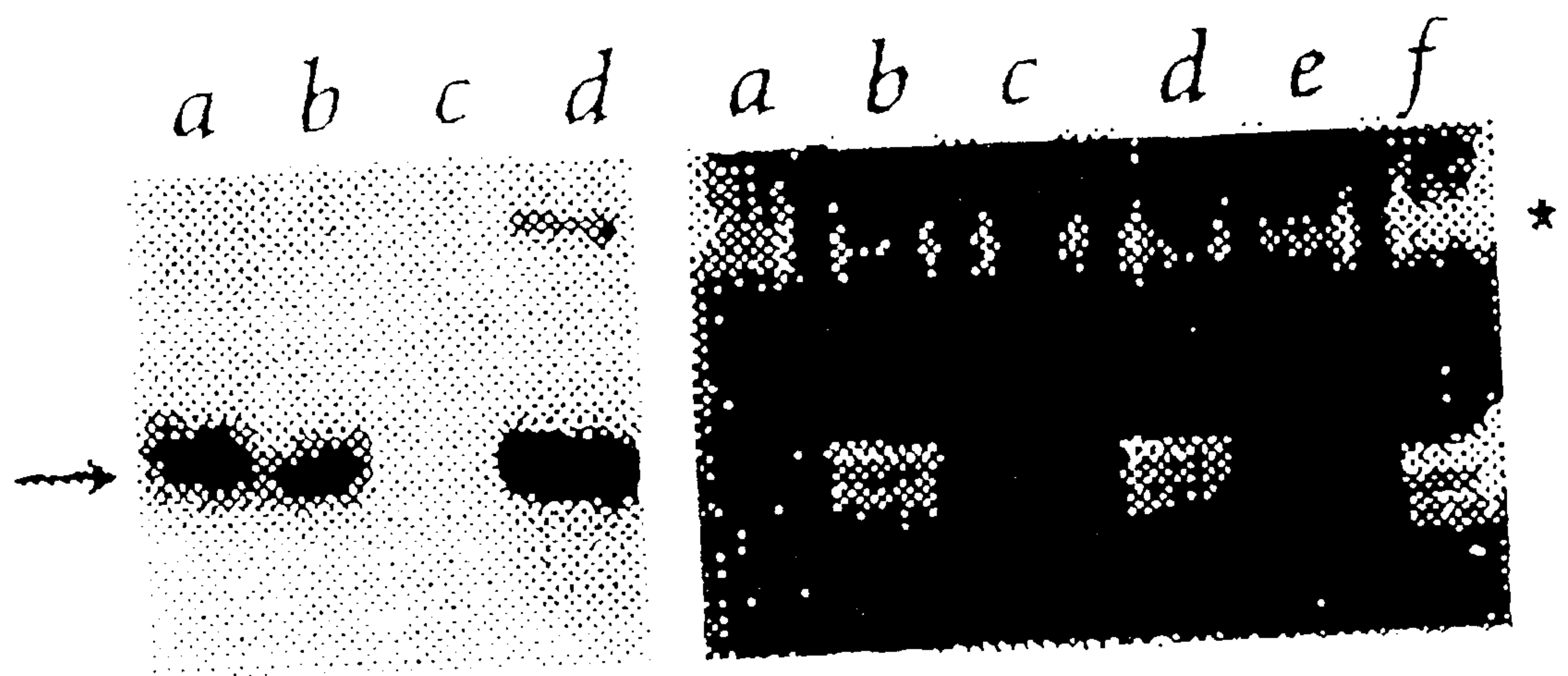


Figure 2

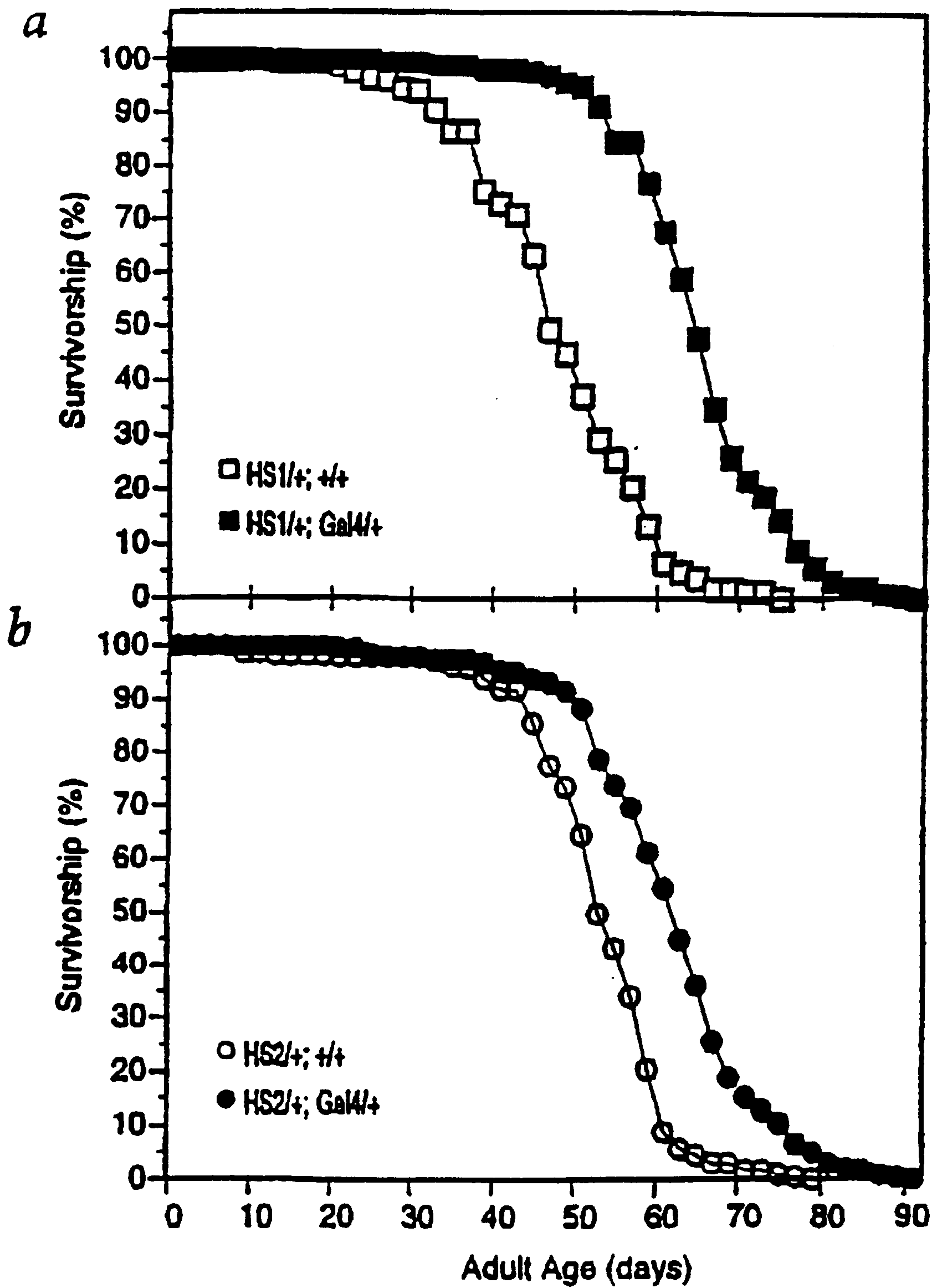


Figure 3

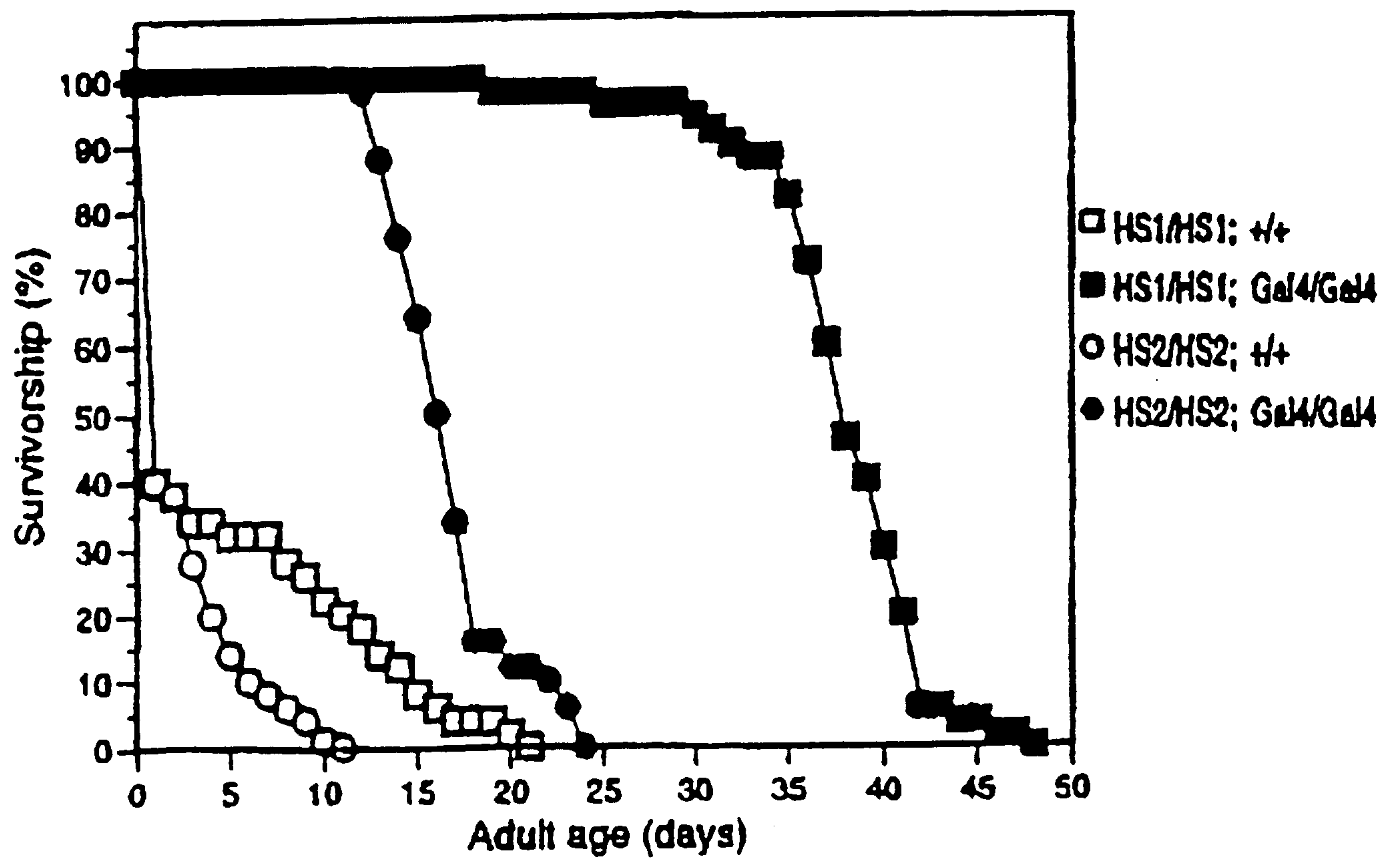


Figure 4a

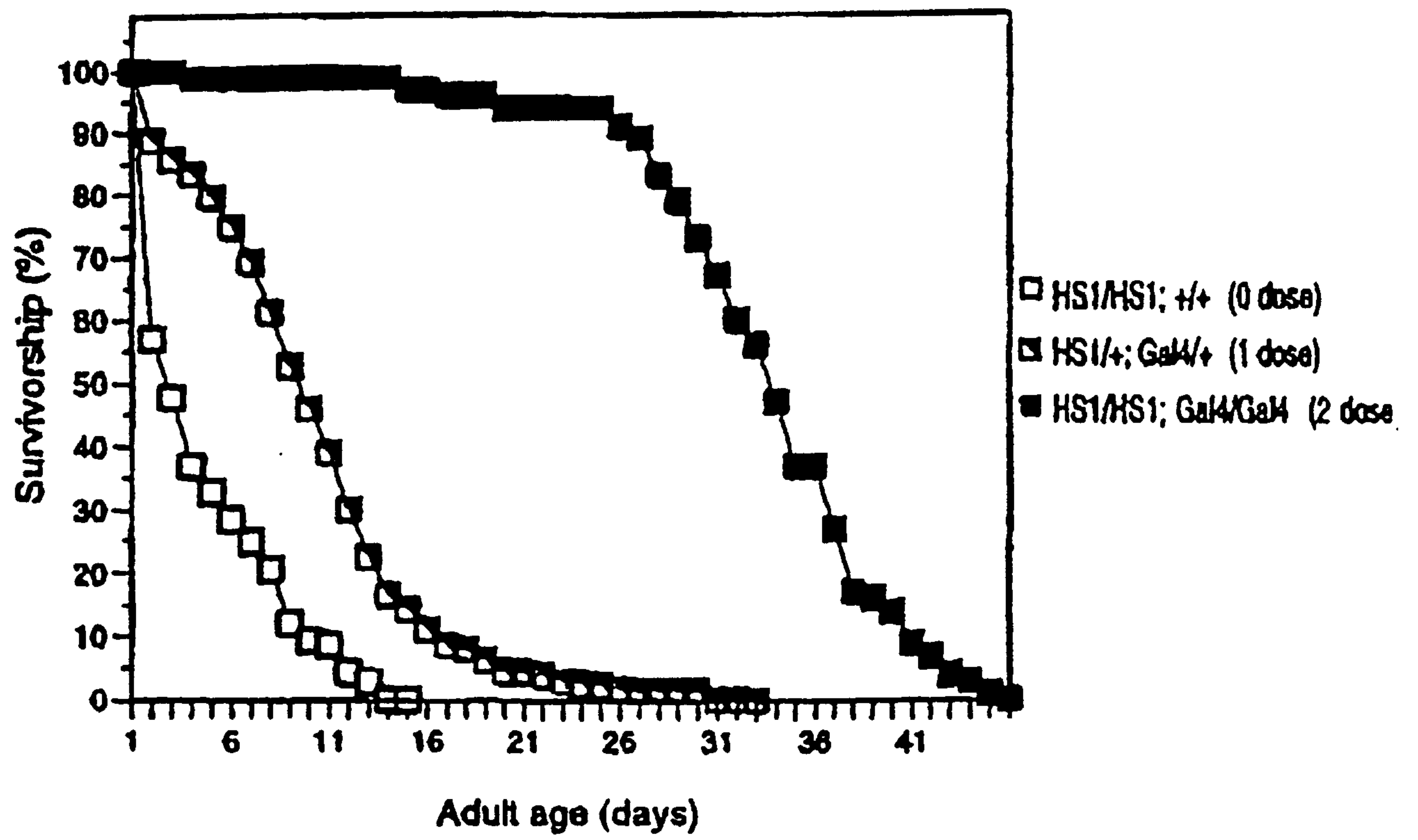


Figure 4b

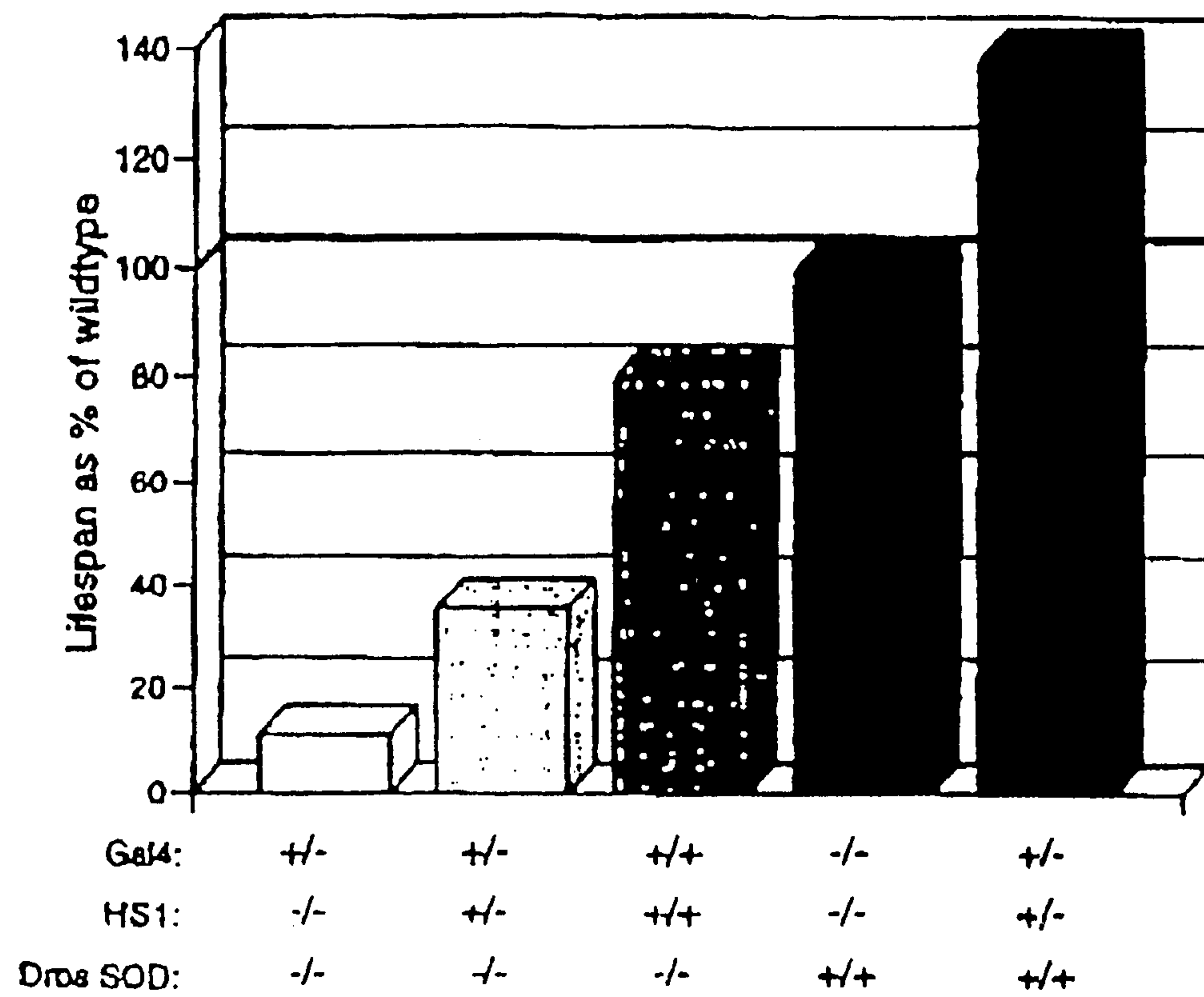


Figure 5

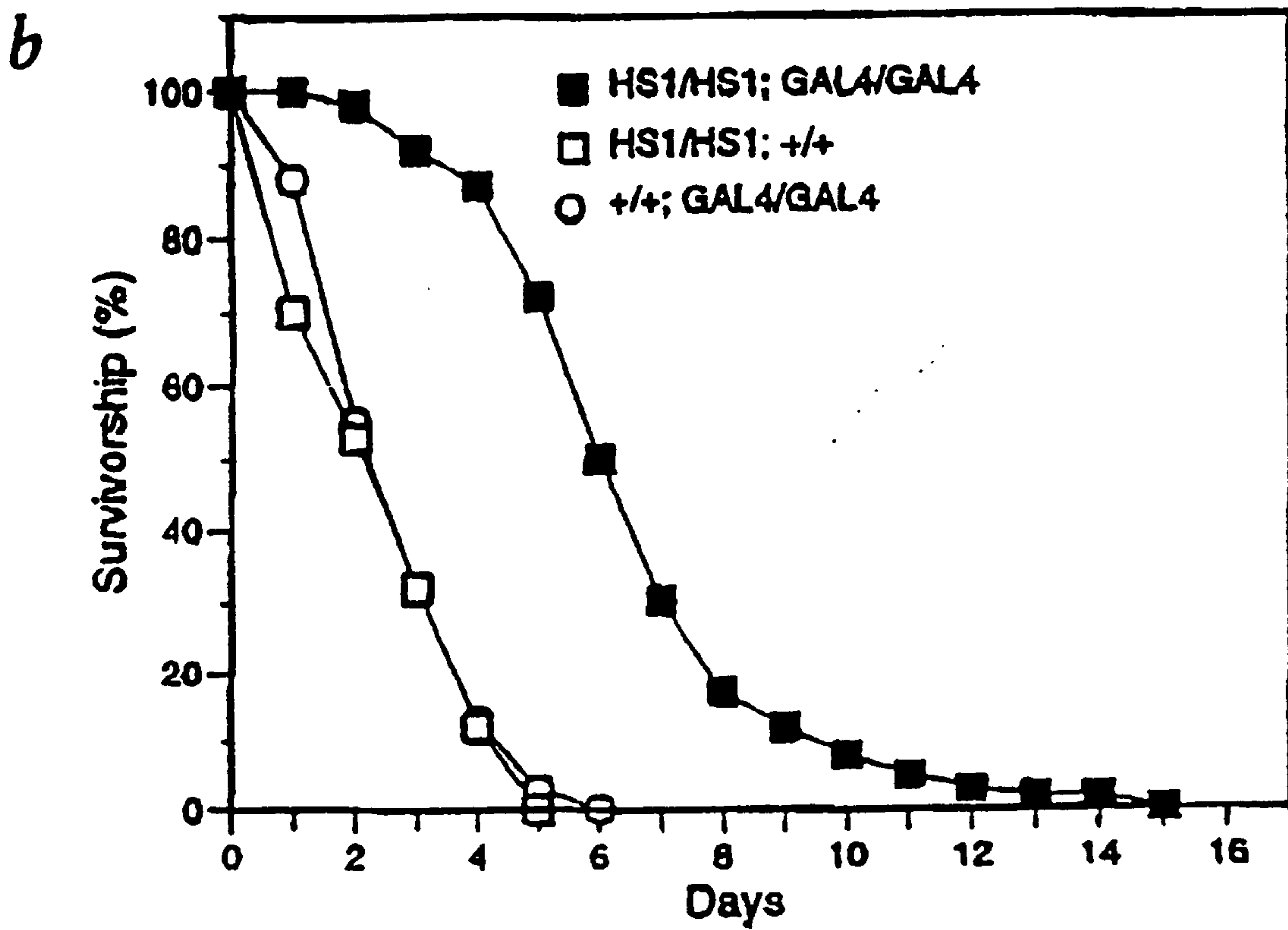
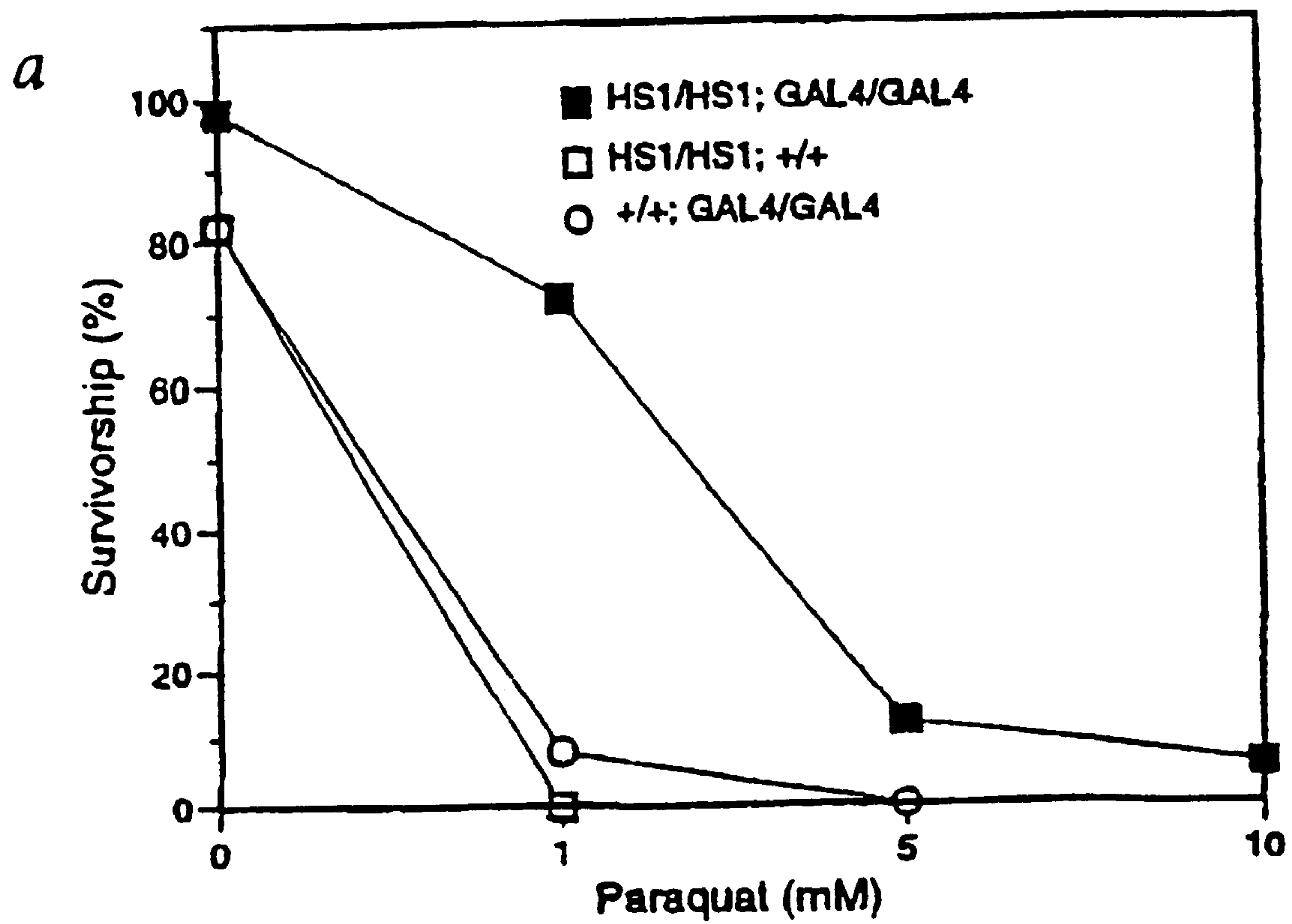


Figure 6