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Influence of Age on Stress Responses to Metabolic Cage Housing in Rats

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SUMMARY

1. We studied the effect of isolation stress in 3- and 12-month-old rats individually housed in metabolic cages for 7 days. Urine (24 hr) was collected daily from one group of animals of each age. The other group was tested in an open field and on a hot plate on days 1 and 7.

2. Total deambulation in the open-field test was lower in young than in older rats both on day 1 (54.7 ± 9.9 vs 80 ± 8.9 crossings/session; $P < 0.04$) and on day 7 (21 ± 9 vs 48 ± 7 crossings per session; $P < 0.04$) and decreased significantly in the two groups when tested on day 7 ($P < 0.03$). Latency to paw-licking in the hot-plate test was longer in young than in older animals on day 1 (14 ± 2 vs 8 ± 4 sec; $P < 0.05$) but was similar in the two groups on day 7.

3. Urinary excretions of norepinephrine (NE) and epinephrine (E) were determined by HPLC with electrochemical detection. Urinary NE in day 1 was similar in young and older animals (2627 ± 828 vs 3069 ± 598 ng/24 hr). In young animals NE excretion decreased along the study and was significantly ($P < 0.02$) lower than on day 1 during the last 3 days of the study. Conversely, in older animals urinary excretion of NE remained similar throughout the study. On day 7 urinary excretion of NE in older animals was about two fold that in young rats. Urinary E was similar in young and older rats (341 ± 127 vs 532 ± 256 ng/24 hr) on day 1 and showed a tendency to increase throughout the study.

4. Urinary monoamine oxidase inhibitory (IMAO) activity was determined by testing the ability of urine extracts to inhibit rat liver MAO activity *in vitro* and was higher in young than in older animals throughout the study (day 1, 54.8 ± 4.2 vs 25.1 ± 5.1%; $P < 0.02$). In young rats excretion of IMAO was significantly higher during the last 3 days of the study than on day 1 ($P < 0.05$). In older animals urinary IMAO showed a tendency to increase at the end of the study.

5. Isolation stress caused by housing rats in metabolic cages results in different behavioral and metabolic responses in young and older animals. Young animals exhibit a lower locomotor and analgesic response and excrete lower amounts of NE and higher IMAO activity in the urine than older rats. The metabolic and behavioral responses to isolation stress are highly dependent on the age of the animals tested. These results should be taken into consideration when designing experiments requiring the use of metabolic cages.

KEY WORDS: rats; metabolic cages; isolation stress; age; locomotor behavior; metabolic response.

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INTRODUCTION

The capacity to maintain homeostasis after exposure to stress declines with age. This decline has been associated with a decreased capacity to adapt to, and in some cases to survive, a changing environment. Stress responses result from coordinated activation of several effector systems including those of sympathoneural and adrenomedullary activation. The adrenal medulla and the sympathetic nerves can be activated independently by different stressors (Kopin, 1995). In rats, the stress-induced increase in circulating catecholamines depends on the age of the animals and on the stressor used. During immobilization, the increase in plasma norepinephrine (NE) was much greater in 11-month-old rats compared to the increase observed in 4-month-old rats, whereas the increase in plasma epinephrine (E) was not significantly different in older and younger animals (Michalikova *et al.,* 1990). However, when stressed for a brief period of time by intermittent footshock for 1 min, 12- and 24-month-old rats exhibited a diminished sympathetic–adrenal medullary response compared to 4-month-old rats (Mabry *et al.,* 1995). After the exposure to hypoxia, plasma NE and E were decreased in young rats and increased in old rats (Mader *et al.,* 1991).

We have previously reported that a brief period of isolation, repeated over 5 days, increases the level of plasma catecholamines and produces changes in endogenous MAO inhibitory activiy in rat tissues (Armando *et al.,* 1989). The presence of endogenous MAO inhibitory activity (IMAO) has been described in human (Glover *et al.,* 1980) and rat urine (Glover *et al.,* 1981) and a wide range of rat tissues (Armando *et al.,* 1986). This putative endogenous modulator of MAO activity has been named tribulin (Sandler, 1982) but its chemical composition is yet to be defined. Endogenous IMAO activity has been shown to increase in several rat tissues in response to cold restraint (Armando *et al.,* 1988; Bhatacharya *et al.,* 1988), footshock (Lemoine *et al.,* 1990), anxiety (Bhattacharya *et al.,* 1995), and audiogenic seizures (Medvedev *et al.,* 1992). Increments in urinary IMAO activity in response to stress have been reported in humans (Glover and Sandler, 1993; Doyle *et al.,* 1996) and rats (Glover *et al.,* 1981). In both tissues and urine, increments in IMAO appear to be associated with states of anxiety and agitation.

We studied locomotor behavior, response to thermal pain, and urinary excretion of catecholamines and IMAO factor in young and older rats, in an attempt to clarify the effect of age on stress responses during isolation induced by housing in metabolic cages, a procedure often necessary for metabolic studies in these animals.

MATERIALS AND METHODS

Three-month ($n = 12$)- and 12-month ($n = 12$)-old male Wistar rats were used in these studies. All animals were inbred in our laboratory, kept at 22°C on a 12 : 12-h dark–light cycle, and given free access to normal rat diet (protein content 25%) and tap water.

Experimental Procedure

Animals were individually housed in standard metabolic cages placed in their usual room (Nalgene, USA) on the morning of day 1. Food and water were supplied ad libitum. Animals of the two age groups were randomly assigned to one of the following experimental groups: in one group 24-h urine samples were collected from day 1 to day 7 ($n = 6$); in the other group animals were behaviorally tested in an open field (OFT) and on a hot plate on days 1 and 7 ($n = 6$).

All urine was collected in plastic tubes, then measured, and an aliquot was frozen until assayed. Urinary epinephrine (E), norepinephrine (NE), and monoamine oxidase inhibitory (IMAO) activity were determined as described below.

Open-Field Test

The test was carried out as described by Bures *et al.* (1976). Animals were placed in one corner of a homogeneously illuminated (100 W) open field ($1 \times 1 \times 0.4$ m). The floor was divided into 25 identical squares, 16 of them peripheral or external (i.e., adjacent to walls) and 9 central or internal (nonadjacent to walls). Each crossing of a dividing line with at least the forelegs was scored; this was considered internal ambulation when it entered an internal square and external ambulation when it entered an external one. The sum of internal and external ambulation was considered as total ambulation and used for evaluation of the results. The test lasted 5 min.

Hot-Plate Test

Animals were placed on a hot plate at 50°C immediately after the open-field test. The time elapsed from 0 (i.e., when placed on the plate) until they first licked their paws was measured using a videocamera and a chronometer. This value is referred to as the paw-lick latency.

Determination of Catecholamines

The E and NE content in 10 μl urine was determined as reported previously (Eisenhofer *et al.,* 1986). Briefly, catecholamines in the samples were partially purified by batch alumina extraction, separated by reverse-phase high-pressure liquid chromatography using a 4.6×250-mm-ODS, 5-mm column (Axxiom Chromatography Inc., USA), and quantified amperometrically by the current produced upon exposure of the column effluent to oxidizing and then reducing potentials in series using a triple-electrode system (ESA, Bedford, MA). Recovery through the alumina extraction step averaged 70–80% for E and NE. Catecholamine concentrations in each sample were corrected for recovery of an internal standard, dihydroxybenzylamine. The limit of detection was about 15 pg/volume assayed for each catechol.

MAO Inhibition Assay

IMAO activity was assayed by its ability to inhibit rat liver MAO activity (Glover *et al.*, 1980). All urine samples were diluted with water to give a constant creatinine concentration of 10 μg/20 μl. Concentrations of creatinine were similar in 3- and 12-month-old animals (85 ± 15 vs 119 ± 30 mg%). Diluted urine was acidified to pH 1 and extracted into 15 vol of HPLC-grade ethylacetate. After centrifugation the organic layer was carefully removed and evaporated to dryness under N_2. Blanks consisting of equal volumes of acidified water to those of the urines were also extracted into ethylacetate and carried throughout the procedure. The residues were taken up in 100 m*M* phosphate buffer, pH 7.4. Aliquots (20 μl) of the solution were incubated with 20 μl of a MAO preparation (1%, w/v, rat liver homogenate) and 10 μl of ^{14}C-tyramine (sp act, 58.9 mCi/mmol; New England Nuclear) diluted with unlabeled tyramine to give a final concentration in the incubation mixture of 83 μM. Samples were incubated at 30°C for 30 min and the incubation was terminated by the addition of 50 μl of 2 *N* HCI. The radioactive product formed was extracted into 3 ml of toluene : ethylacetate (1 : 1), and after centrifugation the organic phase was transferred to vials for liquid scintillation spectrometry. Assays were performed in duplicate.

Data Analysis

Data are means ± SE. One-way ANOVA for repeated measures followed by Newman–Keuls test was used to assess the significance of differences among serial determinations in 3- and 12-month-old animals. Paired *t* test was used to assess differences between day 1 and day 7 in the open-field and the hot-plate tests. $P < 0.05$ defined statistical significance.

RESULTS

Urinary excretion of NE throughout the 7 days of the study in the two groups of rats is shown in Fig. 1. Urinary NE on day 1 was similar in both young and older animals (2627 ± 828 vs 3069 ± 598 ng/24 hr). In young animals, NE excretion decreased during the study and was significantly ($P < 0.03$) lower than on day 1 in the last 3 days. In 12-month-old animals urinary excretion of NE remained similar throughout the study. Urinary NE on days 2 to 7 was significantly higher in older animals than in the younger group ($P < 0.03$). On day 7 urinary excretion of NE in older animals was about twofold that in young ones (3028 ± 685 vs 1458 ± 353 ng/24 hr).

Urinary excretion of E throughout the study is shown in Fig. 2. On day 1 urinary E in the younger group was not significantly different from that in the older rats (341 ± 127 vs 532 ± 256 ng/24 hr). In young rats there was a tendency to higher urinary E levels at the end of the study, although the changes were not statistically significant. In older animals urinary E concentrations remained similar during the study.

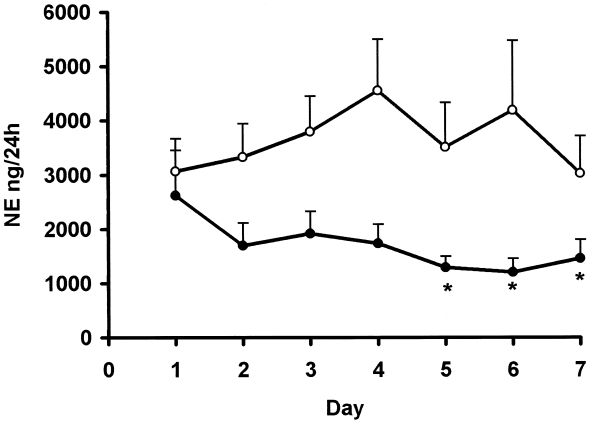

Fig. 1. Daily urinary excretion of NE in 3-month (●)- and 12-month (○)-old animals. Results are means ± SE. *Significantly different from 3-month-old rats on day 1 (ANOVA).

Urinary IMAO activity was higher in 3-month-old rats than in 12-month-old animals throughout the study (day 1, 54.8 ± 4.2 vs 25.1 ± 5.1%; $P < 0.02$) (Fig. 3). In young rats excretion of IMAO was significantly higher in the last 3 days of the study than on day 1 ($P < 0.05$). In older animals urinary IMAO did not change significantly through the study, although there was a tendency to higher IMAO excretion on day 7. In young rats pooled days 1 to 4 IMAO levels were significantly lower than those from day 5 to day 7 (54 ± 2 vs 64 ± 2%; $P < 0.001$). In older animals pooled days 1 to 4 levels were also higher, although not significantly, than those from day 5 to day 7 (28 ± 2 vs 35 ± 3%).

Total deambulation in the open-field test was significantly lower in young than in older rats both on day 1 (54.7 ± 9.9 vs 80 ± 8.9 crossings/session; $P < 0.04$) and on

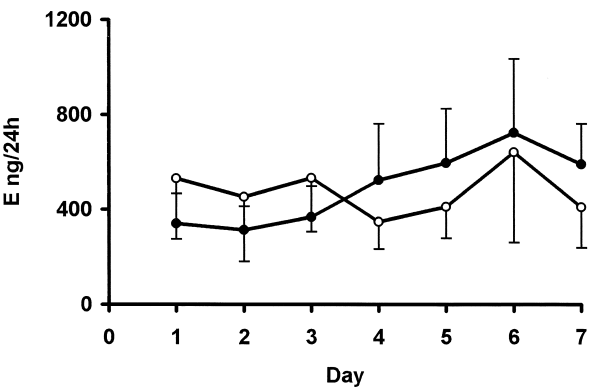

Fig. 2. Daily urinary excretion of E in 3-month (●)- and 12-month (○)-old animals. Results are means ± SE.

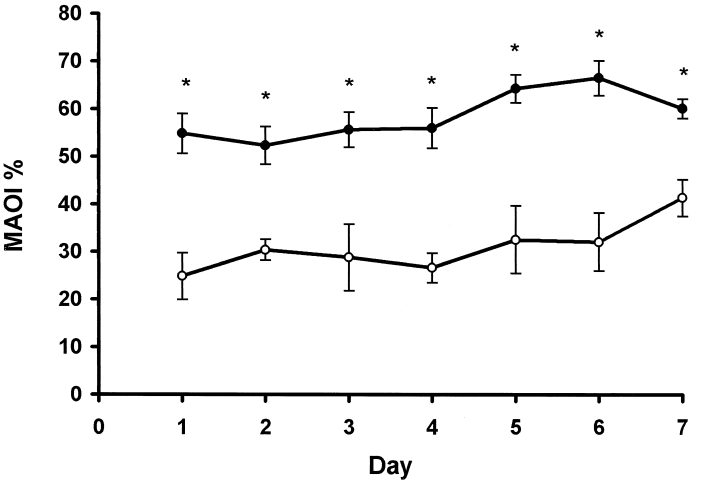

Fig. 3. Daily urinary excretion of IMAO, expressed as percentage inhibition produced by a volume of urine equivalent to 10 μg of creatinine in the assay, in 3-month (●)- and 12-month (○)-old animals. Results are means ± SE. *Significantly different from old rats (ANOVA).

day 7 (21 ± 9 vs 48 ± 7 crossings per session; $P < 0.04$) (Fig. 4). Total deambulation decreased significantly in both groups of animals when tested on day 7 ($P < 0.03$).

Latency to paw-lick in the hot-plate test was significantly higher on day 1 in young than in older rats (13 ± 2 vs 8 ± 1 sec; $P < 0.03$). Latencies on day 7 were similar to those on day 1 in both groups.

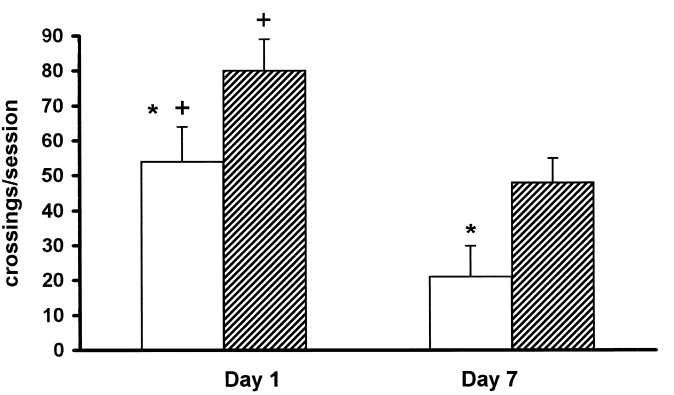

Fig. 4. Scores of ambulation in the open-field test in 3-month (open bars)- and 12-month (hatched bars)-old animals. Rats were tested on days 1 and 7. Results are means ± SE. *Significantly different from 12-month-old animals; $^{+}$Significantly different from day 7 value.

DISCUSSION

This study shows age-dependent metabolic and behavioral responses in rats during isolation-induced stress resulting from housing in metabolic cages.

Sympathetic activity, as reflected by basal plasma NE, is usually increased with age in rats as well as in humans (Ziegler *et al.,* 1976; Chiueh *et al.,* 1980; Michalikova *et al.,* 1990). Conversely, adrenomedullary activity, as reflected by basal plasma E, is apparently not subjected to significant age-related changes, at least in rats (Chiueh *et al.,* 1980; Irwin *et al.,* 1992). Both sympathetic and adrenomedullary activities are, however, profoundly influenced by stress.

Previous work suggested that there might be age-related differences in the sympathetic and adrenomedullary responses to stress. The increase in plasma NE and E after stressors such as immobilization (Chiueh *et al.,* 1980) and cold exposure (Avakian *et al.,* 1984) was similar in young and older animals. On the other hand, the increase in plasma NE and E after acute restraint stress (Mabry *et al.,* 1995) was higher in older rats. Conversely, aged animals had increased plasma E, but not plasma NE, levels in response to immobilization (Cizza *et al.,* 1995). It appears that the age-related influences in the sympathetic and adrenomedullary responses to stress, as determined by measurement of plasma catecholamines, are dependent on the condition of the experiments and the different stress protocols.

We have determined urinary NE and E as indirect measures of sympathetic and adrenomedullary activation, respectively, during stress. Because of the characteristics of our study, 24-hr collection of basal urinary catecholamines could not be determined, and our results represent the excretion of catecholamines beginning during the first 24 hr of isolation stress and thereafter.

During the first day of isolation, urinary NE excretion was similar in young and older animals. However, urinary NE concentrations decreased during the study in young animals but remained unaltered in the older group. This may suggest that, in response to isolation stress, sympathetic activity is increased for a few days in young rats, returning to ''basal'' thereafter. Whether the unaltered urinary NE excretion observed throughout the experiment in old animals reflects a persistent increased sympathetic activity throughout the study—suggesting a lack of adaptation to the environmental change—or a lack of response to the stress of isolation remains to be established. In any case, it is clear that age has a profound influence on the sympatheic activation during isolation.

On the other hand, our results suggest that the adrenomedullary activation after isolation, as determined by urinary E excretion, is not dependent on the age of the animals.

Urinary IMAO activity was higher in young than in older rats throughout the study. Since raised IMAO activity appears to be associated with conditions of anxiety and agitation (Glover and Sandler, 1993), our findings suggest that isolation stress is a more aversive situation for young than for older rats. This is also suggested by the results of ambulation in the open-field test. Changes in exploratory behavior reflect changes in the amount of fear elicited by exposure to the open field. This, in turn, depends on the relative aversiveness of pretreatment (Royce, 1977), and reduced exploration in the open field reflects a more pronounced state of fear or

anxiety. Thus, the lower scores of exploration that we observed may reflect a more profound emotional disturbance in young rats, provoked by the environmental changes, than in older animals. Similarly, the higher paw-lick latencies in the hot-plate test suggest that isolation is a more stressful situation for young animals, because stress causes a reduction in pain sensitivity, a phenomenon known as stress-induced analgesia (Bodnar *et al.,* 1980).

In the last days of the study urinary IMAO increased, or showed a tendency to increase, compared to that in the earlier phase of the study, in both young and older rats. This fits well with the decreased exploration scores in the open-field test, indicative of a lack of adaptation to the stressful situation, and suggests a heightened perception of the aversive situation. The close relationship observed between changes in output of urinary IMAO and in changes in exploratory behavior supports the proposed role of this factor as a parameter of stress and raises the question of whether one of its components is an anxiogenic rather than an anxiolytic substance. Although most of the IMAO activity in the urine remains to be chemically defined, one of its components has been found to be isatin (2,3-dioxindole), reported to be anxiogenic in a range of rodent models (Bhattacharya and Acharya, 1993).

The present results demonstrate that metabolic studies in rodents include an important component of isolation-induced stress and that some of the metabolic and behavioral changes are age dependent. Because age affects sympathetic and adrenomedullary activation, the possible excretion of anxiogenic factors, and the perception of aversive conditions differently in young and older animals, the profile of metabolic and behavioral alterations during stress and the influence of age in each of the individual parameters should be analyzed with caution.

REFERENCES

Armando, I., Glover, V., and Sandler, M. (1986). Distribution of endogenous benzodiazepine receptor ligand-monoamine oxidase inhibitory activity (tribulin) in tissues. *Life Sci.* **38:**2063–2067.

Armando, I., Lemonie, A. P., Ferrini, M., Segura, E. T., and Barontini, M. (1989). Repeated (isolation) stress increases tribulin-like activity in the rat. *Cell. Mol. Neurobiol.* **9:**115–122.

Armando, I., Levin, G., and Barontini, M. (1988). Stress increases endogenous benzodiazepine receptor ligand-monoamine oxidase inhibitory activity (tribulin) in rat tissues. *J. Neural Transm.* **71:**29–37.

Avakian, E. V., Horvath, S. M., and Colburn, R. W. (1984). Influence of age and cold stress on plasma catecholamine levels in rats. *J. Auton. Nerv. Syst.* **10:**127–133.

Battacharya, S. K., Glover, V., McIntyre, I., Oxenkrug, G., and Sandler, M. (1988). Stress causes an increase in endogenous monoamine oxidase inhibitor (tribulin) in rat brain. *Neurosci. Lett.* **92:**218–221.

Bhattacharya, S. K., and Acharya, S. B. (1993). Further investigations on the anxiogenic effects of isatin. *Biogenic Amines* **9:**453–463.

Bhattacharya, S. K., Chakrabarti, A., Sandler, M., and Glover, V. (1995). Rat brain monoamine oxidase A and B inhibitory (tribulin) activity during drug withdrawal anxiety. *Neurosci. Lett.* **199:**103–106.

Bodnar, R. J., Kelly, D. D., Brutus, M., and Glusman, M. (1980). Stress-induced analgesia: Neural and hormonal determinants. *Neurosci. Biobehav. Rev.* **4:**87–100.

Bures, J., Buresova, O., and Huston, J. (1976). *Techniques and Basic Experiments for the Study of Brain and Behavior,* Elsevier Scientific, Amsterdam.

Chiueh, C. C., Nespor, S. M., and Rapoport, S. I. (1980). Cardiovascular, sympathetic and adrenal cortical responsiveness of aged Fischer-344 rats to stres. *Neurobiol. Aging* **1:**157–163.

Cizza, G., Gold, P. W., and Chrousos, G. P. (1995). Aging is associated in the 344/N Fischer rat with decreased stress responsitivity of central and peripheral catecholaminergic systems and impairment of the hypothalamic-pituitary-adrenal axis. *Ann. N.Y. Acad. Sci.* **771:**491–511.

Doyle, A., Hucklebridge, F., Evans, P., and Clow, A. (1996). Urinary output of endogenous monoamine oxidase inhibitory activity is related to everyday stress. *Life Sci.* **58:**1723–1730.

Eisenhofer, G., Goldstein, D. S., Stull, R., Keiser, H. R., Sunderland, T., Murphy, D. L., and Kopin, I. J., (1986). Simultaneous liquid chromatographic determination of 3,4 dihydroxyphenylglycol, catecholamines and 3,4 dihydroxyphenylalanine in plasma and their responses to inhibition of monoamine oxidase. *Clin. Chem.* **32:**2030–2033.

Glover, V., and Sandler, M. (1993). Tribulin and isatin: An uptdate. In Yasuhara, H., Parvez, S., Oguchi, K., Sandler, M., and Nagatsu, T. (eds.), *Monoamine Oxidase: Basic and Clinical Frontiers,* VSP, Utrecht, pp. 61–71.

Glover, V., Reveley, M., and Sandler, M. (1980). A monoamine oxidase inhibitor in human urine. *Biochem. Pharmacol.* **29:**467–470.

Glover, V., Bhattacharya, S. K., Sandler, M., and File, S. (1981). Benzodiazepine reduce stress-augmented increase in rat urine monoamine oxidase inhibitor. *Nature* **292:**347–349.

Irwin, M., Hauger, R., and Brown, M. (1992). Central corticotropin-releasing hormone activates the sympathetic nervous system and reduces immune function: Increased responsitivity of the aged rat. *Endocrinology* **131:**1047–1053.

Kopin, I. J. (1995). Definitions of stress and sympathetic neuronal responses. *Ann. N.Y. Acad. Sci.* **771:**19–30.

Lemoine, A., Armando, I., Brun, J., Segura, E., and Barontini, M. (1990). Footshock affects heart and brain MAO and MAO inhibitory activity and open field behavior in rats. *Pharmacol. Biochem. Behav.* **36:**85–88.

Mabry, T. R., Gold, P. E., and McCarty, R. (1995). Age-related changes in plasma catecholamine responses to chronic intermittent stress. *Physiol. Behav.* **58:**49–56.

Mader, S. L., Downing, C. L., and Van Lunteren, E. (1991). Effect of age and hypoxia on beta-adrenergic receptors in rat heart. *J. Appl. Physiol.* **71**(6):2094–2098.

Medvedev, A., Gorkin, V., Fedotova, I., Semiokhina, A., Glover, V., and Sandler, M. (1992). Increase of brain endogenous monoamine oxidase inhibitory activity (tribulin) in experimental audiogenic seizures in rats: Evidence for a monoamine oxidase A inhibitory component. *Biochem. Pharmacol.* **44:**1209–1210.

Michalikova, S. H., Balazova, D., Jezova, D., and Kvetnansky, R. (1990). Changes in circulating catecholamines levels in old rats under basal conditions and during stress. *Bratisl. Lek. Listry* **91:**689–693.

Royce, J. R. (1977). On the construct validity of open-field measures. *Psychol. Bull.* **84:**1098–1106.

Sandler, M. (1982). The emergence of tribulin. *Trends Pharm. Sci.* **3:**471–472.

Ziegler, M. G., Lake, C. R., and Kopin, I. J. (1976). Plasma noradrenaline increases with aging. *Nature* **261:**333–335.