Published in final edited form as:

Exp Gerontol. 2013 February ; 48(2): 240–249. doi:10.1016/j.exger.2012.09.006.

Chemical regulation of mid- and late-life longevity in *Drosophila*

Philip McDonald¹, Brian M. Maizi², and Robert Arking^{*}

Department of Biological Sciences, Wayne State University, Detroit, MI 48202, United States

Philip McDonald: pmcdonal@med.wayne.edu; Brian M. Maizi: bmaizi@umich.edu; Robert Arking: aa2210@wayne.edu

Abstract

We tested the effects of a Class I histone deacetylase inhibitor (HDACI), sodium butyrate (NaBu), on the longevity of normal- and long-lived strains of *Drosophila melanogaster*. This HDACI has mixed effects in the normal-lived Ra strain as it decreases mortality rates and increases longevity when administered in the transition or senescent spans, but decreases longevity when administered over the health span only or over the entire adult lifespan. Mostly deleterious effects are noted when administered by either method to the long-lived La strain. Thus “mid- to late-life” drugs may have different stage-specific effects on different genomes of a model organism. A different HDACI (suberoylanilide hydroxamic acid, SAHA) administered to the normal-lived strain showed similar late-life extending effects, suggesting that this is not an isolated effect of one drug. These data also show that the use of an HDACI can significantly alter the mortality rate of the senescent span by decreasing its vulnerability, or short-term risk of death, in a manner similar to that of dietary restriction. These studies may help to shed light on the frailty syndrome affecting some aging organisms.

Keywords

Sodium butyrate; SAHA; HDAC inhibitors; Longevity regulation; *Drosophila*; Stage-specific drug sensitivity; Healthy senescence

1. Introduction

The adult life span in *Drosophila* and other gradually aging organisms consists of a health span, a transition phase, and a senescent span (Arking et al., 2002). Analysis of these life stages in model organisms showed that they are characterized by different patterns of gene expression such that the health span is defined by tightly regulated gene expression which maximizes tissue function while minimizing inflammatory and other damage responses; the transition phase is characterized by a qualitative decrease in the regulatory ability of the cell, and the senescent span is characterized by a stochastic pattern of degradation of the gene expression network (Arking, 2009).

A common approach to administering experimental pro-longevity drugs is to give the drug over the entire adult life span. Some drugs act by providing the animal with exogenous protection against various stressors, such as oxidative stress (Li et al., 2007; Zhang et al., 2009). Other drugs act by altering the gene-expression patterns and so protect the animal by

altering its endogenous defenses (Park et al., 2009). The first such genotropic compound demonstrated to have a beneficial effect on *Drosophila* longevity was 4-phenylbutyrate (4PB) (Kang et al., 2002) which was shown to function as a histone deacetylase (HDAC) inhibitor (Chen et al., 2002). They also showed that 4PB feeding during the first 12 days of the adult life span (e.g., including a portion of the health span) was not as effective as feeding during the time period from 12 days to the end of life (e.g., including the transition and senescent periods). It was later shown that this optimal 4PB feeding period resulted in an elevated level of histone H3 acetylation and an increased level of hsp22 and hsp70 expression in both short- and long-lived strains (Zhao et al., 2005a,b). These researchers also reported that NaBu fed to larvae of a short lived line increased the life span of the resultant adults, but had no effect on the longevity of a longer lived strain, observations which we here generally confirm and extend. Zhang et al., 2009) showed that NaBu extended the longevity of *Caenorhabditis elegans*, suggesting that whatever mechanism is involved may well be conserved.

Given the stage-specific patterns of gene expression known to exist in model organisms (Park et al., 2009; Pletcher et al., 2002; Zhao et al., 2005b), then genotropic compounds may well have stage-specific positive effects in one part of the life span but negative effects in another part. This difference could stem from the stage-specific presence or absence of key target molecules for the particular drug. If so, then it would be difficult to discern from whole-life treatments alone if a particular compound showed no effect because it really was not an effective pro-longevity drug, or because there was a masking effect such that deleterious effects in the health span, for example, preceded beneficial effects in the senescent span (or vice-versa). Temporally targeted investigations should yield insights into important patterns of stage-specific gene activity; and such experimental protocols are widespread in genetic tests (e.g., Duffy, 2002). Prolongevity drug discovery experiments would benefit from the inclusion of stage-specific protocols since misclassification of beneficial compounds as being without effect may actually be due to competing positive and negative effects of the drug in different life stages. Even though aging per se is not presently recognized as a medical condition requiring treatment, these data nonetheless lend empirical support to the idea that future drugs should be able to mimic the effects of endogenous gene regulatory mechanisms causing significant increases in longevity of model organisms (Kenyon, 2010), particularly those capable of bringing about effective late-life interventions in aging (Rae et al., 2010).

The question is whether such anti-aging drugs will be rare or common, and whether their discovery will be serendipitous or expected. Drug experiments have shown that the midlife initiation of oral rapamycin treatment enhances the healthy late-life survival of mice (Harrison et al., 2009). Even short term feeding of particular drugs can mimic dietary restriction (DR) induced patterns of health gene expression (Barger et al., 2008). DR experiments have also shown that DR diets induce a healthy late-life survival in many model organisms, including primates, but usually have their maximum effects only when started early in life (Lipman et al., 1995). DR experiments have also shown that multiple variables, such as mitochondrial type, affect the organism's response (Soh et al., 2007). Drug-induced alteration of gene expression patterns so as to induce a healthy survival phenotype is certainly feasible. What is not yet known is whether stage-specific feeding of genotropic drugs can in fact induce a healthy and/or an extended late-life survival of a model organism.

In this report, we tested the effects of sodium butyrate (NaBu) and suberoylanilide hydroxamic acid (SAHA) on the longevity of our normal-lived Ra and long-lived La strains. Butyrate was chosen because it is a broad spectrum, if relatively insensitive, histone deacetylase inhibitor (HDACI) representative of the Class I, II and IV zinc binding enzymes (Witt et al., 2009). SAHA has similar effects on cells as does NaBu but at much lower doses

(Zhou et al., 2011), and so was used to verify and extend the NaBu data. The fly strains have been previously described (Arking et al., 2002), but in summary the La strains were derived by selection from the Ra strains over a period of 21 generations (Arking, 1987; Luckinbill et al., 1984). They live longer due to a delayed onset of senescence (~93% increase in age at onset) which effectively doubled the health span while keeping the senescent span almost unchanged. The longevity extension in this strain is thus due almost entirely to the extension of the health span only. The delayed onset is brought about by multiple physiological changes (Arking et al., 2002).

We now report that NaBu has beneficial effects in the normal-lived Ra strain in that it increases longevity when administered in the transition or senescent spans, but decreases longevity when administered over the health span only or over the entire larval and/or adult life span. Only deleterious effects are noted when administered by either method to the long-lived La strain. Thus “mid- to late-life” drugs may have different stage-specific effects on different genomes of a model organism. The mid to late-life beneficial effects are also seen in the SAHA treated normal-lived animals. Experiments designed to identify such “mid- or late-life” drugs should take that genome and stage specificity into account. These considerations encourage the use of targeted searches for effective pro-longevity drugs (Sierra, 2010).

2. Materials and methods

2.1. Animals and doses

Two strains of *Drosophila melanogaster* were used in this investigation, the normal-lived Ra strain and the La strain selected for extended longevity as described in Arking (1987, 2002). Only female flies were utilized for all experiments, in part because it appears that female mortality kinetics drive the longevity of the species (Novoseltseva et al., 2008). The low dosage of 10 mM NaBu in the adult food media has been widely used (Chen et al., 2002; Zhao et al., 2005a,b). The high dose of 100 mM NaBu has also been established in the literature as appropriate for work with *Drosophila* (Dey-Guha and Kar, 2001). Soh et al., (2007) previously reported varying DR responses in male and female flies and demonstrated that the 2× concentration of the standard sucrose–yeast–agar medium (Luckinbill et al., 1984) was the equivalent of an ad libitum (AL) diet for the Ra strain. That diet was used throughout the experiment. Thus the experimental hypothesis is that NaBu treatment would significantly alter the overall and/or stage specific longevity observed on this AL diet relative to the longevity observed in the absence of the drug.

2.2. Experimental design

The basic design of the overall experiment was an initial screen of 16 different dose/strain/stage regimens to detect those sensitive to the drug, followed by two different types of replication experiments to confirm our initial findings. The screening experiments were done once, confirmation experiments were done on the most interesting phenomenon. Details may be found in Supplementary Table 1 as well as in the relevant figure captions and text.

For the first experiment using NaBu, cohorts of 140 or 160 animals from each strain (seven or eight vials of 20 animals each) were fed low or high doses of NaBu throughout their adult life span. Adult treatment consisted of feeding from ~2 days after eclosion to death on AL standard sucrose–yeast–agar medium (Luckinbill et al., 1984) with 0 mM NaBu, or on AL media with 10 mM NaBu, or on AL media with 100 mM NaBu added.

For the second experiment using NaBu, cohorts of 160 animals from each strain (eight vials of 20 animals each) were exposed to one of three stage-specific treatments. These treatments

were designed to determine the differential effect of NaBu on *Drosophila* longevity when administered during one of three phases of the animal's adult life span: the health span, transition, and senescent span. Using control data, these phases in the Ra strain were determined to correspond to consecutive three-week periods, from about 1–21 days, 21–42 days, and 42–63 days respectively. Adult treatment consisted of feeding on AL + 10 mM NaBu, or AL + 100 mM NaBu, during the indicated time period. The animals were fed normal AL medium during the rest of their adult life spans. The La animals were treated in the same manner.

The data from the second experiment were verified by independent experiments testing the effect of NaBu on the transition and/or senescent span, using the same protocols as described above, save that 25 flies/vial and 10–13 vials were used in the SAHA experiments. In addition, the generality of our experimental observations was tested by using a different HDAC inhibitor, SAHA, known to have a similar spectrum of effects as does NaBu albeit at a much lower effective dose in vitro (Zhou et al., 2011). A stock solution of 500 mg SAHA dissolved in 50 ml DMSO (0.378 M) was prepared. Appropriate aliquots were taken from this to yield effective doses of 1, 10, or 20 μ M/ml of AL food (e.g., 2 $\times$ media, see above) in $\sim$ 4 ml food per vial. Vehicle DMSO controls were used. The effects of the SAHA treatments are presented by both survival curves and mortality curves, using procedures as described above.

2.3. *Drosophila* husbandry and longevity experiments

All stocks were maintained in incubators at 25 °C and a 12 h/12 h light/dark cycle. Adults were transferred to new vials three times weekly and survival data was recorded for each vial. The data for each treatment group was compiled, and when all flies had expired, survival curves were plotted using the GraphPad Prism 5 software. Age-specific mortality rate (Gompertz) data was calculated using the Winmodest program (Pletcher et al., 2000) and graphed using Prism GraphPad v5 software. A total of 5653 animals were used in these experiments (Supplementary Table 1).

3. Results

We present our results organized by the portion of the life span during which the animals were exposed to NaBu, and by the strain (Ra or La) tested.

3.1. Effect of continuous exposure to NaBu

3.1.1. Continuous adult exposure

3.1.1.1. Ra strain: It is evident from the survival curves (Fig. 1A) that continuous high dose NaBu treatment of the normal-lived Ra adult *Drosophila* significantly decreases their maximum longevity compared to the control ($p < 0.0001$; see figure captions for complete statistical results). This appears to occur via a decreased length of late life (i.e., post 45 day longevity). At low dose, an obvious but borderline significant ($p = 0.0589$) shortening of the late life span is also noticed. We conclude that the Ra animals are negatively affected by this treatment.

3.1.1.2. La strain: The response of the long-lived La animals to continuous adult exposure to NaBu was similar to that of the Ra animals in that the long-lived La strain showed a significant decrease in its longevity relative to the control at high doses ($p = 0.0244$) but not at low doses ($p = 0.2869$) (Fig. 1B). Taken together, the three La survival curves are not significantly different from one another (log-rank test, $p = 0.1177$). We conclude that the La animals are significantly and negatively affected by this treatment, without any indication of a positive effect on their mortality or longevity.

3.1.2. Continuous larval + adult exposure

3.1.2.1. Ra strain: Ra animals raised on AL+NaBu food from the time of egg-laying until death showed significant dose-dependent longevity effects (Fig. 2A). Both treatments showed a short (~6 day) delay in the onset of senescence relative to the control; however they differ in their effects on the senescent span itself. In the low dose group, the treatment also induced a significantly longer life span ($p > 0.0001$) which involves an alteration of the survival pattern such that there is a 9 day increase in the median life span. In the high dose group, the treatment induced a significant decreased longevity which involved a 10 day decrease in the median life span. Neither dose affected the maximum longevity. These data suggest that the low dose slowed the mortality rate during the transition and senescent periods while the high dose accelerated it in these same life stages.

3.1.2.2. La strain: Exposure of the long-lived La strain to the drug continuously during its larval and adult stages leads to a set of survival curves which are statistically different from one another (Fig. 2B) ($p=0.1177$). The high dose group shows a significant decrease in its longevity relative to the controls ($p=0.0244$), and this decrease also seems to begin at the approximate end of the health span. However, the low dose group is not statistically different from the control ($p=0.2869$). The La animals are apparently not as severely affected by NaBu, for reasons put forth in the discussion.

3.2. Effect of stage specific exposure to NaBu

3.2.1. Stage specificity of the Ra strain

3.2.1.1. Exposure during the adult health, transition, or senescent span exposure: The results of stage-specific NaBu treatment of adult Ra *Drosophila* are quite different compared to the results of the continuous exposure data above in that beneficial effects are observed with mid- or late-life intervention in the Ra strain. Both doses of the drug decreased longevity when applied during the health span only (1–21 days) (Fig. 3). However, treatment with NaBu during the transition spans (21–42 days; Fig. 3) yielded a highly significant increase in longevity ($p<0.0001$) at either dose. The low dose yielded a 12.5% increase in median life span, while the high dose yielded a 4.1% increase in median longevity, even though the drug was not applied until the mid-life stage of the control cohort's life span. Treatment with NaBu during the senescent span (43–64 days) also yielded a highly significant increase in longevity ($p<0.0001$) at either dose (Fig. 3). The low dose yielded a 8.3% increase in median and a 2.6% increase in maximum longevity, while the high dose yielded a 8.3% increase in median but a 13.2% decrease in maximum longevity. Overall, the largest increase in median life span (+12.5% to 54 days) was induced by NaBu feeding at low dose during the transition phase. An independent experiment was done to replicate this last finding. That data, shown in Supplementary Fig. 1, shows that the median longevity was increased by 7.8% and the maximum longevity was increased by 9.2% in the NaBu treated cohort. A different form of replication experiment is described in Section 3.3 below.

Fig. 4A focuses on the effects of low dose, mid- or late-life intervention on survival during the senescent span (days 43 ff) only. It is clear that intervention during mid-life (21–42 days) is significantly more effective than that done during late life (43 ff days) ($X^2=10.31$, 1 df, $p=0.0013$). Not only are the median life spans increased as indicated, but also are the maximum life spans (LT_{10}) which are estimated as being ~55 days for the control, ~60 days for the senescent low dose, and ~61.6 days for the transition low dose (Fig. 4A).

The data of Fig. 4A implies that the intervention probably decreased the late-life mortality rates of the treated animals. A test of this prediction was done using the Winmodest program (Pletcher et al., 2000) to analyze changes in the age-specific mortality rates (see

Acknowledgments). Fig. 5A shows that a Gompertz-logistic curve is the best fit to the observed age-specific mortality data derived from the data of Fig. 4A. It also shows that both the low and high dose mid-life interventions significantly reduced the age-specific mortality rate, but that the low dose intervention, which yielded the greatest increase in median longevity (Fig. 3), seems to have accomplished this by decreasing the mortality rate mostly during the first half of the senescence period. This results in both a reduction in the long term aging rate as indicated by the significant differences in the slopes, and in a reduction of the initial vulnerability, or short term risk of death, due to the significant lowering of the intercepts.

3.2.2. Stage specificity of the La strain

3.2.2.1. Adult health, transition, or senescent span exposure: NaBu had a deleterious effect on the life span of the La strain of *Drosophila* when administered at a low dose during each of its three physiological spans (Fig. 6A). At a high dose, however, NaBu treatment was significantly deleterious only when administered during the health or senescent spans (Fig. 6B). NaBu treatment was most detrimental during the health span, where there is an obviously negative dose-response in median longevity beginning shortly after the administration of the drug at both the low dose (a ~ 15% decrease) and high dose (a ~21% decrease) cohorts (Fig. 5A,B). There is no significant difference between the low and high dose health span curves ($p=0.1787$). The immediately deleterious effect of NaBu on the long-lived adults suggests that this drug is inhibiting some essential process in this strain at this stage. Note that these health span data of Fig. 6 appear to contradict the adult stage data of Fig. 1B; there is no obvious reason for this discrepancy.

Treatment during the transition span also yielded an apparent dose response effect, but the two transition survival curves are not significantly different ($p=0.1081$) from each other. Treatment during the senescent span of the La animal also leads to significant decreases in survival curves for both doses as evidenced by a decreased survival in late life relative to controls ($p<0.0001$ for the low dose, $p=0.0081$ for the high dose) (Fig. 5). The two senescent survival curves are each significantly different from their controls ($p<0.0001$), and from each other ($p=0.0091$).

Mid-life treatment with NaBu results in an increase in their age-specific mortality rates during the senescent span (Fig. 5B). This increased late-life mortality gives rise to the shorter life spans induced by NaBu in every experimental situation involving the long-lived strains (Fig. 6).

3.3. HDAC inhibitor specificity of the late life response in Ra animals

The generality of the experimental observations reported in Section 3.2.1.1 above was tested by using a different HDAC inhibitor, SAHA, known to have a similar spectrum of effects as does NaBu albeit at a much lower effective dose in vitro (Zhou et al., 2011). The survival results of dose-response tests of SAHA dissolved in DMSO and added to the food administered to Ra strain females at age 42 ff days are shown in Fig. 7, along with that of the DMSO controls. The three SAHA doses altered the late life survivorship so as to yield small but highly significant increases in both median and maximum life (LT_{10}) spans relative to the control ($p=0.0002$). There is no significant difference between the three dose-response curves detected by the log rank test ($p=0.5400$). There is, however, an observable dose-dependent difference in the age at which the maximum difference in survivorship between the control and experimental cohorts. The 1 μ M dose yields a 23.2% difference at age 65, the 10 μ M dose yields a 16.6% difference at age 62, and the 20 μ M dose yields a 21.6% difference at age 60 days. We have no information at present regarding the gene expression data underlying these changes in survivorship.

3.3.1. Effect of SAHA on mortality kinetics of senescent Ra animals—The changes in the age-specific mortality rates arising from SAHA treatment of older animals were calculated using the Winmodest program (Pletcher et al., 2000) as described (see Acknowledgments). Fig. 8A–C shows that the smoothed age-specific mortality line (5 day smoothing of the $\ln q_x$ observed data points) for each cohort switched to SAHA-containing food at day 42 shows no obvious visible alteration for about a week. However, beginning at about 50 days, the age-specific mortality line consistently and obviously falls below that of the control mortality line; suggesting that it takes the ingested SAHA about a week before its effects on mortality can be seen. Presumably it is this reduced mid-life mortality that accounts for the extended longevity of each of the SAHA cohorts relative to the control. Fig. 8D shows that a Gompertz-logistic curve is the best fit (data not shown) to the observed control and experimental post-50 day age-specific mortality data derived from the data of Fig. 7.

Since control and experimental cohorts are best fit by a logistic Gompertz, one can test whether they have the same or different parameters using a likelihood-ratio test which yields a X^2 value. Comparing the three different experimental cohorts to the control shows that they are significantly different via this test ($X^2=28.18$, 3 df, $p<0.0001$). Thus mid-life treatment with SAHA has significantly affected both their mortality rate and their survival (Fig. 7) relative to controls. If one compares the control to the 10 μM SAHA cohort, then the control is significantly different from the experimental cohort in the slope ($X^2=6.66$, 1 df, $p=0.01$), but is not significantly altered with respect to the intercept ($X^2=0.6$, 1 df, $p=0.4$) nor in the 's' term of the logistic curve ($X^2=3.04$, 1 df, $p=0.08$). We conclude that the mortality curves post-50 days of age for the SAHA treated cohorts are significantly different from that of the untreated control, and that the difference is driven predominantly by a change in its slope. Finch et al. (2000) have pointed out that changes in the slope of the Gompertz curve are equivalent to changes in the rate of aging. We estimate the mortality rate doubling time (MRDT) of the control cohort to be ~ 4.73 days and that of the 10 μM SAHA cohort to be ~ 5.55 days. Thus SAHA has induced a decrease of $\sim 15\%$ in the MRDT.

The fact that low dose NaBu treatment of mid-life Ra animals also resulted in a statistically significant reduction in late-life age-specific mortality rates (Fig. 5) lends credence to our interpretation that late-life mortality lowering may be a general effect of HDACi's when properly applied.

3.4. Summary of the data

Our data shows that continuous exposure of either normal- or long-lived *Drosophila* to high or low doses of NaBu during the entire adult phase led to a significant shortening of longevity in both strains. Treating either strain for the entire larval + adult stages also adversely affected longevity; with the exception of the Ra strain treated with the low dose which showed an extension of their transition + senescent phases. Stage specific exposure of either strain during the health span also led to deleterious effect in both strains. However, exposure of the normal-lived Ra strain to either NaBu or SAHA during the transition span, or the senescent span, led to a significant increase in median longevity and corresponding decreases in the late-life mortality rates. This beneficial effect of NaBu was not seen in the long-lived La strain. NaBu and SAHA, both of which are HDAC inhibitors with a similar spectrum of effects (Zhou et al., 2011) have the characteristics of a mid- or late-life drug which brings about a healthy senescence in normal-lived animals as indicated by lower levels of age-specific mortality and a significant extension of the senescent span. We conclude that these data, taken together, present proof of principle for the hypothesis that mid-life treatment with these HDACi's can significantly reduce late-life mortality and lead to a healthier senescence.

4. Discussion

4.1. Do these chemicals work via epigenetic effects?

Epigenetic effects on gene expression involve alterations of the chromatin conformation around genes which then activate or repress their transcription (Swaminathan et al., 2012; Vasanthi and Mishra, 2008). One such epigenetic mechanism is the addition or removal of an acetyl group to particular histone residues. Histone deacetylases (HDACs) catalyze the removal of acetyl groups from specific lysine residues of histone proteins, leading to a repression of the affected genes (Witt et al., 2009). HDAC inhibitors oppose the deacetylation of specific sites, and lead to a transcriptionally active chromatin state. The classical Class I, II and IV HDACs all rely on Zn^{2+} as a cofactor, while the Class III sirtuin HDACs rely on NAD^+ as an essential cofactor. Some of these molecules are known to have effects on longevity. The role of the Class III Sir2 gene in organismal longevity is currently being debated (Canto and Auwerx, 2011; Lombard and Pletcher, 2011). However, the gene is known to have a delaying effect on cellular level senescence (Li and Tollefsbol, 2011) which may affect age-related disease. Both Sir2 overexpression and resveratrol treatment yield a significant overlap of longevity genes (Antosh et al., 2011). Altering NAD^+ levels via up-regulation of a *Drosophila* nicotinamidase also resulted in increased longevity (Balan et al., 2008) without, however, having any effect on oxidative stress resistance (Miller and Arking, in preparation). Suppression of the *rp43* gene via mutation resulted in extended longevity (Rogina et al., 2002; Zhao et al., 2005b). Thus the several classes of HDACs may well work via different genetic networks.

Chen et al. (2002) showed that NaBu caused hyperacetylation and unwinding, or puffing, of the *Drosophila* polytene chromosome at the *hsp70* locus. Zhao et al. (2005a) showed that one-time NaBu treatment increased heat shock protein (HSP) expression considerably in a short lived strain of flies but had less of an effect on long-lived flies. Kang et al. (2002) showed that phenylbutyrate fed to flies resulted in an extension of longevity, particularly when fed during the later phase of the health span and the transition phase. This latter result was the first to present proof of principle that a drug could significantly increase longevity in a model organism. Our results are consistent with their findings and extend them to include both other HDAC inhibitors as well as the importance of stage-specific screening for efficient detection of potential candidate “mid-life” drug molecules. It must be noted that at the moment, we have no direct molecular evidence that these drugs really act in an epigenetic manner in our flies and so rely on the literature for the evidence on which this discussion is based.

The sensitivity of the adult Ra animal to lifetime feeding of NaBu suggests that such HDAC activity must be taking place during at least part of the adult span (Fig. 1), and during some part of the larval stage as well (Fig. 2). Stage-specific exposure of the normal-lived Ra animals to NaBu resulted in significantly decreased longevity when administered during the health span; but resulted in significantly increased longevity following administration in the transition or senescent spans (Fig. 3A,B). The longevity of the long-lived La animals to NaBu was significantly reduced following administration at either dose in the health, or transition, or senescent spans (Fig. 6A,B). The simplest way to interpret these data is that inhibition of HDACs during the health span must interfere with the epigenetic effects normally taking place in the adult animal at that time, and so results in harmful effects in both normal- and long-lived strains. However, the beneficial effects observed in the transition- and senescent-span normal-lived Ra animals implies that those epigenetic effects do not normally occur during these life span stages in that strain; their presence in the comparable stages of the long lived strain may be one result of the artificial selection regimes employed, and so the application of the drug in the Ra strain brings about a partial restoration in late life of patterns of gene expression characteristic of a healthy senescence.

The fact that two different HDAC inhibitors, NaBu and SAHA, had similar beneficial effects on mortality and survivorship during the senescent span, suggests that this may be a general effect of this class of HDAC inhibitors. The negative effects of this HDAC inhibitor on the long-lived La strain suggest that the added drug interferes in some way with the presumably high levels of specific acetylation already present in this strain. Similar observations on the differing reaction of shorter- and longer-lived *Drosophila* strains to NaBu were reported by Zhang et al. (2009). These predictions regarding the mode of action of this mid-life drug will need to be critically tested in future experiments.

The main conclusion to be drawn from these data is that NaBu negatively affects longevity when applied continuously in the adult stage (Fig. 1), but has a dose-dependent effect if given during the entire larval + adult life span (Fig. 2). In addition, both NaBu and SAHA have significant effects on longevity and mortality when applied to the transition or senescent phases of normal-lived *Drosophila* strains (Figs. 3, 4A, 5A, 7-8) but not of long-lived strains (Figs. 4B, 5B, 6). This apparently contradictory set of results is, however, what would be expected if the gene regulatory mechanisms affected by NaBu were those intimately involved in inducing gene expression patterns characteristic of a healthy senescence. Applying the drug during life span stages when such epigenetically induced gene expression patterns were naturally present might well lead to a drug-induced interference with the endogenous signals. Such interference would probably be disruptive to the gene-based state of health and manifest itself in a decreased survivorship, as we have observed. On the other hand, applying the drug to life span stages when the endogenous gene expression patterns are not present would likely induce such healthy gene expression patterns and thus increase the state of health of the individual. This would result in a lower mortality rate for that life span stage. One conclusion to be drawn from our data is that the observed stage-specific ability of an HDACI such as NaBu or SAHA to adversely affect the survival of the Ra and/or La animals implies that the organism's sensitivity to the drug is due to the presence of stage specific HDAC-dependent gene expression patterns. Interfering with those patterns may well cause a shortening of longevity. Inducing such gene expression patterns during a stage when they are not normally present could underlie our observed extensions of longevity. Any discussion of 'mid-life drugs' carries this mechanistic paradigm with it (Kenyon, 2010; Rae et al., 2010).

The significantly different dose-dependent effects of Fig. 2A suggest that the presence of NaBu during the developmental period alters the subsequent adult gene expression patterns into an extended survival mode. Treatment during the adult stage only does not do this (Fig. 1A). Together, these data suggest that there is a developmental component to adult longevity and aging. The existence of such a developmental component has been previously shown by us (Buck et al., 1993; Jung et al., in review as well as by Zhao et al. (2005a,b).

4.2. Antagonistic pleiotropy

One can view these results of HDACI sensitivity as describing an example of antagonistic pleiotropy in that a given drug has harmful effects at one stage and beneficial effects at another. This is a somewhat unusual example in that the antagonistic pleiotropy varies not only over the life span of a normal-lived strain, but also varies across strains depending on their longevity. We suggest that these effects are due to differential gene expression such that the NaBu/SAHA target genes/products are only available at some, but not all, stages. The patterns of this antagonistic pleiotropy in a later stage can be changed by earlier treatment with the drug, which then shifts the later stage gene expression patterns from a harmful to a beneficial pattern (or vice-versa). An example of this may be seen by comparing Figs. 1A and 2A. Feeding NaBu only during the adult stage has detectable negative effects on both Ra and La animals. However, feeding NaBu at low doses during

both the larval + adult stages presumably alters the larval gene expression patterns such that the adult Ra animal now responds positively to the drug. If this interpretation is correct, then antagonistic pleiotropy is not necessarily a fixed attribute but may well be an outcome of earlier exogenous and endogenous events. Given the increasing attention being paid to characterizing various epigenetic mechanisms, then it may be possible in the near future to be able to describe the genetic and molecular mechanisms underlying this particular example of antagonistic pleiotropy.

4.3. States of senescence and frailty

Decreasing late life mortality rates and increasing late life survival lead inevitably to a reconsideration of the senescent state. Our data demonstrates that senescence is not necessarily a fixed trajectory, but may now be considered a variable process. We know that there are differences in gene expression patterns in young animals destined to live long relative to those destined to live a normal life (Antosh et al., 2011; Soh et al., in press). Such data raises the question as to whether there are healthy and non-healthy states of senescence as well. If there are, then it might be possible that NaBu and SAHA have the ability to switch older animals from one state to the other if properly administered.

Mouse studies have shown that there are at least five different genes which serve as biomarkers of aging in multiple mouse strains (Park et al., 2009). Various dietary supplements are as effective as is dietary restriction in opposing the increased expression of the aging biomarkers in the heart and the cerebellum, and presumably decreasing morbidity in the old mice. Thus these biomarker genes likely constitute part of a gene expression pattern associated with the onset of a normal (i.e., chronic disease prone) senescence. A recent human genome-wide-association-study by Walter et al. (2011) showed that there was a correlation between SNPs in a panel of neural-related genes with the presence or absence of a healthy senescence. Eight SNPs were associated with a disease free senescence while 14 were associated with the presence of chronic disease(s) during senescence. If SNPs are to exert an effect on the phenotype, then this means that they must somehow be expressed in the animal. In addition to the presence of unique SNPs, a healthy mid- or late-life survival is likely to also involve characteristic stage-specific patterns of gene expression perhaps dependent on the effects of those SNPs. Jazwinski et al. (2010) have shown that combinations of particular haplotypes are significantly associated with a healthy senescence and increased survival in humans. The Ra animals treated with NaBu or SAHA during their transition or senescent spans show a decreased mortality rate (Figs. 5, 8) which results in a longer senescent span and thus an increase in the maximum (LT₁₀) longevity. (Figs. 6–8).

4.4. Dietary restriction

Dietary restriction (DR) has been reported to decrease the organism's short-term risk of death (Mair et al., 2003). NaBu or SAHA treatment also decreases the organism's short-term risk of death (Figs. 5A and 8). It is in this context that NaBu or SAHA treatment may be viewed as a type of DR mimetic but with mid- to late-life specificity (Figs. 3, 4, 8, 9). The slower aging in these animals during the senescent span is indicative of a healthy senescence. It would be interesting to determine the difference in gene expression patterns during the senescent span of treated and control animals, and to see if orthologs of the gene products identified in these prior studies are preferentially found in the treated animals with the extended senescent span. Antosh et al. (2011) have recently used a comparative genomics approach to point out that there exists a common health span genetic signature in *Drosophila*. We suggest that it is plausible that there may also exist common senescent span signatures, one associated with a healthy senescence and one associated with a chronic disease-based senescence; and that epigenetically active molecules such as NaBu or SAHA may allow organisms to shift from one to the other if given at the appropriate stage.

4.5. Conclusion

Further work will be necessary to critically test these predictions regarding the mechanisms underlying mortality and survival changes induced in normal-lived, but not long-lived, animals by these two HDACIs.

Our data suggests that choosing regulatory genotropic candidate compounds from the list of chemicals known to have epigenetic effects may be a useful search strategy, especially when combined with a stage-specific screening protocol. These considerations further encourage the use of targeted searches for effective pro-longevity drugs (Sierra, 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

It is a pleasure to acknowledge the supportive efforts of Melissa Dick, Jung-Won Soh, Thomas J. Nichols, and Nicholas Marowsky during the course of these experiments. We thank the anonymous reviewers for their constructive criticisms. The work was supported in part by a WSU Research Stimulation Program for Tenured Faculty grant to RA and in part by personal funds. The SAHA was a generous gift from Prof. Michael Tsainy of the WSU Karmanos Cancer Institute. The analysis of the mortality kinetics in Figs. 5 and 8 was graciously performed by Prof. Scott Pletcher and the *Drosophila* Aging Core (DAC) of the Nathan Shock Center of Excellence in the Biology of Aging funded by the National Institute of Aging (P30-AG-013283).

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Abbreviations

HDACI	histone deacetylase inhibitors
NaBu	sodium butyrate
SAHA	suberoylanilide hydroxamic acid or vorinostat

Fig. 1.

Feeding of NaBu only during the adult stage decreases longevity of Ra and La adults. The lifetime feeding of NaBu at either dose does not increase longevity in either the Ra (Panel A) or La (Panel B) strains relative to controls. The low dose (10 mM) has no statistically significant effect relative to controls (log-rank test, $p=0.0589$ (Ra) or $p=0.2869$ (La)). The high dose (100 mM) yields significant decreases relative to controls in Ra ($p<0.0001$) and La ($p=0.0244$). The median life spans of the several cohorts are listed in the legends of Panels A and B. The N for each Panel A cohort is: control=159; low dose=148; high dose=150. The N for each Panel B cohort is: control=160; low dose=154; high dose=152.

Fig. 2.

Feeding of NaBu during the larval + adult stages has dose-dependent effects on longevity of Ra and La adults. Feeding NaBu at either dose throughout the developmental and adult stages has different effects on the Ra (Panel A) or La (Panel B) strains relative to controls. In the Ra strains, the low dose results in a significant extension of median, but not maximum, age relative to controls (log-rank test, $X^2=26.81$, 1 df, $p<0.0001$) while the high dose results in a significant decrease of median but not maximum age ($X^2=20.21$, 1 df, $p<0.0001$). In the La strains, the low dose has no effect ($p=0.9415$) while the high dose significantly decreases the median but not maximum age ($X^2=13.04$, 1 df, $p=0.0003$). The median life spans of the several cohorts are listed in the legends of Panels A and B. The N for each Panel A cohort is: control=169; low dose=136; and high dose=132. The N for each Panel B cohort is: control=160; low dose=135; and high dose=135.

Fig. 3.

Effects of stage-specific feeding of NaBu on Ra adults at different doses. Feeding NaBu to Ra adults only during their health, transition or senescent spans leads to significant stage-specific effects on longevity. The dotted lines in the Figure indicate the approximate boundaries of the three spans. At a low dose (Panel A), there is a non-significant decrease in median longevity relative to controls (log-rank test; $p=0.2781$) for intervention in the health span. However, a significant increase in the median and late-life longevity is observed following feeding NaBu during the transition ($X^2=52.98$, 1 df, $p<0.0001$) or senescent ($X^2=38.52$, 1 df, $p<0.0001$) spans. Note that NaBu feeding added 12.5 % to the median life span of the control even though the treatment did not start until 54 % (41/76) of the control longevity was already over at the treatment start. At a high dose (Panel B), the same qualitative, but different quantitative, longevity effect is observed. There is a non-significant decrease in the median life span following treatment in the health span ($p=0.1594$), and a significant increase as a result of intervention in the transition ($X^2=23.81$, 1 df, $p<0.0001$) and senescent ($X^2=20.65$, 1 df, $p<0.0001$). The maximum life span is reduced following treatment in the health or senescent spans but not noticeably in the transition span. The median life spans of the several cohorts are listed in the legends of Panels A and B. The N for each Panel A cohort is: control=152; health=147; transition=153; and senescent=155. The N for each Panel B cohort is: control=152; health=151; transition=155; and senescent=155.

Fig. 4.

Late-life survival. In Panel A, the Ra survival data from Fig. 3A were rescaled so as to examine the details of extended survival during the senescent span (days 43 ff.). It is clear that low dose NaBu treatment during the transition span has a greater positive effect on survival and longevity in the senescent span than does intervention during the senescent span itself (log-rank test, $X^2=10.31$, 1 df, $p=0.0013$). Other N and p values are as noted in Fig. 3. In Panel B, the La survival data from Fig. 6A is rescaled so as to examine the details of decreased survival during the senescent span (days 43 ff.). It is clear that NaBu treatment at low dose during the senescent span has a greater deleterious effect on survival and longevity than does intervention during the transition span. The long-lived La animals differ from the normal-lived Ra animals in both their negative response to the drug and their periods of greatest sensitivity. N and p values as noted in Fig. 6.

Fig. 5.

Analysis of NaBu effects on mortality kinetics during the senescent span. Mortality rates were calculated from the survival data used in Fig. 4A (for the Ra animals in Panel A) and from the survival data of Fig. 6 (for the La animals in Panel B) as $ux = \ln(px)$, where px is the probability of survival from age x to age $x + 1$. They are presented on the logarithmic scale. The Winmodest program was then used to obtain the most appropriate mortality model of the Gompertz family and determine parameter estimates associated with the best fit. In both cases, a logistic model provides the best fit (data not shown); and the treated cohorts are significantly different from the control cohort (data not shown). Panel A: shows that NaBu intervention at both doses in the mid-life (21–42 day) period yields significant decreases in the mortality rates of Ra animals during the senescent span relative to the control. The treated animals live longer than controls. See text for details. Panel B shows that NaBu intervention at both doses in the mid-life (21–42 days) period yields significant increases in the mortality rates of La animals during the senescent span relative to the control. The treated animals live shorter than controls. See text for details.

Fig. 6.

Effects of stage-specific feeding of NaBu on La adults at different doses. The dotted lines in the Figure indicate the approximate boundaries of the health, transition, or senescent spans. Feeding NaBu at low doses (Panel A) to La adults only during their health (log-rank test; $X^2=49.83$, 1 df, $p<0.001$), transition ($X^2=6.72$, 1 df, $p=0.0095$) or senescent ($X^2=25.91$, 1 df, $p<0.0001$) spans leads to significant decreases relative to controls in their median but not maximum longevity following treatment in each stage. Feeding NaBu at high doses (Panel B) to La adults only during their health ($X^2=16.33$, 1 df, $p<0.001$) or senescent ($X^2=7.022$, 1 df, $p=0.0081$) spans leads to significant decreases relative to controls in their median longevity following treatment in each stage. However, treatment at the high dose in the transition span has a non-significant effect relative to the control ($X^2=1.364$, 1 df, $p=0.2446$). The median life spans of the several cohorts are listed in the legends of Panels A and B. The N for each Panel A cohort is: control=159; health span=154; transition=151; and senescent=151. The N for each Panel B cohort is: control=159; health=129; transition=158; and senescent=152. The Gompertz curves for the senescent span cohorts are shown in Fig. 4B.

Fig. 7.

Late life treatment with SAHA increases late life survival. Sister cohorts of Ra females were treated with DMSO only (N=225), or 1 μ M SAHA in DMSO (N=301), or 10 μ M SAHA in DMSO (N=325) or 20 μ M SAHA in DMSO (N=288). The three experimental cohorts are highly significantly different from the control (log rank test, $X^2=19.50$, 3 df, $p=0.0002$), but do not statistically differ between themselves (log-rank test, $X^2=1.233$, 2 df, $p=0.5400$). The vertical arrow approximates the age (42 days) at which the experimental cohorts were introduced to SAHA. See text for additional details.

Fig. 8.

Analysis of SAHA effects on mortality kinetics during the senescent span. For each of the three SAHA dose–response curves of Fig. 7, the observed data points for mortality (i.e., age-specific death rate ($\ln q_x$); circles for controls; triangles for experimentals) and the smoothed mortality lines (5 day window; black for control; red for experimental) are shown for the control cohort relative to the 1 μ M (A), 10 μ M (B), and 20 μ M (C) SAHA cohorts. The black arrows in each panel indicate the approximate time (day 42) when each experimental cohort was shifted to the SAHA-containing food. Note that in all three experimental cohorts, there is no obvious effect of the drug until ~50 days. After that time point, each of the three experimental cohorts shows an obvious and consistent lowering of the smoothed mortality line relative to the control. Panel D shows that a logistic Gompertz curve best fits the observed data points in each cohort from 50 days until the final death. Note that the post-50 day mortality curves for the SAHA cohorts are all significantly lower than the control cohort (likelihood-ratio tests, $X^2 = 6.66$, 1 df, $p = 0.01$). See text for further discussion.