

# Defensive Behavior against Noxious Heat Stimuli Is Declined with Aging Due to Decreased Pain-Associated Gene Expression in *Drosophila*

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## Abstract

Aging is defined as a collective process that alters organism's functional capacity and appearance over the course of life. Apart from an increase in susceptibility to many diseases, aging affects the cellular system that is responsible for decoding painful stimuli. Yet, aging-associated molecular mechanisms of pain perception remains elusive. Using *Drosophila*, we showed a decrease in temperature tolerance and a reduction in high temperature thermal avoidance with aging. Locomotor activity assay demonstrated that the age-dependent changes in heat nociception did not stem from the general decline in muscular activity. However, we identified pain-related gene expression alteration with aging. We anticipate that our findings would help opening a new window onto developing the optimal pain treatment for the elderly.

**Key Words:** Aging, Pain, *Drosophila*

## INTRODUCTION

Aging is a combination of processes that alters the functional capacity and appearance over time. Apart from harmless changes like wrinkles, aging increases the susceptibility to diseases such as atherosclerosis, cancer, diabetes and Alzheimer's disease (Stern *et al.*, 2003; Finkel *et al.*, 2007; Sue Kirkman *et al.*, 2012; Hebert *et al.*, 2013). Aging also affects the cellular system that is responsible for decoding environmental stimuli (Freiher *et al.*, 2013). An important aging-associated alternation happens in pain sensation (Lautenbacher *et al.*, 2005; McCleane and Smith, 2006; Huang *et al.*, 2010; Yezierski, 2012). Despite the indispensable role in survival, the unpleasant feeling of destructive stimuli or tissue damages is interpreted differently as the organism becomes older. Several studies have shown changes in pain threshold with advancement of age (Lautenbacher *et al.*, 2005; McCleane and Smith, 2006). For instance, heat pain sensitivity is slightly diminished while pressure pain sensitivity is increased in the elderly. However, we do not know the molecular mechanisms of aging that affect sensation of painful stimuli.

In this study, we aimed to explore age-dependent changes in pain perception at the molecular level. In particular, we focused on heat nociception, as it is the most thoroughly char-

acterized painful stimulus to date (Julius, 2013). Many cellular components should work in concert via an exquisite intricate process to adequately and efficiently decode the meaning of noxious thermal assaults. Complexity of heat nociception interpretation is drastically increased with aging because all cellular components that are associated with pain sensation are subject to age-related anatomical and functional changes. Therefore, we decided to use *Drosophila* as a relatively simple organism to uncover the age-dependent modifications in heat nociception.

*Drosophila* is inexpensive to keep in the laboratory yet sufficiently sophisticated to exhibit effective negative reinforcing behavioral responses in exposure to heat stimuli. This characteristics allowed many researchers to successfully use the fruit fly as an animal model in antinociception research. (Spatz *et al.*, 1974; Heisenberg *et al.*, 2001; Tobin and Bargmann, 2004; Manev and Dimitrijevic, 2005) In this study, aged *Drosophila* demonstrated a decrease in temperature tolerance and a reduction in high temperature thermal avoidance. At the molecular level, our findings were explained, at least in part, by altered pain-related gene expression. With a staggering increase in the aged population who suffers from complications of persistent pain, we anticipate that our study would advance our understanding of the pathophysiology of pain perception and pave

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the road for developing optimal pain treatments in the elderly.

## MATERIALS AND METHODS

### Fly husbandry

w1118 strain *Drosophila* was obtained from Bloomington *Drosophila* Stock Center (Bloomington, IN, USA). Flies were maintained in plastic bottles containing standard *Drosophila* diet. The diet was prepared by mixing 8.5 g corn meal, 5 g sugar, 1.5 g live yeast and 0.46 g agar in 100 ml distilled water. Then, the mixture was boiled and cooled down to approximately 80°C. 1% v/v propionic acid was added to the mixture to prevent mold growth. The bottles housing flies were kept in 12-hours light/dark cycles, at 25°C and 60% humidity controlled fly-incubator as previously described. (Kim, 2013) Flies were transferred to bottles containing freshly prepared diet approximately every 3 weeks to refresh the stock.

### Crossings, diet preparation

10 virgin female flies and 3-5 male flies were kept together inside bottles containing standard *Drosophila* diet for 4 days. Then, parents were separated and eggs were hatched to larvae, which metamorphose into the adult fly through the pupae stage. 14 days after crossings, female flies were sorted as age-associated phenotypic changes are more extensively characterized in female flies. (Partridge *et al.*, 2005) CO<sub>2</sub> was used to briefly anesthetize the flies for sorting, which was completed within 3 min to minimize CO<sub>2</sub> toxicity. Sorted female flies were fed on *ad libitum* (AL, 5% YE) diet. The recipe for preparation of AL was same as the standard *Drosophila* diet with the exception of replacement of 1.5% live yeast with 5% yeast extract.

### Temperature tolerance assay

At the age of 1 or 15 days, 5 flies were transferred to 14 ml round-bottom polystyrene tubes (17×100 mm, SPL life sciences, Korea), which were immersed in a preheated water bath in the range of 36°C to 46°C. Exposure to high temperature initiated the defensive behavior that ended with incapacitation. Lag time (seconds) until paralysis of all flies was recorded.

### Thermal avoidance assay

At the age of 1 or 15 days, 7 flies were transferred to a petri dish (60 mm in diameter, 10 mm in height, with a demarcated horizontal median line), which was floated on water bath for 4 min. The number of flies that move to the cooler top part of the dish (above the horizontal median line) was counted and avoidance percentage was calculated. Water bath temperature was set from 40°C to 46°C.

### Measurement of locomotor activity

At the age of 1, 15 and 30 days, flies were transferred to new polystyrene vials (25 mm diameter) that contain freshly made AL diets. Then, the vials were placed into the *Drosophila* LAM25 Locomotor Activity Monitor (Trikinetics, Waltham, MA, USA) and data were acquired and processed with DAMSystem 308 software (Trikinetics, Waltham, MA, USA).

### RNA preparation, cDNA synthesis and quantitative polymerase chain reaction (qPCR)

According to the manufacturer's protocols, total RNA was

isolated from five flies using RNeasy mini kit® (Qiagen, Valencia, CA, USA) and cDNA was prepared from the isolated total RNA with QuantiTect Reverse Transcription Kit® (Qiagen, Valencia, CA, USA). To quantify the amount of transcripts, SYBR Green based qPCR was performed with SensiFast SYBR NO-Rox Kit® (Taunton, MA, USA) using a Rotor-Gene Q thermocycler (Qiagen, Valencia, CA, USA) under the following conditions: 95°C, 5 seconds for denaturation, 55°C, 20 seconds for annealing and extension. Following gene specific primer sets were used: *β-tubulin* (CG9277) F, ACA-TCC-CGC-CCC-GTG-GTC, R, AGA-AAG-CCT-TGC-GCC-TGA-ACA-TAG; *wengen* (CG6531), F, ATC-TTC-CAG-CCA-CAA-CAC-G, R, AGG-ATT-CGT-CGC-TCC-TGA-T; *painless* (CG15860), F, CCA-GGT-TGT-CGT-GAC-TTC-ATT, R, CTT-GTC-CAG-CTT-CTT-GTT-GAT-G; *dTRPA1* (CG5751), F, ATA-CAC-GAA-GCG-GCC-AAG, R, GCC-CTC-CGA-GTC-GTA-GAA-G; *eiger* (CG12919), F, CGA-CGA-GTT-CCA-AAA-GGA-GT, R, GTC-GTC-GTC-CTC-CTC-ATC-C; *straightjacket* (CG12295), F, GGC-ATG-GAG-CTG-TTT-CAT-CT, R, CCT-TTG-CAT-CCT-TGA-AAC-TCT-C; *hedgehog* (CG4637), F, GGA-TTC-GAT-TGG-GTC-TCC-TAC, R, GGG-AAC-TGA-TCG-ACG-AAT-CT. The specificity of amplicons was verified with melting curve analysis and the messenger levels were normalized using  $\beta$ -tubulin, as the internal control, and calculated according to the  $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001).

### Reagents

*Drosophila* diet ingredients (corn meal, sugar, live yeast, yeast extract and agar) were purchased from Hansol Tech Inc. (Seoul, Korea). Propionic acid (cat # 64655-0430) was purchased from Junsei Chemical Co. Ltd. (Tokyo, Japan).

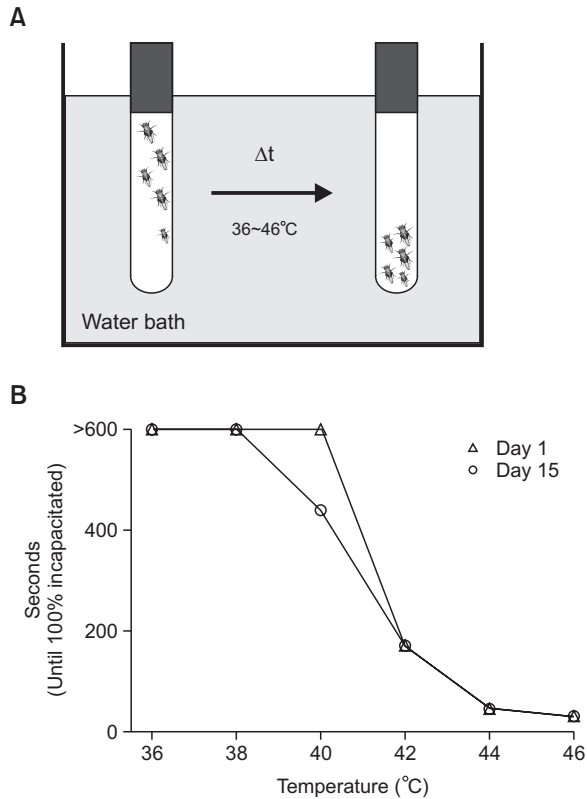
### Statistics

Unpaired two-tailed Student's *t*-test and ANOVA with *Bonferroni post-hoc* test were used for the statistical comparison between two independent groups and more than two independent groups, respectively.

## RESULTS

### Temperature tolerance was decreased with age

To investigate changes in pain sensation with aging, temperature tolerance was tested on *Drosophila* as previously described. (Neely *et al.*, 2011) Plastic tubes housing young (Day 1) or middle-aged (Day 15) flies were immersed in water bath with preset temperature ranging from 36°C to 46°C. Since heat is rapidly disseminated through the tubes, flies sensed an increase in temperature and showed defensive behaviors on exposure to a noxious heat assault. As flies were confined within completely immersed tubes, sustained exposure to elevated temperature ultimately incapacitated the flies. Therefore, we decided to measure the lag time to incapacitation of all flies as an index of temperature tolerance. The spiking frequency begins to increase at around 38°C in the nerve of *Drosophila* (Tracey *et al.*, 2003), so we set the lowest water bath temperature to 36°C. At 36°C, notable changes in behavior was not observed in young or middle-aged flies. Both groups were not incapacitated before 600 seconds. Therefore, the temperature was gradually increased by 2°C to determine the optimal condition to examine age-dependent heat-associated pain behavior changes. Interestingly, it was found that at 40°C,

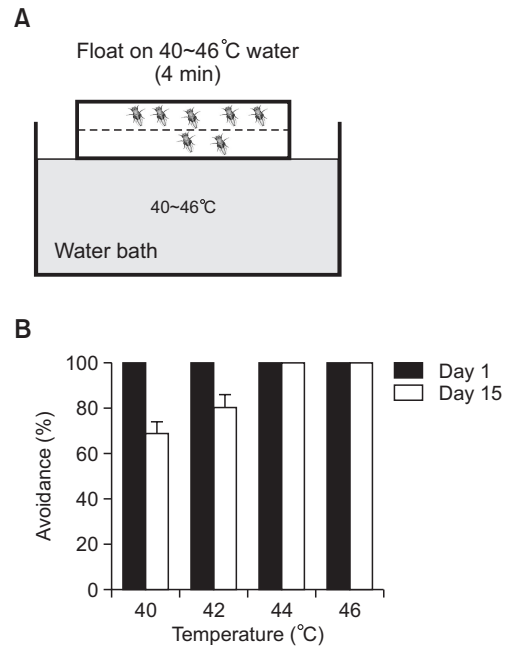


**Fig. 1.** Temperature tolerance was decreased with age. (A) Schematic diagram depicting the protocol of temperature tolerance assay. Plastic tubes entraining 5 flies were immersed in water bath and lag times ( $\Delta t$ ) until all flies became incapacitated were measured. (B) By increasing temperature of water bath from 36°C to 46°C with 2°C increments, the lag times were recorded with young (Day 1, open triangle) and middle-aged (Day 15, open circle) flies. Each symbol presents mean value calculated from 3 independent experiments.

young flies survived (>600 sec) while middle-aged flies were all incapacitated by 438.3 sec (Fig. 1B). Further increase in temperature rapidly incapacitated flies without revealing any difference in temperature tolerance between young and middle-aged groups. These observations indicated altered ability to resist a thermal assault with age.

**High temperature thermal avoidance responses were reduced with age**

Despite the clear demonstration of age-dependent reduction of temperature tolerance, cellular mechanisms that underlie these changes are not completely investigated yet. We hypothesized that middle-aged flies are less sensitive to changes in temperature, which prevents them from rapidly avoiding a noxious heat assault, thereby facilitating incapacitation. To test this hypothesis, high temperature thermal avoidance was performed as described previously (Neely *et al.*, 2011; Milinkeviciute *et al.*, 2012). In this assay, water bath temperature was preset to range from 40°C to 46°C. Young or middle-aged flies were entrained in a clear polystyrene chamber, which was floated on the water bath for 4 min. Since a noxious heat assault triggers thermal avoidance behavioral responses, we counted the number of flies remaining on the

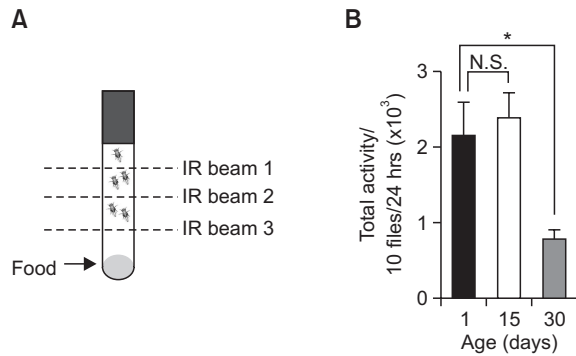


**Fig. 2.** High temperature thermal avoidance responses were reduced with age. (A) Schematic representation of thermal avoidance assay. Plastic chambers housing 7 flies were floated on water bath which was set at 40-46°C for 4 min. Flies staying below the designated median line (dotted line) were considered to have defects in noxious heat sensation. Number of flies avoiding the hot plate (staying on the top half) is divided by total fly number to calculate avoidance percentage. (B) By increasing water bath temperature from 40°C to 46°C in 2°C increments, thermal avoidance was tested on young (Day 1, black bars, n=5 for each temperature point) and middle-aged flies (Day 15, white bars, n=5 for each temperature point). Data are presented as mean  $\pm$  S.E.M.

lower half of the chamber in which temperature is higher than the upper half. It was based on the assumption that reduction of thermal pain sensitivity will restrain flies from moving to the cooler upper half. Total number of transferred flies was used as the denominator to calculate thermal avoidance percentage using this formula: % avoidance=[(total number-number in the lower half of the chamber)/total number] $\times$ 100. Young (Day 1) flies were found to be very sensitive to changes in temperature. All flies moved to the upper half at all tested temperatures. In a stark contrast, only 68.6% and 80% of middle-aged (Day 15) flies showed thermal avoidance response at 40°C and 42°C, respectively (Fig. 2B). Further increase in the temperature of the water bath to 44°C or 46°C elicited 100 % thermal avoidance response (Fig. 2B). These observations imply that although a motivating force that drives avoidance responses against painful thermal stimuli remains intact, the temperature threshold triggering avoidance responses may be altered with aging.

**Spontaneous locomotor activity remained unchanged with age**

To investigate cellular mechanisms underlying the changes associated with thermal pain behavior, we first tested if age-dependent decline of locomotor activity is responsible for the reduction of high temperature thermal avoidance response. Specifically, it is possible that despite unaltered nociception,



**Fig. 3.** Monitoring age-dependent total spontaneous locomotor activity. (A) Clear plastic vials entraining 10 flies were placed on the *Drosophila* activity locomotor monitor. The machine counts how many times flies cross lines separated by IR (infrared) beams. (B) Total spontaneous locomotor activity was recorded from 4 pm to next day's 4 pm with young (Day 1, black bar,  $n=4$ ), middle-aged (Day 15, white bar,  $n=5$ ) and old flies (Day 30, gray bar,  $n=5$ ). Data are presented as mean  $\pm$  S.E.M. \* $p<0.05$ , N.S. stands for not significant. One-Way Anova with Bonferroni post-hoc test.

the decline in muscular functions hinders flies from moving away from noxious heat. To test this possibility, spontaneous locomotor activity was monitored in young (Day 1) and middle-aged (Day 15) flies for 24 hours (Fig. 3A). No significantly different movement activity was observed. As a control experiment, old (Day 30) flies were subjected to the same assay, which revealed a considerable reduction of spontaneous locomotor activity compared to those of young and middle-aged flies. These findings suggest that muscular function is not impaired at least until the middle age. Besides, this result implied that utilizing old flies (Day 30) was not suitable for pain behavior assays affected by locomotor activity. Therefore, we ruled out the possibility of age-dependent decline in locomotor activity as the basis for a diminished thermal avoidance response with aging.

#### Pain-associated gene expression levels were altered with age

Flies express sets of genes that are known to play an important role in thermal pain sensation. (Milinkeviciute *et al.*, 2012) Among them, we examined expression of 6 genes whose functions in pain sensation are relatively well characterized. These genes are *painless*, *dTRPA1*, *straightjacket*, *eiger*, *wengen* and *hedgehog*. *painless* and *dTRPA1* code for ion channels involved in triggering action potentials when nerves are exposed to harmful thermal stimuli in *Drosophila*. (Tracey *et al.*, 2003; Bautista *et al.*, 2006; Kang *et al.*, 2010). *straightjacket*, a peripheral component of different  $Ca^{2+}$  channels, has been demonstrated to be critical in heat nociception. (Neely *et al.*, 2010) *eiger* and *wengen* are TNF-like factor and its receptor, respectively. *hedgehog* regulates the threshold of thermal pain sensation by sensitizing pain signaling (Babcock *et al.*, 2009). Quantitative PCR revealed much less transcripts of *painless*, *straightjacket*, *wengen* in middle-aged flies than young flies. Messenger levels of *eiger* and *hedgehog* remained unchanged but *dTRPA1* expression was found to be slightly increased with age.

## DISCUSSION

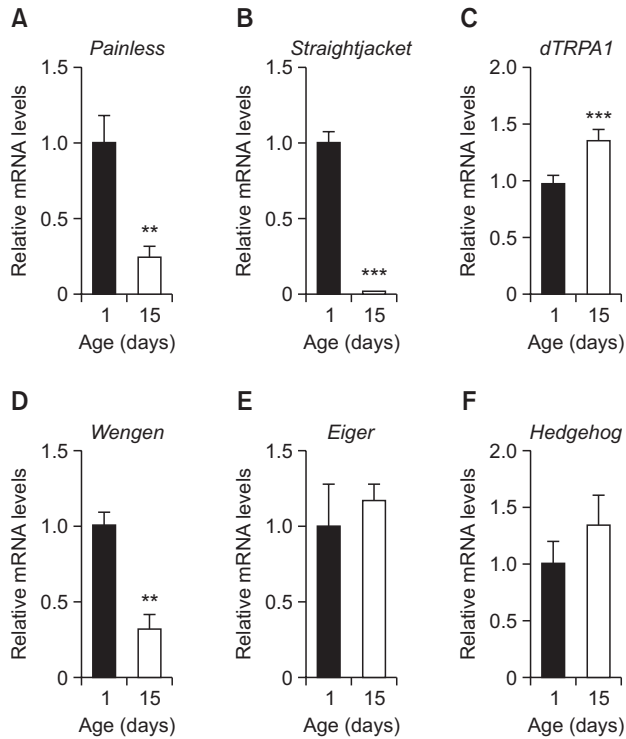
Socioeconomic burden of pain is estimated to be over \$560 billion in United States, which is similar to the combined costs of heart diseases and cancer (Institute of Medicine (US) Committee on Advancing Pain Research, 2011, *Relieving Pain in America: A Blueprint for Transforming Prevention, Care, Education, and Research*, Washington (DC)). Moreover, an overwhelming increase in the elderly population who suffer from chronic diseases that are inevitably associated with persistent pain demands a breakthrough in the field of pain research for better pain management. Yet, rapid achievements in understanding the underlying mechanisms of pain sensation is often hindered by ethical issues and difficulties in executing well-controlled experiments on mammalian animal models. To circumvent these issues, it has been proposed to utilize the fruit fly as an alternative *in vivo* pain model (Manev and Dimitrijevic, 2005).

Traditionally, *Drosophila* represents a preferred animal model for aging research owing to inexpensiveness to maintain colonies, ease in genetic manipulation and short lifespan (He and Jasper, 2014). These advantages are self-explanatory by studies that revealed important signaling pathways involved in aging process (Katewa and Kapahi, 2011; Partridge *et al.*, 2011). Therefore, we envisioned *Drosophila* as a captivating *in vivo* model for studying the relationship between aging and reaction to pain.

The importance of our findings is twofold. First, we found that aging significantly affected fly's ability to trigger defensive behaviors against heat stimuli (Fig. 1, 2). When exposed to heat, middle-aged flies were quickly incapacitated (Fig. 1) and only a small fraction of the old flies moved away from the heat source (Fig. 2). These age-associated changes in behavioral responses against a thermal assault could be the result of several factors. One simple explanation would be an overall age-dependent decline in general health, which may facilitate the incapacitating processes (Fig. 1) and decelerate cellular signaling necessary to trigger a thermal avoidance response (Fig. 2). However, the movement assay did not support this postulation, failing to reveal an obvious difference in general muscular capacity between young and middle-age flies (Fig. 3), providing indirect evidence that age-related weakening of general health may not be sufficient to explain the observed behavioral changes. Alternatively, we hypothesized that aging increases the threshold for heat pain, which may leave the aged flies exposed to a thermal assault for an extended period without triggering appropriate defensive responses (Fig. 2), thereby accelerating incapacitation (Fig. 1). Our findings are in agreement with previous reports showing an increase in pain threshold in the elderly (Kaye *et al.*, 2010). However, there is a paucity in the literature focusing on age-related molecular mechanisms underlying changes in pain threshold.

That being said, the second important aspect of our study is that we provide, to the best of our knowledge, the first molecular insight on age-related changes in pain threshold. Pain perception is affected not only by sensory discriminative components such as location, intensity and duration of tissue damages but also by motivation affective components including emotional aspects and reaction to painful stimuli. Although pain perception is a subjective experience, it is known that pain signal is initiated in the specialized primary afferent sensory neurons named nociceptors. In case of nociception, pungent chemical

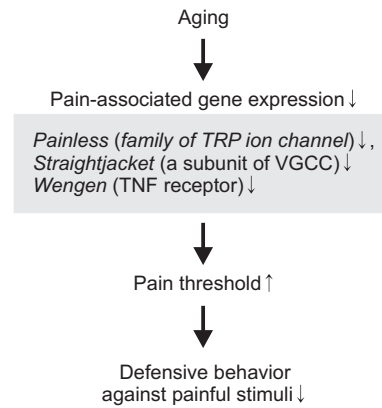




**Fig. 4.** Changes in pain-associated gene expression profile with age. (A-F) SYBR Green based qPCR was performed to compare levels of pain-related gene expression between young (Day 1) and middle-aged (Day 15) flies.  $\Delta\Delta Ct$  method was used to calculate relative gene expression with  $\beta$ -tubulin being the internal control. Consistent data were obtained with 2-3 biological replications. Data are presented as mean  $\pm$  ranges. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , Student's t-test.

mediators originating from outside (pepper, mustard and etc.) or inside the cells (NGF, bradykinin and ATP) activate their corresponding receptors to transmit the information to the spinal cord, and then to the brain via generation of unique patterns of action potentials (Julius, 2013). Consequently, much effort has been put to elucidate the molecular identity of special receptors that recognize painful mediators. These efforts have uncovered key pain-associated molecules that can be roughly categorized into ion channel family and nociceptor sensitizing signaling modulators (Willis, 2001; Julius, 2013; Bennett and Woods, 2014).

It is estimated that *Drosophila* conserves up to 75% of human disease genes (Bier, 2005). As such, mammalian homologues of pain-related genes are expressed in *Drosophila*. In the ion channel family, *painless* and *dTRPA1*, members of TRP ion channels, were characterized as the heat pain transducer in *Drosophila* (Tracey *et al.*, 2003; Neely *et al.*, 2011). Besides, *straightjacket*, a subunit of voltage-gated  $Ca^{2+}$  channel, is recently identified to be involved in heat nociception by genome-wide screening. (Neely *et al.*, 2010) We found a dramatic decrease in the expressions of *painless* and *straightjacket* with increasing age (Fig. 4A and D). These findings are in agreement with our hypothesis of increased pain threshold with aging that decreases the probability to trigger appropriate signaling in response to increased temperature. Intriguingly, *dTRPA1* expression level was slightly but consistently



**Fig. 5.** Summary of results. Aging decreases expression of pain-associated gene expression such as *painless* (family of transient receptor potential (TRP) ion channel), *straightjacket* (a subunit of voltage-gated  $Ca^{2+}$  channel (VGCC)), and *wengen* (tumor necrosis factor (TNF) receptor), which could increase pain threshold, thereby declining defensive behavior against painful stimuli.

increased with aging (Fig. 4E). Although *Drosophila* TRPA1 preferentially functions as a heat sensor, its physiological roles are not confined to thermal sensing as its mammalian TRPA1 ortholog detects a wide array of distinct physical, chemical and thermal stimuli. Thus far, *dTRPA1* has been linked to many other cellular functions such as embryogenesis, (Hunter *et al.*, 2014) circadian activity, (Lee and Montell, 2013) avoidance responses against citronellal vapor -a plant-produced insect repellent- (Kwon *et al.*, 2010) and chemical avoidance in gustatory receptor neurons. (Kim *et al.*, 2010) Therefore, it is plausible that *dTRPA1* needs to remain at a relatively constant level to play its versatile cellular functions despite advancing in age, which could be tested in future projects.

In addition to aforementioned ion channels, which are considered as direct heat pain sensors, cells harbor signaling molecules to modify sensitivity of sensors as an alternative way to regulate heat pain sensation. Indeed, *eiger* and *wengen* are *Drosophila*'s homologues of mammalian tumor necrosis factor (TNF) and its receptor, respectively. *hedgehog* (hh) is known to be involved in UV-induced thermal allodynia (Cunha *et al.*, 1992; Babcock *et al.*, 2009; Babcock *et al.*, 2011). Our findings show that the transcript level of *wengen* decreased significantly with aging while the transcript levels of *eiger* and *hh* remained unchanged. *Painless* is reported to be sensitized by both TNF and hh signalings. (Babcock *et al.*, 2011) Therefore, a decrease in *wengen* expression may contribute to a reduction in *painless*-mediated thermal pain sensitivity. On the contrary, *dTRPA1* is only affected by hh signaling (Babcock *et al.*, 2011). Previously, we showed that *dTRPA1* level is slightly up-regulated with aging. Consistent with this observation, an unchanged *hh* level indicates that *dTRPA1* signaling in *Drosophila* has to remain active despite aging, probably for fulfilling its versatile cellular functions.

In the future, we imagine it would be a highly interesting project to examine age-associated changes in pain behavior with genetically modified flies. For instance one may expect knocking-out *painless* or/and *straightjacket* or/and *wengen* would abolish the observed alterations in pain behavior with advancing age. However, we could not completely eliminate the possibility that compensatory up-regulation of unidentified

genes involved in pain signaling during certain developmental stages could mask the effect of knock-out. Nonetheless, such genetic approaches would be worthy of executing to provide additional evidence supporting the idea that *painless*, *straight-jacket* and *wengen* play an important role in altering pain sensation with aging.

Finally, as aged flies are quickly incapacitated (Fig. 1) and exhibit diminished ability to escape from the heat source (Fig. 2), altered pain-associated gene expression could prevent the elderly from prompting appropriate response to painful stimuli, which could lead to irreversible tissue damage. Actually, older people are less likely to vocalize their experience despite the importance of pain assessment for effective management (Kumar, A. and Allcock, N. 2008, Pain in Older People; Reflections and experiences from an older person's perspective, Age UK). Therefore, we hope that our findings unraveling age-associated changes in pain sensation at the molecular level would be informative for health professionals to provide optimal pain care in the elderly.

## ACKNOWLEDGMENTS

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