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## Title page

# Melatonin treatment reverts aged-related changes in guinea pig gallbladder neuromuscular transmission and contractility

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**Running Title Page**

**Running Title:** Melatonin and aged gallbladder

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**Abbreviations:**

EFS, electrical field stimulation; CGRP, calcitonin gene-related peptide; CCK, cholecystokinin; GI, gastrointestinal tract; ACh, acetylcholine; BSA, bovine serum albumin; DTT, 1,4-dithio-DLthreitol; Phosphate-buffered saline solution , PBS; Nonidet

P-40, octylphenyl-polyethylene glycol; FITC-phalloidin, fluorescein isothiocyanate-labeled phalloidin; L-NAME, Nω-nitro-L-arginine methyl ester; $Bt_3$-Ins(1,4,5)$P_3$-PM, 2,3,6-Tri-O-Butyryl-myo-Inositol-1,4,5-trisphosphate-Hexakis(propionoxymethyl) Ester; mN, milliNewtons; ANOVA, Analysis of variance; CCE, capacitative calcium entry; $IP_3$, D-*myo*-inositol 1,4,5-triphosphate,;

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**Abstract**

The incidence of gallbladder illness increases with age but the altered mechanisms leading to gallbladder dysfunction are poorly understood. Here we determine the age-related alterations in gallbladder contractility and the impact of melatonin treatment. Isometric tension changes in response to electrical field stimulation (EFS) and to agonists were recorded from guinea pig gallbladder muscle strips. $[Ca^{2+}]_i$ was determined by epifluorescence microscopy in fura-2 loaded isolated gallbladder smooth muscle cells, and F-actin content was quantified by confocal microscopy. Aging reduced neurogenic contractions, which was associated to the impairment of nitrergic innervation and to increased responsiveness of capsaicin-sensitive relaxant nerves, possibly involving calcitonin gene-related peptide (CGRP). Melatonin treatment for four weeks restored neurogenic responses to normal values, with an associated recovery of nitrergic function and the disappearance of the capsaicin-sensitive component. Aging also reduced the contractile responses to cholecystokinin (CCK) and $Ca^{2+}$ influx. The impaired contractility only correlated with diminished $Ca^{2+}$ mobilization in response to activation of $Ca^{2+}$ influx. Melatonin improved contractility and increased smooth muscle F-actin content without changing $Ca^{2+}$ homeostasis. In conclusion, aging impairs gallbladder function as the result of changes in the inhibitory neuromodulation of smooth muscle contractility and the reduction in the myogenic response to contractile agonists. Impaired contractility seems to be related to decreased $Ca^{2+}$ influx and damage of contractile proteins. Melatonin significantly ameliorated these age-related changes.

## INTRODUCTION

Population aging was one of the most distinctive demographic events of the 20th century and according to the increase in the mean life expectancy, the elderly population will constitute the 20% of the world population in the next 50 years (Centers for Disease Control And Prevention (CDC), 2003). Aging is understood mainly as a slow, gradual, and passive process influenced by interplay of multiple genetic and environmental factors. It is widely accepted that aging is not a disease but the borderline between pathological and normal aging is quite narrow. Although there are many kinds of 'non-disease' aging manifestations including, blood vessel stiffening, skin slacking, joint stiffening, etc, the molecular structures of aged organisms are abnormal (Bailey, 2001). These molecular abnormalities could be responsible of the increase in the incidence and prevalence in the elderly of the so called age-related diseases such as neurodegenerative disorders (Troulinaki and Tavernarakis, 2005), cardiovascular disease (Ferrari et al., 2003), diabetes (Winer and Sowers, 2004), incontinence (Nasr and Ouslander, 1998), etc.

Regarding the gastrointestinal tract (GI), the prevalence of dysfunctions related to motility is higher in older than in younger adults (Camilleri et al., 2000). However, there are very few studies concerning the cellular and molecular mechanisms whose alteration could be responsible for such us dysfunctions in aging. Thus, it has been reported that neuronal loss occurs with age in the myenteric plexus of the GI in several species including man (Gomes et al., 1997). However, it is unclear whether cell death affects all classes of myenteric neurons nonselectively or is confined to specific phenotypes (Wade and Cowen, 2004). Another important component that could be affected by aging, leading to contractility impairment, is smooth muscle itself. In this regard, information regarding the putative changes in cellular mechanisms of smooth muscle contraction is

scanty (Bitar and Patil, 2004) . Acetylcholine-induced contractile responses are impaired in colon (Roberts et al., 1994) which could be due to the limited cell length distribution found in aged-animals but also to alteration of the intracellular cell signalling pathways related to activation of protein kinase C and cytoskeleton reorganization (Bitar, 2003). There is little information regarding the possible changes in $Ca^{2+}$ homeostasis during aging in smooth muscle in general and gastrointestinal smooth muscle in particular, where it has been described an aged-related decline in the L-type $Ca^{2+}$ currents both in rat and human colon smooth muscle cells (Xiong et al., 1993; Xiong et al., 1995).

Knowledge of the molecular mechanisms involved in aging is required to develop strategies to preserve the quality of life of the increasingly aging population. Degenerative changes associated with aging have been related to progressive damage by reactive oxygen and nitrogen species in those situations where the anti-oxidative defence systems fails to eliminate them (Sohal and Allen, 1990). Recently, melatonin, the main hormone of the pineal gland was proposed as a protective agent against macromolecular destruction associated with longevity (Reiter et al., 1996). The protective effects of melatonin could be related to the ability of the hormone to synchronize circadian rhythms and thereby to reduce biological stress, as well as to its direct free radical scavenging activity and its indirect antioxidants properties (Reiter et al., 2002).

In the current study we explored the aged-related alterations of neuromuscular transmission, gallbladder contractility and smooth muscle $Ca^{2+}$ homeostasis. Our results show that aging-induced gallbladder impairment is primarily related to changes in the inhibitory innervation of the organ and to a decrease in the myogenic response to excitatory hormones and neurotransmitters. Melatonin treatment reversed the functional impairment caused by aging at the level of both the neuromuscular transmission and the myogenic contractility despite being ineffective in the restoration of $Ca^{2+}$ homeostasis.

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## Materials and Methods

### Tissue preparation

Gallbladders were removed from 4-and 20-mo-old female guinea pigs after deep halothane anaesthesia and cervical dislocation, and were immediately placed in cold Krebs-Henseleit solution (for composition see *Solutions and drugs*) at pH 7.35. The gallbladder was opened from the end of the cystic duct to the base, and trimmed of any adherent liver tissue. After the preparation was washed with the nutrient solution to remove residual bile, the mucosa was carefully dissected away.

A group of aged animals was treated orally with melatonin (2.5 mg/Kg/day). Melatonin was dissolved in glucose solution (0.5%) and placed in the oropharynx by a syringe. This treatment was applied daily at the same time, just before the light in the animal house was switched off (7 p.m.). All the experiments were conducted according to the guidelines of Animal Care and Use Committees of the University of Extremadura.

### Measurement of melatonin in serum

Melatonin levels were determined in the different age groups by using a commercial radioimmunoassay kit (IBL, Hamburg), according to the manufacturer's instructions. The kit consisted of $^{125}$I-melatonin (0.68 μCi/ml), rabbit anti-melatonin serum, melatonin standards, delipidizing agent, assay buffer, precipitating antiserum, and controls (lyophilized plasma samples). Results were expressed in pg/ml.

### Contraction recording of guinea pig gallbladder smooth muscle strips

Gallbladder strips (measuring ~3 x 10 mm) were placed vertically in a 10 ml organ bath filled with Krebs-Henseleit solution maintained at 37 °C and gassed with 95% $O_2$ - 5% $CO_2$. Isometric contractions were measured using force displacement transducers that

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were interfaced with a Macintosh computer using a MacLab hardware unit and software (ADInstruments; Colorado Spring, CO, USA). The muscle strips were placed under an initial resting tension equivalent to 1.5 g load and allowed to equilibrate for 60 min, with solution changes every 20 min. Every strip coming from a given animal was used in a different experimental protocol.

Intrinsic nerves were activated by electrical field stimulation (EFS) with a pair of external platinum ring electrodes (0.7 mm in diameter) connected to a square wave stimulator (Cibertec CS9/3BO) programmed through Scope software application from MacLab (AD Instruments). Trains of stimuli (0.3 ms duration, 5-40 Hz, 350 mA current strength) were delivered for 10 s at 3 min intervals. After construction of a frequency response curve and in order to pharmacologically dissect the neurogenic responses, antagonists were added to the organ bath for 20 min, and then the EFS protocols were repeated.

In some experiments the contractile effects of acetylcholine (ACh), cholecystokinin (CCK), KCl and $Ca^{2+}$ restoration after intracellular $Ca^{2+}$ store depletion were tested..

**Cell isolation**

Gallbladder smooth muscle cells were dissociated enzymatically using a previously described method (Pozo et al., 2002). Briefly, the gallbladder was cut into small pieces and incubated for 34 min at 37°C in enzyme solution (for composition see Solutions and drugs) supplemented with 1 mg/ml BSA, 1 mg/ml papain, and 1 mg/ml DTT. Next, the tissue was transferred to fresh enzyme solution containing 1 mg/ml BSA, 1 mg/ml collagenase, and 100 μM $CaCl_2$ and incubated for 9 min at 37°C. The tissue was then washed three times using cold enzyme solution, and the single smooth muscle cells were isolated by several passages of the tissue pieces through the tip of a fire-polished glass Pasteur pipette. The resultant cell suspension was kept in enzyme solution at 4°C until use, generally within 6 h. All experiments involving isolated cells were performed at

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room temperature (22°C). The cell length was similar in the three age groups (young adult: 44.68 ± 1.62; aged: 45.49 ± 1.83; melatonin: 43.07 ± 1.36 μm, n = 98, 61 and 96 cells, respectively)

**Cell loading and $[Ca^{2+}]_i$ determination**

$[Ca^{2+}]_i$ was determined by epifluorescence microscopy using the fluorescent ratiometric $Ca^{2+}$ indicator fura 2 as previously described (Morales et al., 2004). Isolated cells were loaded with 4 μM fura 2-AM at room temperature for 25 min. An aliquot of cell suspension was placed in an experimental chamber made with a glass poly-D-lysine treated coverslip (0.17 mm thick) filled with $Na^+$-HEPES solution (for composition see Solutions and drugs) and mounted on the stage of an inverted microscope (Eclipse TE2000-S; Nikon). After cell sedimentation, a gravity-fed system was used to perfuse the chamber with $Na^+$-HEPES solution in the absence or presence of the experimental agents. Cells were illuminated at 340 and 380 nm by a computer-controlled monochromator (Optoscan, Cairn Research) at 0.3 cycles/s, and the emitted fluorescence was selected by a 510/40-nm band-pass filter. The emitted fluorescence images were captured with a cooled digital charge-coupled device camera (ORCAII-ER; Hamamatsu Photonics) and recorded using dedicated software (Metafluor, Universal Imaging). The ratio of fluorescence at 340 nm to fluorescence at 380 nm ($F_{340}/F_{380}$) was calculated pixel by pixel and used to indicate the changes in $[Ca^{2+}]_i$. A calibration of the ratio for $[Ca^{2+}]_i$ was not performed in view of the many uncertainties related to the binding properties of fura 2 with $Ca^{2+}$ within smooth muscle cells.

**F-actin Content Measurement**

The F-actin content of gallbladder smooth muscle cells was determined according to a previously published procedure (Morales et al., 2005b). Briefly, samples of gallbladder smooth muscle cell suspensions (200 μl) in $Na^+$-HEPES solution were transferred to 200

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µl ice-cold 3% (w/v) formaldehyde in PBS (for composition see Solution and drugs) for 10 min. Fixed cells were permeabilised by incubation for 10 min with 0.025 % (v/v) Nonidet P-40 detergent dissolved in PBS. Cells were then incubated for 30 min with fluorescein isothiocyanate-labeled phalloidin (FITC-phalloidin; 1 µM) in PBS supplemented with 0.5 % (w/v) bovine serum albumin (BSA). After incubation, the cells were collected by centrifugation for 2 min at 10000 x *g* and resuspended in PBS. Staining of actin filaments was measured using a confocal laser-scanning system (model MRC-1024, Bio-Rad) with excitation wavelength of 488 nm and emission at 515 nm. The cell F-actin content was quantified as arbitrary units of fluorescence using the ImageJ software (NIH, Bethesda).

**Solutions and drugs**

The Krebs-Henseleit contained (in mM): 113 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 25 $NaHCO_3$, and 11.5 D-glucose. This solution had a final pH of 7.35 after equilibration with 95% $O_2$-5% $CO_2$. The enzyme solution used to disperse cells was made up of (in mM): 10 HEPES, 55 NaCl, 5.6 KCl, 80 sodium glutamate, 2 $MgCl_2$, and 10 D-glucose, with pH adjusted to 7.3 with NaOH. The $Na^+$-HEPES solution contained (in mM): 10 HEPES, 140 NaCl, 4.7 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 10 D-glucose, with pH adjusted to 7.3 with NaOH. The $Ca^{2+}$-free $Na^+$-HEPES solution was prepared by substituting EGTA (1 mM) for $CaCl_2$. The PBS used in F-actin studies contained (in mM): NaCl 137, KCl 2.7, $Na_2HPO_4$ 5.62, $NaH_2PO_4$ 1.09 and $KH_2PO_4$ 1.47 with pH adjusted to 7.2. Drug concentrations are expressed as final bath concentrations of active species. Drugs and chemicals were obtained from the following sources: acetylcholine (ACh), atropine, caffeine, CGRP fragment 8-37 ($CGRP_{8-37}$), CCK fragment 26–33 (CCK-8) sulfated, DTT, glibenclamide, ionomycin, L-NAME, melatonin, thapsigargin, and FITC-phalloidin were from Sigma Chemical (St. Louis, MO); *E*-capsaicin was from

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Tocris (Bristol, UK), $Bt_3$-Ins(1,4,5)$P_3$-PM was from SiChem (Bremen, Germany); fura 2-AM was from Molecular Probes (Molecular Probes Europe, Leiden, Netherlands); collagenase was from Fluka (Madrid, Spain); and papain was from Worthington Biochemical (Lakewood, NJ). Other chemicals used were of analytical grade from Panreac (Barcelona, Spain). Stock solutions of atropine, capsaicin, fura 2-AM, thapsigargin, ionomycin, were prepared in DMSO, and FITC-phalloidin was prepared in ethanol. The solutions were diluted such that the final concentration of DMSO was ≤0.1% vol/vol. This concentration of DMSO did not interfere with fura 2 fluorescence. Melatonin was prepared in 80 mM glucose solution.

**Quantification and statistics**

Results are expressed as means ± SEM of n cells or gallbladder strips. Gallbladder tension is given in milliNewtons (mN)/mg of tissue. All results from $[Ca^{2+}]_i$ determinations are given as $\Delta F_{340}/F_{380}$.Statistical differences between means were determined by Student's *t*-test. Differences between multiple groups were tested using two-way analysis of variance (ANOVA) followed by Bonferroni´s post hoc test. Differences were considered significant at $P < 0.05$.

## Results

**Aged related changes in efferent innervation in EFS-induced gallbladder responses**

EFS induced a frequency-dependent gallbladder contraction (a representative trace is shown in Fig. 1*A*) with a maximum amplitude of 2.23 ± 0.18 mN/mg tissue at the frequency of 40 Hz (n = 26, Table 1). When gallbladder strips from aged animals were electrically stimulated, a significant decrease in the EFS-induced contraction at all the frequencies tested was recorded (Fig. 1*A*, Table 1). The diminished response was reflected by reductions in both the amplitude of the peak and the duration of the

contraction ($P$ < 0.001, two-way ANOVA for both parameters, Fig. 1*B*, Table 1). In addition, there was a long lasting off-relaxation (amplitude of 0.245 ± 0.025 mN/mg at 40Hz, n = 22) in the strips from older animals, but not in the younger animals.

A group of old animals was treated orally with melatonin (2.5 mg/Kg/day) for four weeks mimicking the circadian rhythm of the naturally secreted hormone. This treatment increased melatonin blood levels in aged animals when measured at 9 am (young adult: 26.24 ± 2.12; aged: 19.6 ± 1.48; melatonin: 27.09± 2.29 pg/ml, n = 10, 7, and 7 animals respectively, $P$ < 0.05 aged vs young adult and aged vs melatonin) Melatonin treatment ameliorated age-induced impairment in the EFS response as both the amplitude and duration of EFS-induced contractions were comparable to those in young adult strips, and the off-relaxations were absent (Fig. 1*A* and *B*, Table1).

The aged-related impairment in gallbladder contractility could involve changes in the intrinsic innervation of the organ, alterations in the smooth muscle contractility itself or a combination of these mechanisms. The gallbladder contractile response to exogenous ACh was significantly reduced in aged animals (young adult: 3.266 ± 0.260; aged: 1.911 ± 0.27; melatonin: 4.232 ± 0.538 mN/mg, n = 29, 15 and 11 strips, respectively, $P$ < 0.001 aged vs young adult and aged vs melatonin), suggesting an age-related impairment in the myogenic response to the neurotransmitter released from intrinsic nerves. Taking into account that ACh is the primary excitatory neurotransmitter in the gallbladder (Parkman et al., 1997), the expression of EFS-induced response respect to ACh-induced contraction would reflect the amount of ACh released. As shown in Fig. 1*C*, EFS-induced response expressed in this way is higher in strips from aged animals ($P$ < 0.001 by ANOVA), which would indicate an increase in the release of ACh or a decrease in the release of inhibitory neurotransmitters. A decrease in the release of ACh does not seem

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probable as the effect of atropine was not different in young adult, aged or melatonin-treated aged strips (Fig. 2, Table 2).

To explore the possibility of an aged-induced increase in the release of inhibitory neurotransmitters, we assayed the effects of L-NAME in each experimental group. The inhibitor of the nitric oxide synthase enhanced EFS-induced contraction in young adult strips (89.3 % of enhancement at 5 Hz, n = 10, $P < 0.01$ by ANOVA) but did not have any effects in the aged group (Fig. 3, Table 2). When the old animals were pretreated with melatonin the nitrergic component was stimulated by EFS and L-NAME enhanced EFS-induced contraction (193.8 % of enhancement at 5 Hz, n = 5, $P < 0.001$, by ANOVA, Fig. 3, Table 2).

**Aged related changes in sensory innervation**

In our model, when the sensory nerves were desensitized by the treatment with a high concentration of capsaicin (10 μM) a transient atropine-sensitive contraction was recorded in both young adult and old strips (adult: 0.081 ± 0.016; aged: 0.100 ± 0.016 mN/mg, n = 8 and 6 strips, respectively) but there was no change in EFS-induced responsiveness of young adult strips (Fig. 4, Table 2). However, when aged strips were desensitized by capsaicin a significant increase in the EFS-induced response was recorded ($P < 0.01$ by ANOVA). As shown in Fig. 4, melatonin treatment restored the excitability of the sensory innervation since capsaicin had no effect on EFS-induced responses (Table 2). CGRP induces gallbladder relaxation through stimulation of $K_{ATP}$ ion channels and glibenclamide has been shown to be an effective blocker of these channels in the gallbladder (Zhang et al., 1994). Thus, we assayed the effects of 10μM glibenclamide in EFS-induced response in aged strips, which enhanced the amplitude of contraction to a similar extent than the desensitization with capsaicin (25 % of increase, n = 7, $P < 0.05$ by ANOVA, Figure 5*A*, Table 2). In fact, after glibenclamide treatment

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capsaicin did not enhance EFS-induced responses ($P$ = 0.18, Figure 5*A*, Table 2). In agreement with the lack of effects of capsaicin in the neuromuscular transmission in adult strips, glibenclamide did not modify EFS-induced responses in control tissue (data not shown). These experiments suggest that, in aging, EFS stimulates CGRP-containing sensory nerves, which could contribute to the reduction of gallbladder responsiveness to electrical stimuli via physiological antagonism. This was confirmed by the use of the fragment $CGRP_{8-37}$ as selective blocker of CGRP receptors. As shown in Figure 5*B*, in senescent gallbladder strips $CGRP_{8-37}$ caused an enhancement of EFS-induced contraction (41.9 % of increase, n = 4, $P < 0.05$ by ANOVA, Fig. 5*B*, Table 2) and converted the effect of capsaicin to an inhibition of the contractile response (37 % of inhibition respect to $CGRP_{8-37}$ alone, n = 4, $P < 0.01$ by ANOVA, Fig. 5*B*, Table 2).

**Aged related changes in gallbladder contractility**

To explore how aging affects $Ca^{2+}$ influx-mediated contractions we activated voltage-activated calcium entry-induced by 60 mM KCl, which induced a sustained contraction that was significantly reduced (24.8 % reduction) when we tested gallbladder strips from aged animals and recovered by melatonin treatment (young adult, 3.77 ± 0.37; aged, 2.84 ± 0.26; melatonin: 4.24 ± 0.32 mN/mg, n = 26, 23 and 9 strips, respectively, $P < 0.05$). We have recently shown that capacitative calcium entry (CCE) is another influx route activating contraction in gallbladder smooth muscle (Morales et al., 2004). As previously reported, following depletion of the stores by incubation of control gallbladder strips with 1 μM TPS in $Ca^{2+}$-free medium for 30 min, reintroduction of extracellular $Ca^{2+}$ induced a sustained contraction (3.69 ± 0.53 mN/mg, n = 12) that was significantly smaller in aged strips (2.68 ± 0.29 mN/mg, n = 9, $P < 0.01$) but similar in melatonin-treated animals (4.41 ± 0.39 mN/mg, n = 8).

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To test whether aging affects $Ca^{2+}$ release from sarcoplasmic reticulum, the main intracellular $Ca^{2+}$ store in gallbladder smooth muscle (Morales et al., 2005a), we used CCK as the tool of election. As was the case for $Ca^{2+}$ influx-induced contractions, CCK-induced contraction was impaired in aged strips and melatonin treatment recovered contractility (young adult: 3.7 ± 0.38; aged: 2.48 ± 0.25; melatonin: 3.67 ± 0.52 mN/mg, n = 30, 23 and 9 strips, respectively, $P < 0.01$).

**Aged related changes in calcium handling**

The diminished contractile response to $Ca^{2+}$ influx could be the result of the reduction in the calcium entry as consequence of aged-mediated calcium channel impairment. To test this possibility, we quantified $[Ca^{2+}]_i$ in response to 60 mM KCl and to the protocol to activate capacitative calcium entry. As represented in Figs. 6*A* and *B*, sustained $[Ca^{2+}]_i$ plateau due to $Ca^{2+}$ entry from the extracellular medium was reduced in aged gallbladder smooth muscle cells (26.2 and 34.2 % reduction for KCl and CCE, respectively, n = 49-18 cells, $P < 0.05$). Interestingly, melatonin did not have any effects on calcium influx (KCl: 0.111 ± 0.008 vs 0.115 ± 0.010 $\Delta F_{340}/F_{380}$ n = 34 and 13 cells ; CCE: 0.063 ± 0.005 vs 0.067 ± 0.007 $\Delta F_{340}/F_{380}$ n = 8 and 6 cells for aged and melatonin treated aged cells, respectively)

When we quantified $[Ca^{2+}]_i$ in response to CCK challenge there was no change in the amplitude of $Ca^{2+}$ transient peak indicative of intracellular $Ca^{2+}$ release (n = 28 and 15 cells, $P = 0.897$, Fig. 6*C*). To confirm that $Ca^{2+}$ release from stores through D-*myo*-inositol 1,4,5-triphosphate ($IP_3$) and ryanodine channels remained unchanged in aging, we exposed the cells to 10 μM $Bt_3$-Ins(1,4,5)$P_3$-PM, a membrane permeable analogue of $IP_3$, and to caffeine. Similar to CCK-induced $Ca^{2+}$ transient, the peak responses to the $IP_3$ analogue were not affected by aging (young adult, 0.426 ± 0.031; aged, 0.379 ± 0.028 $\Delta F_{340}/F_{380}$, n = 13 and 7 cells, respectively, $P = 0.272$). When we used caffeine to induce

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$Ca^{2+}$ release through ryanodine receptors we did not detect a difference in the $Ca^{2+}$ transient in young adult vs. aged gallbladder smooth muscle cells (n = 31 and 36 cells, *P* = 0.412, Fig. 6*D*). Collectively, these results suggest that both $Ca^{2+}$ release channels and calcium content in the stores are not affected by aging. The latter was confirmed by application of a low level of ionomycin in $Ca^{2+}$ free solution to the cells, a treatment that releases the $Ca^{2+}$ stores while bypassing channels and receptors. This treatment caused a similar increase in $[Ca^{2+}]_i$ in both adult and aged cells (young adult, 0.188 ± 0.029; aged, 0.180 ± 0.015 $\Delta F_{340}/F_{380}$, n = 14 and 24 cells, respectively, *P* = 0.821), indicating that the integrity of calcium stores is preserved in aging. Melatonin treatment did not have any effect on $Ca^{2+}$ release mechanisms nor $Ca^{2+}$ store content (data not shown)

**Aged-related changes in contractile machinery**

Taken together, the results described above indicate that the aged impairment in contractility is, at least in part, the result of alteration in $Ca^{2+}$ influx, but this physiological condition could also alter gallbladder contractility independently of the calcium signal. This possibility was tested by application of 1μM of ionomycin in presence of extracellular $Ca^{2+}$ (at this concentration ionomycin raises $[Ca^{2+}]_i$ independently of channels and receptors). Ionomycin caused similar elevation in adult and aged and melatonin-treated cells (young adult: 0.355 ± 0.050; aged: 0.419 ± 0.052; melatonin: 0.376 ± 0.057 $\Delta F_{340}/F_{380}$, n = 32, 30 and 16 cells, respectively, *P* > 0. 05), but induced much lower contraction in aged strips than in adult and melatonin-treated aged tissues (young adult: 4.23 ± 0.57; aged: 2.12 ± 0.201; melatonin: 5.39 ± 0.87 mN/mg, n = 7, 14 and 8 strips, respectively, *P* < 0.01 young adult vs aged), suggesting that ageing can also alter the contractile machinery independently of the calcium signal.

To investigate one of the possible causes of this impaired contractility, we determined the total amount of the contractile protein F-actin labelling gallbladder smooth muscle cells

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with FITC-stained phalloidin. We found that aging resulted in a statistically significant decrease in the F- actin content that was recovered by melatonin treatment (young adult: 33.59 ± 0.86; aged: 9.92 ± 0.49; melatonin 28.95 ± 0.87, data as arbitrary units of fluorescence, n = 100, 63 and 92 cells, respectively, $P < 0.05$ young adult vs aged, Fig. 7*D*).

## Discussion

At a time when the world population is ageing at a rapid rate, there is an emergent need to understand the biology of aged GI tract and to translate this knowledge to therapeutics to improve GI function and quality of life of the elderly. The present study was conducted to elucidate functional changes in gallbladder motility during aging and to explore the potential use of melatonin to ameliorate the onslaught of aging on GI structures.

We found that ageing impairs the muscular contraction and modifies the neurological control of the muscle. The possibility that altered GI function in the elderly is related to neurodegeneration of the enteric nervous system is supported by studies conducted both in animals and humans (for revision see Wade and Cowen, 2004). Age-related neuronal loss in the small and large intestines occurs exclusively in the cholinergic population (Roberts et al., 1994; Phillips et al., 2003), with little change, if any, in nitrergic neurons. In the gallbladder, however, cholinergic transmission seems very well maintained with age since the atropine-sensitive contractile response to EFS remains the same in the adult and aged groups. Our finding of a decrease in the functionality of nitrergic fibers in the aged group can be related to previous reports of morphological alterations in nitrergic neurons (Phillips et al., 2003) and impairment nitrergic function by ageing (Smits and Lefebvre, 1996), which is consistent with our functional studies.

Interestingly, in our model, the loss of nitrergic inhibitory function was compensated by an over-reactivity of sensory inhibitory nerves, as demonstrated by the increase in the

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EFS-induced contraction after desensitization with capsaicin. The ganglionated plexus of the gallbladder is rich in afferent fibers that are immunoreactive for both substance P and CGRP (Mawe and Gershon, 1989). Application of capsaicin induced a contractile response that was the result of the substance P-induced contraction and CGRP-induced relaxation (Maggi et al., 1989), similar to our finding of capsaicine-evoked contraction sensitive to atropine. However, capsaicin-desensitization of sensory nerves increased the EFS-induced responses in aged strips without affecting adult strips. The fact that capsaicin effects were mediated by CGRP1 receptor (blocked by $hCGRP_{8-37}$) and its downstream target $K_{ATP}$ channels (blocked by glibenclamide) clearly suggest that hypersensitivity of inhibitory sensory fibers containing CGRP, contribute to gallbladder motility disorders in aging. It is now clear that hypersensitivity of sensory neurons contributes to functional bowel disorders such as irritable bowel syndrome, Hirschsprung's disease, rectal hypersensitivity and faecal urgency (Grundy, 2006).

Motility disorders during aging can also involve age-dependent changes in the response of smooth muscle to neurotransmitters and hormones (for review see Bitar, 2003).due to inhibition of transduction pathways or to alterations of contractile proteins (Bitar, 2003; Bitar and Patil, 2004). We report here both changes in $Ca^{2+}$ signals and a decrease in the F-actin content in aged gallbladder smooth muscle cells. Our study shows that ageing affects $Ca^{2+}$ entry through both L-type $Ca^{2+}$ channels and store-operated $Ca^{2+}$ channels, although the content of intracellular $Ca^{2+}$ stores and its release through $IP_3$ and ryanodine receptors is not influenced. Age-related changes in $Ca^{2+}$ homeostasis appear to be tissue-specific. Thus, reduction of voltage-dependent calcium channels has been described in rat brain cortices (Iwamoto et al., 2004), canine atria (Dun et al., 2003) and colon (Xiong et al., 1993; Xiong et al., 1995) but voltage-activated $Ca^{2+}$ influx is increased in mammalian CA1 hippocampal neurons (Thibault and Landfield, 1996) and heart (Josephson et al.,

2002) during aging. There are no published data related to the effects of ageing on CCE channels. Our data are consistent with the hypothesis that in gallbladder smooth muscle aging mainly affects membrane L-type and CCE $Ca^{2+}$ channels, which could contribute to the reduction in the contractility in response to KCl, CCE, but also to CCK, as this hormone induces $Ca^{2+}$ entry after releasing $Ca^{2+}$ from intracellular stores (Morales et al., 2004; Morales et al., 2005a).

One of the most compelling findings reported here is the beneficial effect of melatonin on gallbladder neuromuscular function and myogenic contractility. Thus, after treating the animals for 4 weeks with melatonin (2.5 mg/Kg/day), there was not only a total recovery of the contractile response to $Ca^{2+}$ influx activation and EFS, but also a reversion of the aged-induced changes in efferent and sensitive innervation, although melatonin did not restore the $Ca^{2+}$ influx. The recovery of the contractility without changes in $Ca^{2+}$ homeostasis can only be explained by effects of melatonin on $Ca^{2+}$-insensitive contractility steps such as contractile proteins or $Ca^{2+}$ sensitization of the contractile machinery. Whereas the former can be related to the increase in the F-actin content described in this study, the later is now under research in our laboratory.

Melatonin is a hormone that is secreted by the pineal gland following a circadian rhythm with minimal blood levels of melatonin during the daytime and maximal levels observed mostly during the middle of the night. Melatonin has special importance in the gastrointestinal tract since the enterochromaffin cells of the gut are the main source of extrapineal melatonin (Kvetnoy et al., 2002). Metabolism of melatonin in liver appears to be the major metabolic pathway for its deactivation (Lane and Moss, 1985) but also active melatonin is secreted in bile and concentrated in the gallbladder (Tan et al., 1999). Although it is well established that pineal melatonin decreases with age (Karasek, 2004), there is no information available regarding gastrointestinal melatonin. If gastrointestinal

melatonin content also decreases with age, the hepatobiliary system in general, and the gallbladder in particular, would suffer the lack of melatonin protective effects. The restoration of gallbladder contractility in response to melatonin treatment in aged guinea pigs is consistent with this hypothesis.

Regarding the GI, there are numerous lines of evidence demonstrating the protective and healing effects of melatonin in gastric ulcers and experimental colitis. Whereas a direct antioxidative effect, activation $CO_3H^-$ secretion by enterocytes, and release of endogenous prostaglandin mediates these protective effects, the healing effect was associated to production of endogenous NO and participation of sensory nerves (for review see (Reiter et al., 2003). The effects of melatonin on symptoms of colitis are related to decrease of NO and PGE2 content, as the result of down-regulated expression of colonic iNOS and cycloxygenase-2 (Dong et al., 2003) in addition to an enhancement of the immune response in the gastrointestinal tract (Mei et al., 2002). The present study is the first report on the beneficial effects of melatonin treatment in aged gastrointestinal tract, but more studies are required to establish the cellular mechanisms through which melatonin exerts its effects in the gallbladder and the optimal pattern of melatonin treatment to ameliorate the effects of ageing. Both lines of research will elucidate the potential use of melatonin in elderly population.

In conclusion, our results indicate that ageing causes impairment of gallbladder function as consequence of neurally-mediated and neurally -independent mechanisms and support a possible beneficial effect of melatonin in gallbladder dysfunction related to ageing.

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## Footnotes:

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## LEGENDS FOR FIGURES

**Fig. 1** N**euromuscular transmission is impaired in ageing and restored by melatonin***A*. Original recordings showing tension responses elicited by EFS (0.3 ms duration pulse, 5-40 Hz, 350 mA, for 10 sec every 3 min) applied to gallbladder strips from youn adult, aged and aged guinea pigs treated with 2.5 mg/Kg/day melatonin. Little bars indicate timing of different frequency stimulations. Traces are typical of 26-15 strips. *B*.- Superimposed recordings of the EFS-induced response to 25 Hz showing in more details the aging related reduction in the peak amplitude and duration of the response. In addition, the long lasting off relaxation can be observed in the recording corresponding to aged strips. Note that melatonin treatment reverted EFS-induced responses to normal, indicating the restoration of neuromuscular transmission in these animals. *C*.- Summary data of EFS induced-responses (peak amplitude) expressed as the percentage of the response to 10 μM ACh. When expressed in this way EFS elicited contractions are higher in aged strips ($P < 0.0001$, by ANOVA).

**Fig. 2. Atropine-sensitive EFS-induced response remains unaltered by ageing**

*A, B* and *C*.- Effects of atropine (1 μM) on the frequency-response curves to EFS (0.3 ms duration pulse, 5-40 Hz, 350 mA, for 10 sec every 3 min) applied to gallbladder strips from young adult (n = 24 strips), aged (n = 21 strips) and aged guinea pigs treated with 2.5 mg/Kg/day melatonin (n = 16 strips). After EFS was performed in control conditions (solid lines) strips were incubated for 30 min with atropine and EFS was repeated again (dotted line). Statistical analysis indicates *P* values < 0.001 (by ANOVA) in all experimental groups. *D*.- Atropine-induced inhibition of EFS responses in the three experimental groups. No significant differences between groups were found at any of the frequencies tested.

**Fig. 3.- Nitrergic function is impaired in ageing and restored by melatonin.**

*A.*- Original recordings of the effects of L-NAME (100 μM) on the EFS (0.3 ms duration pulse, 5-40 Hz, 350 mA, for 10 sec every 3 min) applied to gallbladder strips from young adult, aged and aged guinea pigs treated with 2.5 mg/Kg/day melatonin . After EFS was performed in control condition s strips were incubated for 30 min with L-NAME and EFS was repeated again. Vertical dotted lines indicate the timing of EFS. *B.*- Sumary data of the frequency-response curves to EFS. Data are mean ± SEM. n = 10, 6 and 5 strips for adult, aged and aged treated with melatonin, respectively. Note that L-NAME does not have effects on aged gallbladder strips, indicating the loss of nitrergic function and that melatonin treatment induces the recovery of the nitrergic responses. $P < 0.01$ & $P < 0.001$ (by ANOVA) control vs L-NAME for young adult and aged + melatonin experimental groups.

**Fig. 4.- Ageing induces sensitization of inhibitory sensory afferent fibers and melatonin normalizes their sensitivity.**

*A.*- Original recordings of the effects of capsaicin (10 μM) on the EFS (0.3 ms duration pulse, 5-40 Hz, 350 mA, for 10 sec every 3 min) applied to gallbladder strips from young adult, aged and aged guinea pigs treated with 2.5 mg/Kg/day melatonin. After EFS was performed in control condition s strips were incubated for 30 min with capsaicin and EFS was repeated again. Vertical dotted lines indicate the timing of EFS. *B.*- Sumary data of the frequency-response curves to EFS. Data are mean ± SEM. n = 8, 6 and 4 strips for young adult, aged and aged treated with melatonin, respectively. Note that capsaicin increases the EFS-induced contraction only in aged gallbladder strips, indicating the

sensitization of the inhibitory component of sensory fibers and that melatonin treatment reverts this sensitization. $P < 0.01$ (by ANOVA) control vs capsaicin in aged strips.

**Fig. 5.- Ageing sensitizes CGRP containing fibers .**

*A.*- Original recordings of the effects of glibenclamide (10 μM) and CGRP8-37 in the absence and presence of capsaicin (10 μM )on the EFS (0.3 ms duration pulse, 5-40 Hz, 350 mA, for 10 sec every 3 min) applied to aged gallbladder strips. After EFS was performed in control conditions, strips were incubated for 30 min with glibenclamide or CGRP8-37 and EFS was repeated again. Then, strips were incubated for 30 min with glibenclamide or $CGRP_{8-37}$ plus capsaicin and EFS was repeated again Vertical dotted lines indicate the timing of EFS. *B.*- Summary data of the frequency-response curves to EFS in the presence and absence of glibenclamide or $CGRP_{8-37}$ and glibenclamide or $CGRP_{8-37}$ plus capsaicin. Data are mean ± SEM. n = 7 and 4 strips for glibenclamide and capsaicin group, respectively. Note that both drugs have a stimulatory effect and after the treatment with glibenclamide or $CGRP_{8-37}$ capsaicin does not increase EFS-induced contractions. $P < 0.05$ (by ANOVA) control vs glibenclamide and control vs $CGRP_{8-37}$.

**Fig. 6. Ageing reduces $Ca^{2+}$ influx but not $Ca^{2+}$ release from intracellular stores**. A.- Representative original traces of changes in the fluorescence ratio in response to a depolarizing solution containing 60 mM KCl and 2 mM $Ca^{2+}$ in young adult (solid line) and aged (dotted line) cells . As observed, ageing reduced the plateau response. In B, fura-2 loaded cells were treated with 1 μM thapsigargin (TPS) in $Ca^{2+}$ free solution to deplete the stores. When indicated, cells were perfused with a 2 mM $Ca^{2+}$ HEPES solution resulting in a sustained $[Ca^{2+}]_i$ increase that was reduced in aged cells (dotted line). In *C* and *D* $Ca^{2+}$ release from intracellular stores was induced by 10 nM CCK ($IP_3$-sensitive stores) (*C*) or caffeine (ryanodine-sensitive stores) (*D*). Note that the peak,

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indicative of $Ca^{2+}$ release, was similar in both young adult and aged cells but the plateau, indicative of $Ca^{2+}$ entry was reduced in senescent cells. Traces are typical of 45-15 cells from at least 4-5 independent experiments Histograms show summary data of $\Delta F_{340}/F_{380}$ from experiments in the above described conditions (mean ± SEM). * $P < 0.05$, ** $P < 0.01$.

**Fig. 7. Melatonin restores gallbladder myogenic contractility**

A.- Representative original traces of changes in isometric tension in response to a depolarizing solution containing 60 mM $KCl^{+}$ in young adult (solid line), aged (dashed line) and aged treated with melatonin (dotted line) gallbladder strips. In B, gallbladder strips were treated with 1 µM thapsigargin (TPS) in $Ca^{2+}$ free solution to deplete the stores. When indicated, the organ bath was perfused with a 2 mM $Ca^{2+}$ K-H solution resulting in a sustained contraction that was reduced in aged strips (dash line). In *C* contraction was induced by 10 nM CCK in all groups. Note that the response to all the stimuli was decreased in aged cells but melatonin treatment induced the recovery of the contractility. Traces are typical of 30-9 strips. Histograms show summary data tension (mN/mg) from experiments in the above described conditions (mean ± SEM). *D.*- Aged related changes in F actin content (expressed as arbitrary units) in young adult, aged and aged + melatonin gallbladder cells. Note the decrease in F-actin content in aged cells and the recovery when the aged animals were treated with melatonin. * $P < 0.05$, ** $P < 0.01$.

**Table 1.** EFS-induced gallbladder contractions: effects of ageing and melatonin treatment

| | Young adult | | | Aged | | | Melatonin | | |
|---|---|---|---|---|---|---|---|---|---|
| Frecuency (Hz) | Amplitude (mN/mg) | Duration (s) | n | Amplitude (mN/mg) | Duration (s) | n | Amplitude (mN/mg) | Duration (s) | n |
| 5 | 0.988 ± 0.117 | 43.08 ± 1.29 | 26 | 0.643 ± 0.063 * | 31.01 ± 1.15 * | 22 | 0.812 ± 0.140 | 42.23 ± 1.66 | 15 |
| 10 | 1.315 ± 0.132 | 48.57 ± 1.88 | 26 | 0.890 ± 0.078 * | 33.73 ± 1.28 * | 22 | 1.170 ± 0.163 | 45.53 ± 2.65 | 15 |
| 15 | 1.609 ± 0.147 | 52.80 ± 1.97 | 26 | 1.120 ± 0.099 * | 37.26 ± 1.50 * | 22 | 1.483 ± 0.178 | 45.93 ± 2.43 | 15 |
| 25 | 1.956 ± 0.164 | 57.30 ± 2.55 | 26 | 1.384 ± 0.120 * | 39.14 ± 2.12 * | 22 | 1.967 ± 0.175 | 52.06 ± 2.97 | 15 |
| 40 | 2.232 ± 0.181 | 55.90 ± 2.70 | 26 | 1.570 ± 0.139 * | 39.47 ± 2.61 * | 22 | 2.351 ± 0.157 | 52.66 ± 3.24 | 15 |

*$P < 0.0001$ adult vs aged.

**Table 2.-** Effects of different drugs on EFS-evoked gallbladder contractions

| | Young Adult | Aged | Melatonin |
|---|---|---|---|
| Atropine (10 μM) | abolition<br>*P* < 0.0001 | abolition<br>*P* < 0.0001 | abolition<br>*P* < 0.0001 |
| L-NAME (100 μM) | increase<br>*P* < 0.01 | no effect<br>*P* = 0.824 | increase<br>*P* < 0.0001 |
| Capsaicin (10 μM) | no effect<br>*P* = 0.338 | increase<br>*P* < 0.01 | no effect<br>*P* = 0.386 |
| Glibenclamide (10 μM) | no effect<br>*P* = 0.913 | increase<br>*P* < 0.05 | no tested |
| Gliblenclamide + Capsaicin | no effect<br>*P* = 0.38 | no effect<br>*P* = 0.178 | no effect<br>*P* = 0.178 |
| CGRP $_{8-32}$ (1 μM) | no tested | increase<br>*P* < 0.05 | no tested |
| CGRP $_{8-32}$ + Capsaicin | no tested | decrease<br>*P* < 0.01 | no tested |

** Significance by two way ANOVA*

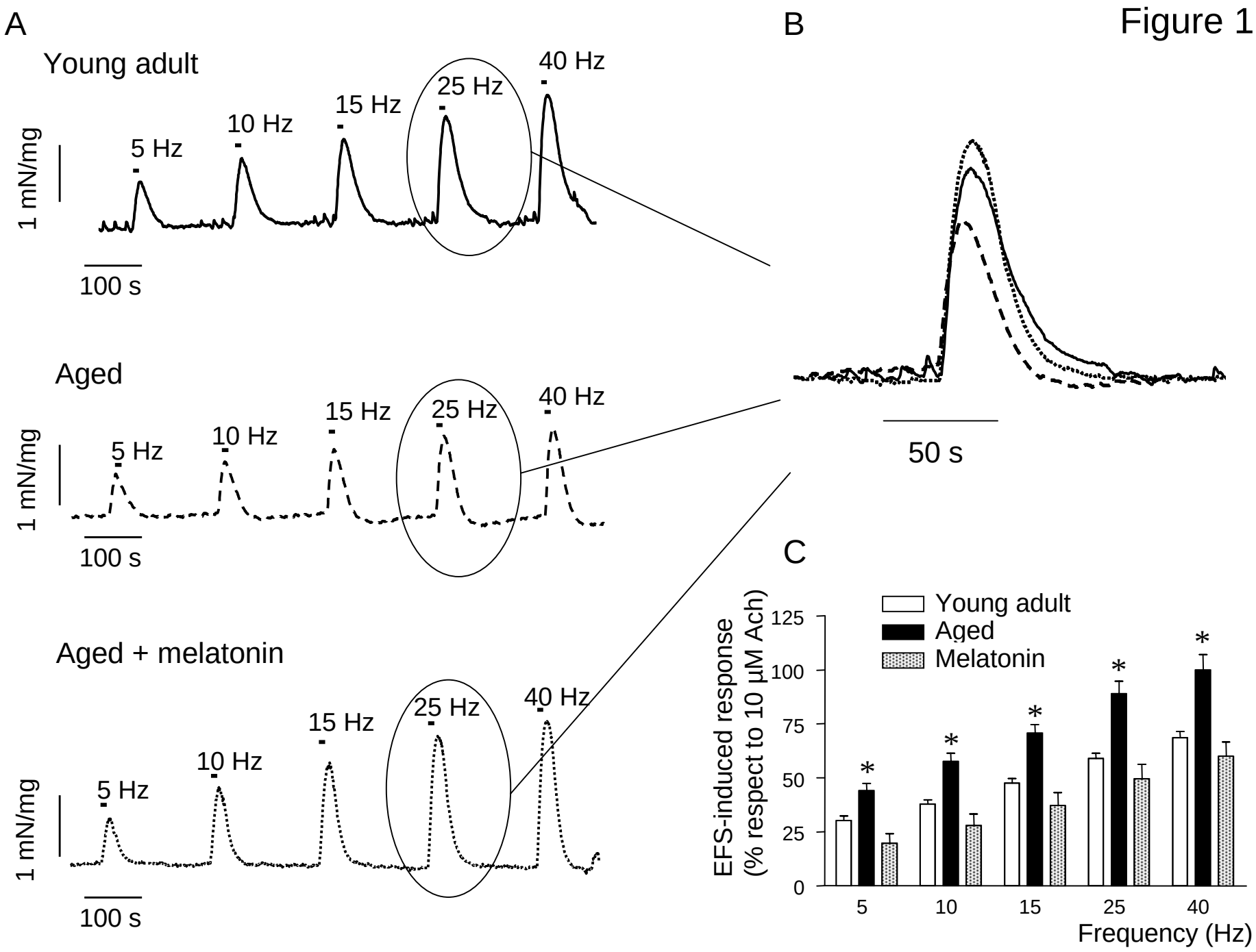



Figure 1

Figure 2

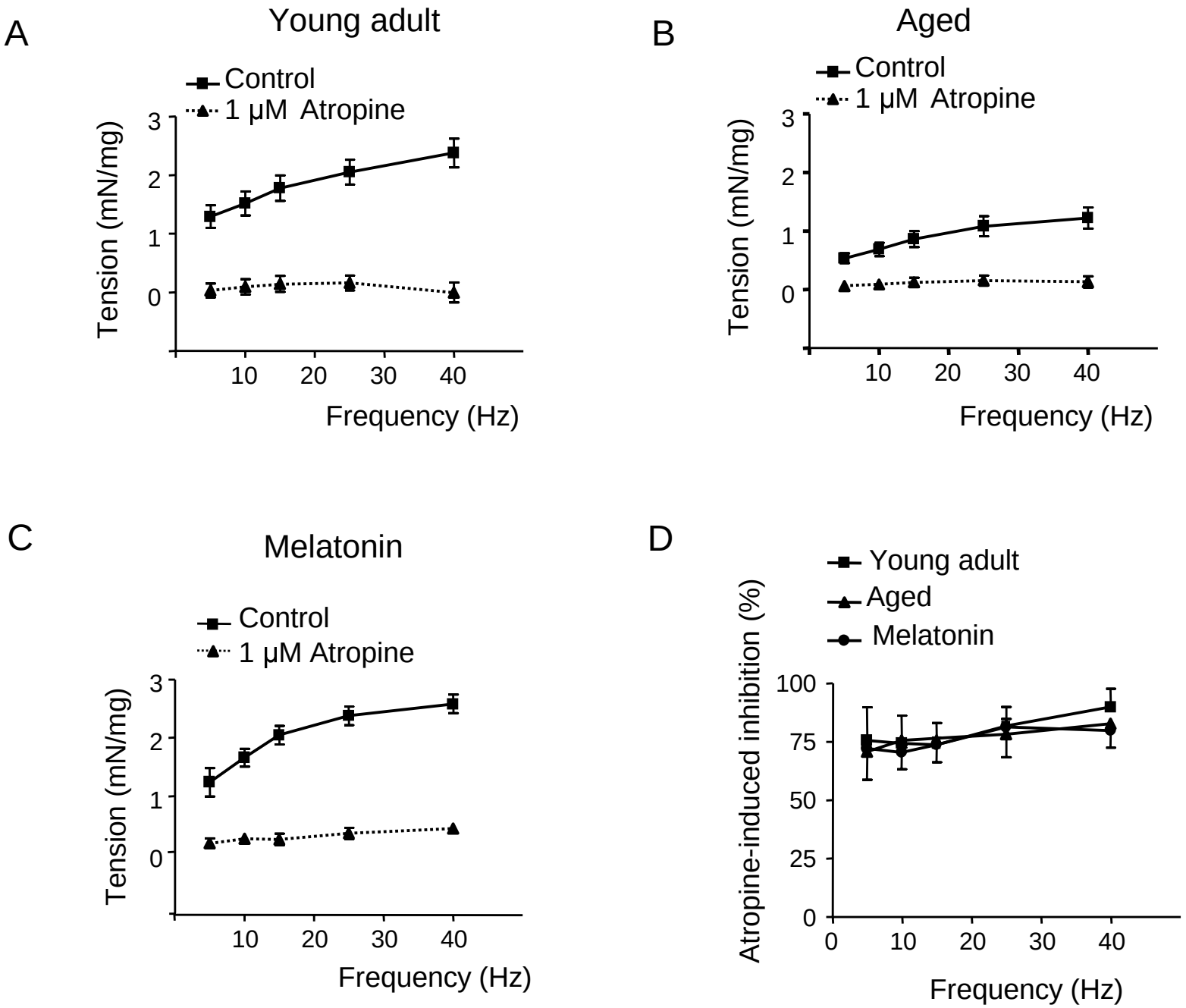
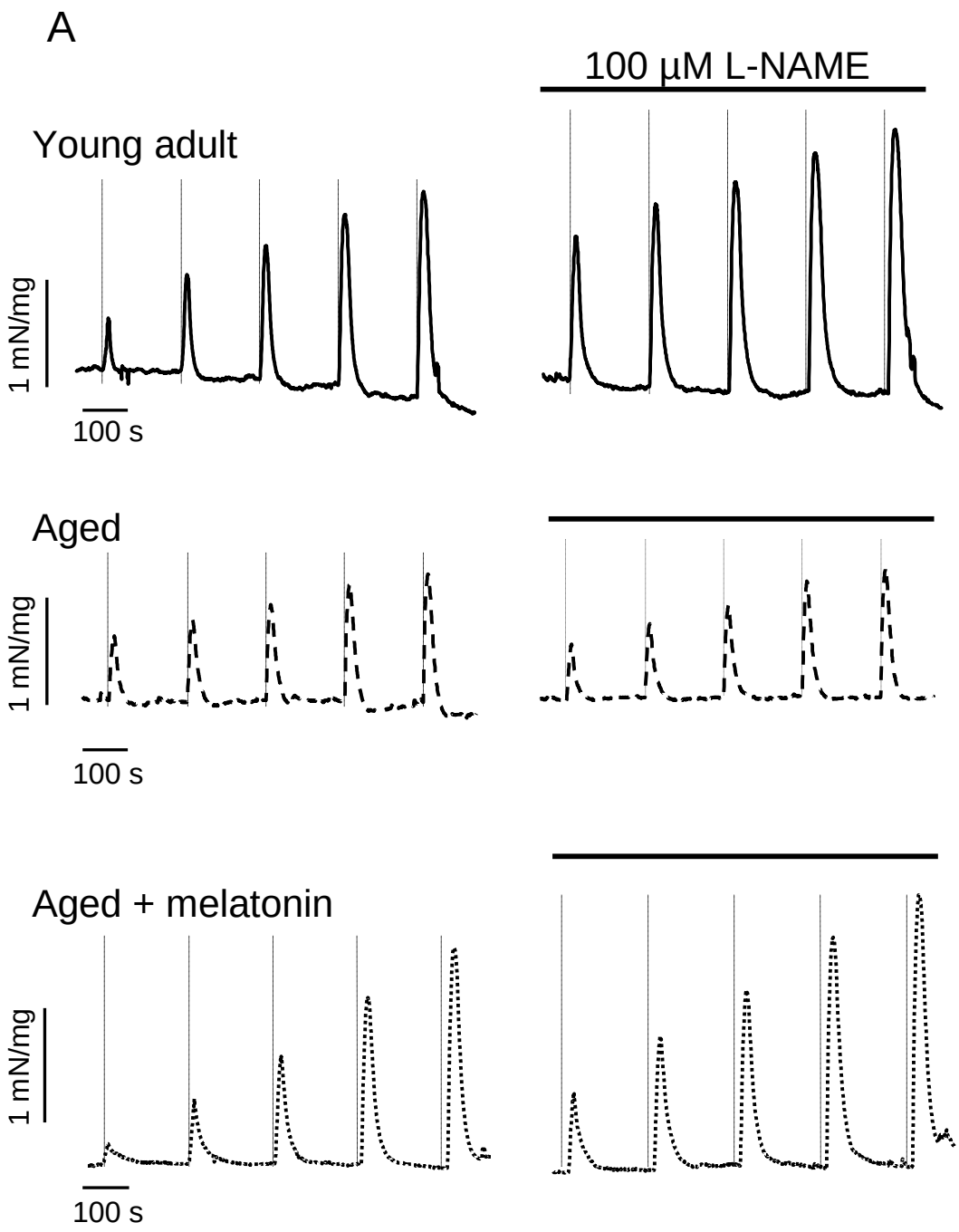
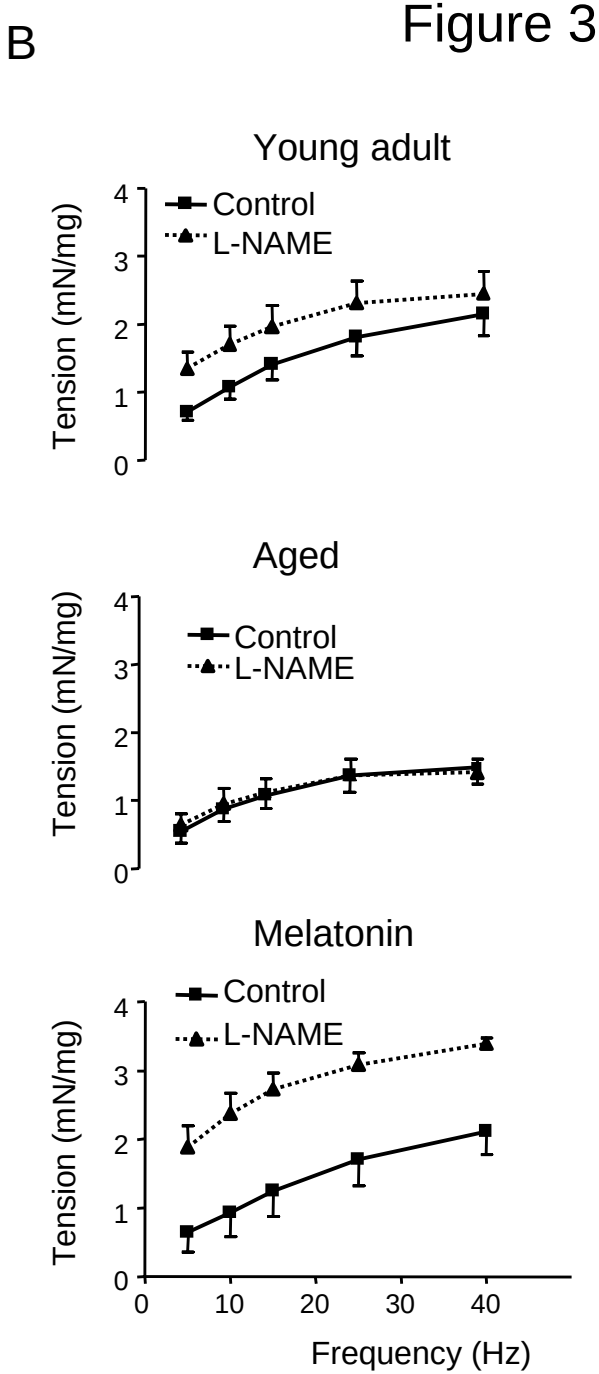


Figure 3



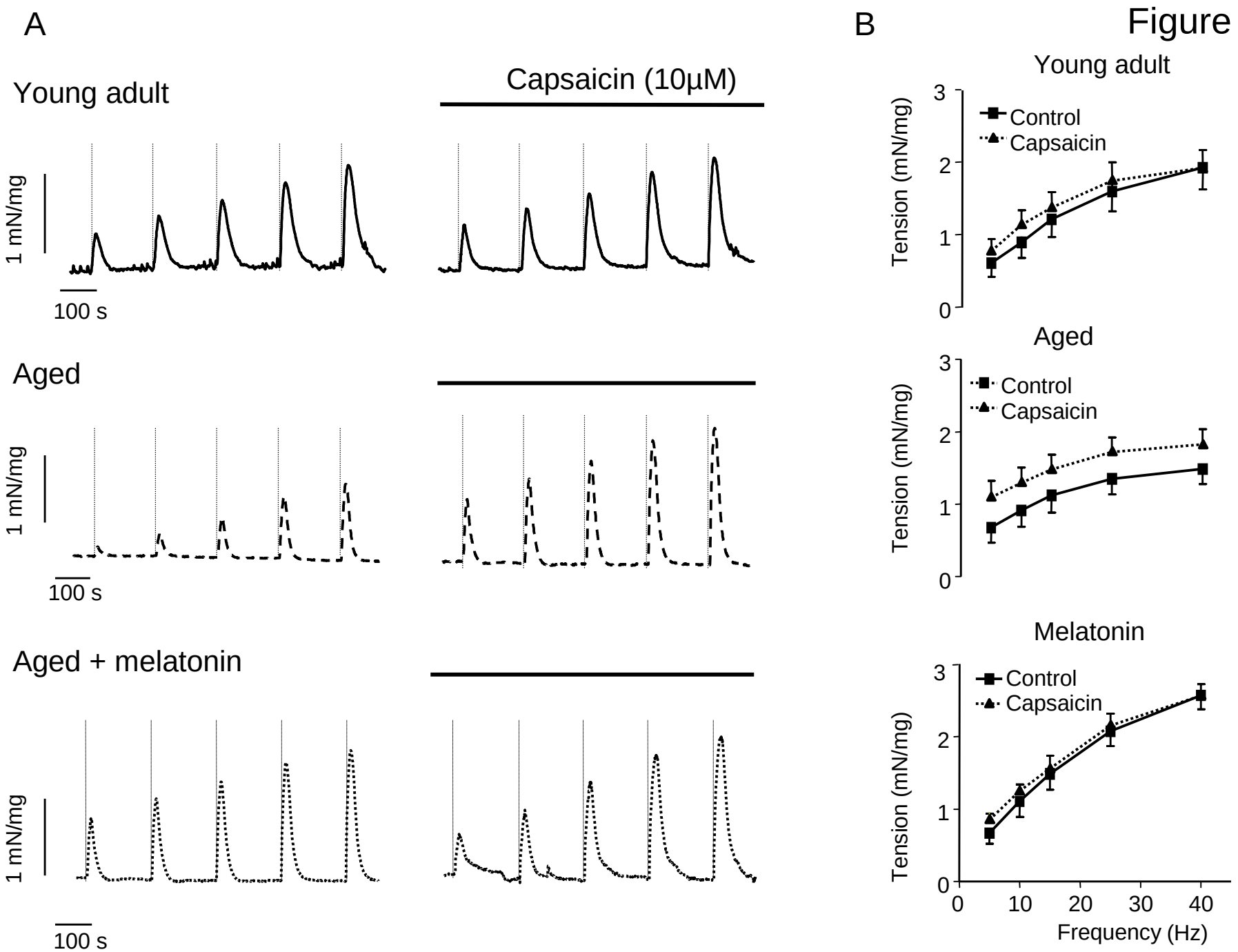
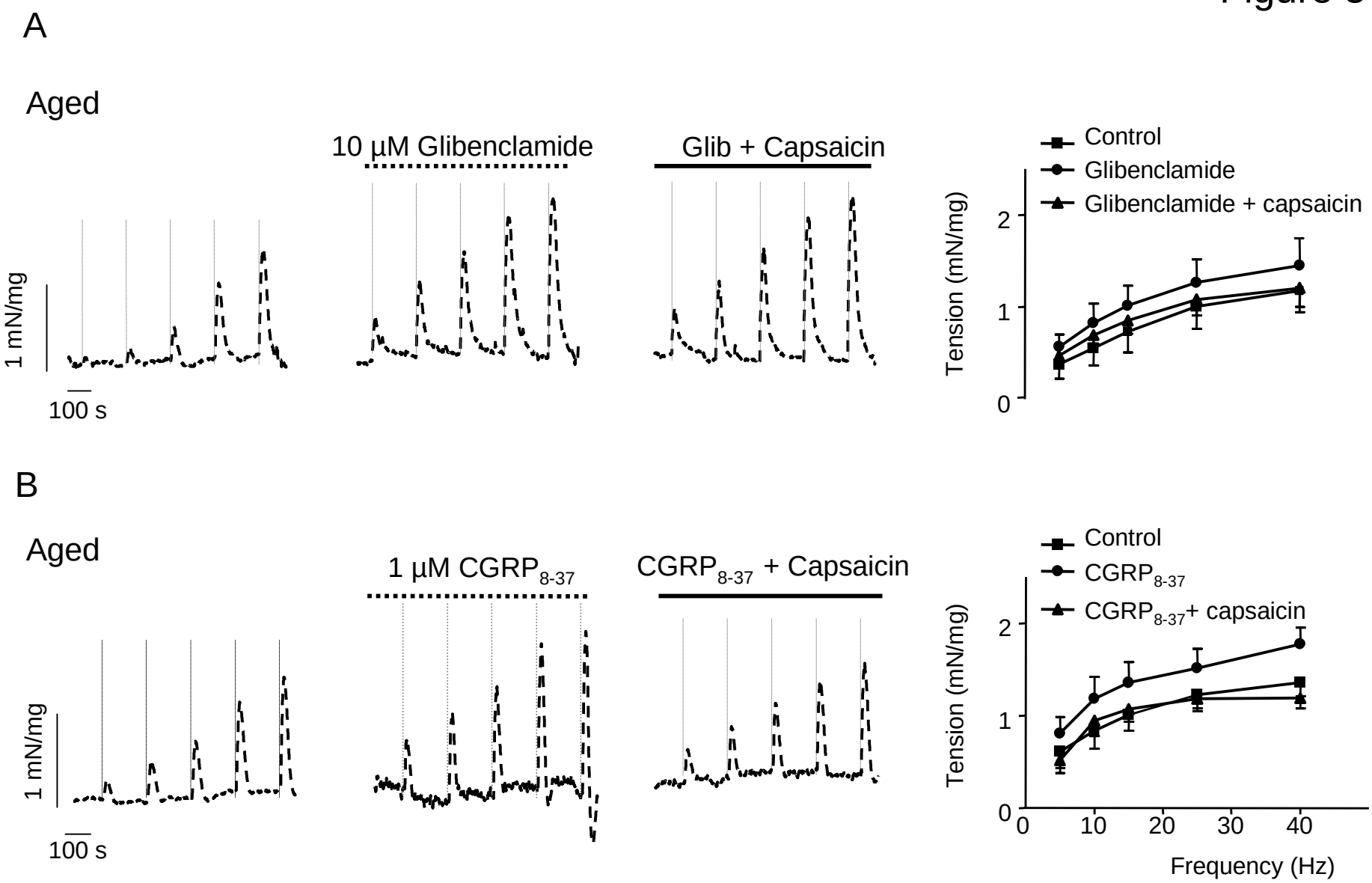



Figure 4

Figure 5



# Figure 6

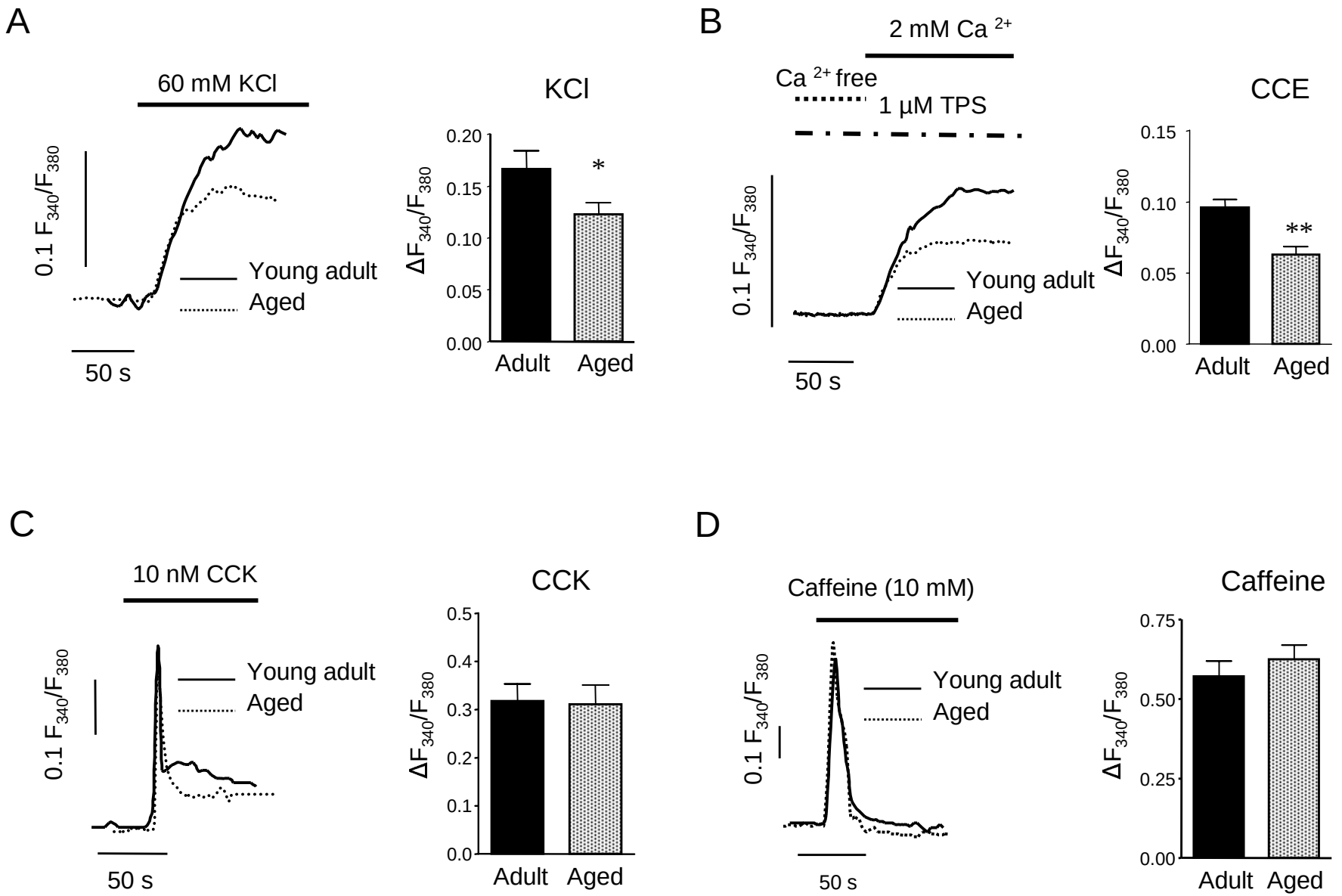


Figure 7