AGING IMPAIRS NEUROGENIC CONTRACTION IN GUINEA PIG URINARY BLADDER. THE ROLE OF OXIDATIVE STRESS AND MELATONIN.

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Running head: melatonin & aged bladder innervation

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ABSTRACT

The incidence of urinary bladder disturbances increases with age, and free radical accumulation has been proposed as a causal factor. Here we investigated the association between changes on bladder neuromuscular function and oxidative stress in aging and the possible benefits of melatonin treatment. Neuromuscular function was assessed by electrical field stimulation (EFS) of isolated guinea pig detrusor strips from adult and aged female guinea pigs. A group of adult and aged animals were treated with 2.5 mg kg⁻¹ day⁻¹ melatonin for 28 days. Neurotransmitter blockers were used to dissect pharmacologically the EFS-elicited contractile response. EFS induced a neurogenic and frequency-dependent contraction which was impaired by aging. This impairment is in part related to a decrease in the detrusor myogenic contractility. Age also decreased the sensitivity of the contraction to pharmacological blockade of purinergic and sensitive fibers, but increased the effect of blockade of nitrergic and adrenergic nerves. The density of cholinergic and nitrergic nerves remained unaltered, but ageing modified afferent fibers. These changes were associated to an increased level of markers for oxidative stress. Melatonin treatment normalized oxidative levels and counteracted the ageing-associated changes in bladder neuromuscular function. In conclusion, these results show that aging modifies neurogenic contraction and the functional profile of the urinary bladder plexus and simultaneously increases the oxidative damage to the organ. Melatonin reduces oxidative stress and improves the age-induced changes in bladder neuromuscular function, which could be of importance to reduce the impact of age-related bladder disorders.
Key Words: melatonin, oxidative stress, detrusor smooth muscle, neuromuscular function, sensory nerves, electrical field stimulation.
INTRODUCTION

Continence and voiding of urine are two important processes controlled by a complex neural network with intrinsic and extrinsic components. In most animal species, bladder contraction is mediated by both cholinergic and nonadrenergic, noncholinergic (NANC) mechanisms (42). Under normal conditions, the micturition contraction in vivo and the contraction evoked by electrical stimulation of nerves in vitro is considered to be mainly mediated by muscarinic receptor stimulation (23). Though M3 receptors are not the more expressed cholinergic subtype, they account for the physiological voiding contraction (8). Adrenergic inputs to urinary bladder have been identified, but their functional role is not well established. The α-adrenergic receptors are mainly expressed in the bladder neck, where they keep the continence, while β-adrenergic receptors are generally distributed in the bladder body, its activation leading to relaxation during the filling phase (14). In the bladder, the second more important excitatory neurotransmitter is ATP, which acts on two families of purinergic receptors: P2X, an ion channel receptor, and P2Y, a family of G protein-coupled receptors (6). ATP is released to bladder smooth muscle by both efferent and afferent synapses, the later serving as a mechanosensor pathway reporting about urinary bladder distension (12). Although nitrergic fibres are present in the urinary bladder direct relaxation of the detrusor smooth muscle in response to NO has not been reported, and therefore the role of NO may be to modulate other transmitters and/or to participate in afferent neurotransmission (2). Other neurotransmitters such as vasoactive intestinal polypeptide, endothelins, tachikinins, angiotensin and prostanoids have been found in the nervous plexus of the urinary bladder, and their functional role has been reviewed in (4).
It is well known that disturbances of bladder function are common in the elderly population and that the incidence of such disorders increases with age, but the altered mechanisms leading to bladder dysfunction are poorly understood. Age-related changes in the innervation of the bladder are of particular interest in understanding how aging impairs contractility given the important role of nerves in the control of bladder function. There are several studies in the literature regarding the effect of aging on the neurotransmitter-induced responses in bladder smooth muscle. However, there is a great variability in the reported results, and some studies indicate age-dependent increases (25, 40) or decreases in ACh-induced responses (44), whereas others report no changes in carbachol-evoked contraction (31, 46) or increased norepinephrine-elicited contractions (32, 40). Information about age-related changes in the neuromuscular function in the bladder is scarce. Yoshida et al reported in humans no changes in the global response to EFS (46), although aging caused a decrease and an increase in the cholinergic and purinergic components of EFS, respectively. In rats, aging decreases EFS-induced neurogenic contractions, but changes in the different components of the neurotransmission were not studied (31). There are no reports on the amelioration of age-related changes of neuromuscular function.

The age-induced damage has been associated to an increased reactive species production and a decrease in the cellular antioxidant mechanisms (17). In this regard, melatonin, a potent endogenous free radical scavenger and antioxidant which declines with age, has been proposed as a good candidate to palliate the ageing associated alterations (22). In fact, we have recently reported that melatonin exerts beneficial effects on the micturition pattern (16) and in the gallbladder neurotransmission of aged guinea pigs (15). However, there is no information about the effects of melatonin on neural alterations induced by aging in the urinary bladder.
The current study was designed to investigate the effect of aging on neurally evoked urinary bladder contraction and the participation of the different neurotransmitters. In addition, we wanted to assess whether the antioxidant properties of melatonin can ameliorate the alterations of bladder neuromuscular function associated to age.

**MATERIALS AND METHODS**

*Solutions and drugs*

The Krebs-Henseleit solution (K-HS) contained (in mM): 113 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, and 11.5 D-glucose. This solution had a final pH of 7.35 after equilibration with 95% O₂-5% CO₂. The phosphate buffer used to homogenize the tissue contained (in mM): NaCl 20, KCl 2.7, Na₂HPO₄ 16, NaH₂PO₄ 4, pH 7.4. Drug concentrations are expressed as final bath concentrations of active species. Drugs and chemicals were obtained from the following sources: melatonin, atropine, guanethidine, L-NAME and suramin were from Sigma Chemical (St. Louis, MO); E-Capsaicin and TTX were from Tocris (Bristol, UK). Other chemicals used were of analytical grade from Panreac (Barcelona, Spain). Stock solutions of atropine and E-Capsaicin were prepared in DMSO. The solutions were diluted such that the final concentration of DMSO was ≤0.1% vol/vol. This concentration of DMSO did not have effect on urinary bladder contractile state.

*Animals and tissue preparation*

Urinary bladders, isolated from 4-and 20-month-old female guinea pigs after deep halothane anaesthesia and cervical dislocation, were immediately placed in cold K-HS (for composition see *Solutions and drugs*) at pH 7.35. The urinary bladder was cleaned of fatty tissue, opened longitudinally, washed with K-HS solution to remove urine
remains and the urothelium was carefully dissected away.

The aged animals used in the study are not senescent according to the life expectancy of these animals (3-4 years). However, at the age of 20-24 months several studies have shown biological differences related to aging (1; 20), although extrapolation of these aging conditions to human age is difficult due to the lack of aging markers and to differences in the development profile of the two species.

A group of adult and aged animal were treated with melatonin (2.5 mg/kg/day, per os) during 28 days just before the start of the dark phase (7 p.m.). Melatonin was dissolved in glucose solution (0.5%) and placed in the oropharynx by a syringe. All the experiments were carried out according to the guidelines of Animal Care and Use Committees of the University of Extremadura.

Contraction recording of guinea pig urinary bladder smooth muscle strips

Strips of detrusor muscle (~4 x 15 mm) were placed vertically in a 10 ml organ bath filled with K-HS maintained at 37 °C and gassed with 95% O₂ - 5% CO₂. Isometric contractions were measured using force displacement transducers interfaced with a Macintosh computer using MacLab hardware and dedicated software (ADInstruments; Colorado Spring, CO, USA). The muscle strips were placed under an initial resting tension of 1.5 g, and allowed to equilibrate for 60 min, with solution changes every 20 min. All the strips obtained from the same animal were 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 Mac Lab. Trains of rectangular pulses (0.3 ms duration, 0.5-40 Hz, 350 mA current strength) were delivered for 10 s at 3 min intervals. After construction of an initial
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 frequency-response curve was repeated. At the end of each experiment the dry weight of the strips was measured to normalization of the contractile responses.

**Western blot analysis**

Detrusor muscle was homogenized in lysis solution (for composition see Solutions and drugs) using a homogenizer (Ika-Werke, Staufen, Germany) and then sonicated for 5 sec. Lysates were centrifuged at 10,000 g for 15 min at 4°C to remove nuclei and unlysed cells and the protein concentration was measured.. Protein extracts (40 µg) were heat-denaturalized at 95°C for 5 min with DTT, electrophoresed on 7.5 or 15 % polyacrylamide-SDS gels and then transferred to a nitrocellulose membrane. Membranes were blocked for 1 h at room Tª using 10% bovine serum albumin (BSA) and incubated overnight at 4°C with affinity-purified polyclonal antibodies for choline acetyltransferase (ChAT, 1:1000, Chemicon International, Inc, Temecula, CA), nitric oxide synthase 1 (NOS1, 1:500, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), calcitonin gene related peptide (CGRP, 1:1000, Abcam, Cambridge, UK) and substance P (SP, 1:1000, Abcam, Cambridge, UK). A mouse anti-α tubulin monoclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) was used as load control. After washing, the membranes were incubated for 1 h at room Tª with anti- IgG-horseradish peroxidase conjugated secondary antibody (anti mouse for α tubulin, 1:10000, Amersham Biosciences, Bucks, UK; anti goat for ChAT,1:10000, and anti rabbit for the rest of primary antibodies, 1:7000, Santa Cruz Biotechnology, Inc, Santa Cruz, CA). The blots were then detected with the supersignal west pico chemiluminescent substrate (Pierce, Rockford, IL). The intensity of the bands was quantified using ImageJ software (NIH, Bethesda, MD) and normalized respect to α-tubulin content.
**Malondialdehyde (MDA) and reduced glutathione (GSH) assays**

Urinary bladder fragments of about 10 mg were emplaced in a cold phosphate buffer at a proportion 1/5 (weigh/volume), homogenized with an homogenizer (Ika-Werke, Staufen, Germany) for two minutes and centrifuged at 10000 rpm for 15 minutes at 4º C. The protein concentration was then quantified with a commercial kit (TPRO-562, Sigma) and the rest of homogenate was treated with cold perchloric acid (7% vol/vol) to eliminate proteins and kept at -80º C until analysis. Malondialdehyde (MDA) level, an index of lipidic peroxidation, was determined based on colorimetric Recknagel’s method (45). Briefly, the samples were incubated with 0.4 % of tiobarbituric acid at 80º C for 20 minutes and later the sample absorbance at 550 nm was measured. Reduced glutathione determination was carried out following the Hissin and Hilf method (18): samples were incubated with 0.005 % of ortophtaldehyde in the darkness at room temperature for 45 minutes and the fluorescent complex formed, indicative of reduced glutathione (GSH) level, was measured with a fluorimeter (excitation 350 nm, emission 425 nm).

**Quantification and statistics**

Results are expressed as means ± SEM of n urinary bladder strips or determinations. Urinary bladder tension is given in millinewtons (mN)/mg of tissue. Inhibition of contraction is calculated as the percentage decrease in the tension evoked by a treatment respect a previous control EFS performed in the same strip. All results from MDA and GSH determinations are given as nmol/mg of protein. Statistical differences between animal groups and drug effects were determined using adequate analysis of variance (two ways ANOVA) followed by a Bonferroni’s post hoc test. Differences were considered significant at \( P < 0.05 \).
RESULTS

Irrespective of the age of animals, electrical field stimulation of guinea pig urinary bladder produced a frequency-dependent contraction of about 10 seconds of duration reaching the maximal amplitude when the strips were stimulated at 25 or 40 Hz. As shown in figure 1, aging decreased the contractile response evoked by EFS, an effect reversed by melatonin treatment (Fig. 1A and 1B). Melatonin did not change significantly the EFS-induced responses in adult animals (Fig. 1A and 1B). To determine whether aging altered the neural origin of the EFS-evoked contraction, we used the nerve Na⁺ channel inhibitor tetrodotoxin (TTX, 1 µM). TTX almost abolished the response to EFS for all the frequencies tested in all the experimental groups (Fig. 1C). In keeping with this, we obtained similar results with a combined treatment with atropine (1 µM), suramin (100 µM) and guanethidine (1 µM), which blocks cholinergic, purinergic and adrenergic neurotransmission, respectively (Fig. 1D).

Ageing-induced alterations in neurogenic contractions can be due to changes in the myogenic response of detrusor, modifications in the intrinsic plexus or both. In order to test the response of bladder smooth muscle, we challenged the strips with the muscarinic agonist bethanecol (100 µM) and the purinergic agonist ATP (100 µM). Ageing reduced the contractile response to both stimuli (bethanecol: adult, 5.8 ± 0.27 mN/mg, aged, 2.5 ± 0.43 mN/mg; ATP: adult, 1.3 ± 0.15 mN/mg, aged, 0.3 ± 0.05 mN/mg; n = 6-9, P < 0.001 adult vs aged for both stimuli). Melatonin treatment (ML) recovered detrusor contractility in aged animals but did not have any effect in adults (bethanecol: ML adult, 5.5 ± 0.37 mN/mg, ML aged, 7.0 ± 0.65 mN/mg; ATP: ML adult, 1.2 ± 0.15 mN/mg, ML aged, 1.3 ± 0.18 mN/mg; n = 6-9, P < 0.001 ML aged vs aged for both stimuli). Depolarization-induced contractility is also impaired by ageing as demonstrated by the observed reduction in KCl-induced response (adult: 5.6 ± 0.41
mN/mg, ML adult: 4.6 ± 0.36 mN/mg, aged, 1.7 ± 0.31 mN/mg, ML aged, 4.8 ± 0.54 mN/mg; n = 6-9, P < 0.001 adult vs aged.

Age-related changes in the excitatory innervation

Similar to several species, the main excitatory neurotransmitter in guinea pig urinary bladder is ACh. The cholinergic contribution to EFS-induced contractile response was determined using atropine (1 μM), which reduced the contraction in a frequency-dependent fashion in all experimental groups (Fig. 2A-D). Although the maximal reduction caused by atropine in aged strips (1.7 mN/mg) was smaller than in the other experimental groups (4 mN/mg), the percentage of inhibition caused by atropine was similar in all groups (Fig. 2E), indicating that the cholinergic component does not change with age. This was confirmed by western blot analysis of the ACh synthesizing enzyme choline acetyltransferase (ChAT), which was expressed at the same extent in all the experimental groups (Fig. 2F).

As shown in Fig. 2, atropine effects ranged from 19% inhibition at 0.5 Hz to 58% inhibition at 40 Hz (Figure 2E), but it was unable to abolish the contraction, indicating the release of additional excitatory neurotransmitters in response to EFS. In order to characterize this non-cholinergic excitatory component we tested suramin, a purinergic antagonist, and capsaicin, which induces a sensory denervation when used at high concentration. In young adult guinea pigs 100 μM suramin reduced the EFS response in a negative frequency-dependent fashion (36% inhibition at 0.5 Hz and 16% inhibition at 40 Hz, Fig. 3A and 3D). By the contrary, suramin was ineffective in aged strips (Fig. 3B and 3D), suggesting that aging evokes a functional purinergic denervation in guinea pig urinary bladder. Melatonin treatment of aged animals restored suramin effects on EFS-evoked response (35% of inhibition at 0.5 Hz and 11% of inhibition at 40 Hz, Fig. 3C and 3D) but did not have any effects on adult animals (Fig. 3D).
As shown in figure 4, desensitization of afferent nerves by a high concentration of capsaicin (10 µM) reduced the response, revealing the contribution of afferent neurones in the contraction induced by EFS. Similar to the purinergic component, the afferent contribution was impaired by aging (Fig. 4B), although capsaicin conserved a slight effect on the contraction. Melatonin treatment also restored the excitability of the afferent innervation, increasing the sensitivity to capsaicin in aged strips to levels similar to those in young adult strips (Fig. 4). However, in young adult animals, melatonin slightly reduced capsaicin inhibitory effects at low stimulation frequencies (Fig. 4D). The smaller inhibition induced by capsaicin on aged strips is in agreement with the increase in calcitonin gene related peptide (CGRP) expression and the decrease in substance P (SP) content found in aged detrusor by western blot analysis (Fig. 4E and F).

To test the presence of interactions between purinergic and afferent innervation, the strips were treated with 100 µM suramin before or after application of 10 µM capsaicin. Both protocols resulted in additive effects of suramin and capsaicin (data not shown) indicating that these neural components do not interact. Following the same approach, we pre-treated the strips with 1 µM atropine before suramin or capsaicin to test respectively cholinergic modulation of purinergic and afferent excitatory neurotransmission. In young adult animals, suramin in the presence of atropine induced an inhibition of the EFS response which was similar to that obtained by suramin alone at low frequencies (suramin alone: 35.8 ± 6.53 % inhibition at 0.5 Hz, suramin after atropine: 29.6 ± 4.46 % inhibition at 0.5 Hz, compare effects of suramin in Fig. 3A & 5A) but it was higher at medium-high frequencies, suggesting that cholinergic nerves negatively modulate purinergic fibers. Surprisingly, in aged animals where application of 100 µM suramin alone did not have effects (see Fig. 3), suramin inhibited the EFS-
elicited contractile response in the presence of atropine (suramin alone: 1.0 ± 3.51 % inhibition at 0.5 Hz, suramin after atropine: 34.2 ± 4.96 % inhibition at 0.5 Hz, Fig. 5B), indicating that aging increases the cholinergic negative modulation of purinergic innervation. In melatonin treated aged animals, the effect of suramin after atropine was similar to that in young animals, showing that at frequencies resembling physiological stimulation (0.5 Hz) there was no apparent cholinergic modulation of the purinergic neurotransmission (suramin alone: 31.7 ± 5.86 % inhibition at 0.5 Hz, suramin after atropine: 27.0 ± 2.33 % inhibition at 0.5 Hz, Fig. 5C). Regarding the afferent sensitive fibers, capsaicin was unable to reduce the neurogenic response in presence of atropine (Fig. 5D, 5E, 5F), suggesting that in urinary bladder afferent innervation collaborates in the total response to EFS through activation of cholinergic motor fibers. In young animals, melatonin treatment did not have any effects on cholinergic modulation of purinergic and sensitive fibers (data not shown).

Age-related changes in the inhibitory innervation

L-arginine-derived nitric oxide (NO) seems to be responsible for the main part of the inhibitory NANC responses in the lower urinary tract (33), although a clear physiological role for this neurotransmitter is not firmly established. To investigate a possible effect of aging on the nitrenergic control of bladder contractility, we tested the effect of the specific NO synthase inhibitor No-nitro-L-arginine methyl ester (L-NAME) on the response to EFS. As shown in Fig. 6, 100 μM L-NAME was ineffective in strips from adult guinea pigs (Fig. 6A) while in aged strips it resulted in an enhancement of the EFS-evoked responses, indicating that EFS releases relaxing NO (Fig. 6B). The effect of L-NAME in strips from aged animals increases with the frequency of stimulation (85.1 ± 4.47 % increase at 0.5 Hz and 21.0 ± 1.91 % increase at 40 Hz) and it is absent when the old animals were treated with melatonin (Fig. 6C).
Despite these results, ageing did not change the expression of nNOS in detrusor strips (Fig. 6E).

To study the participation of adrenergic innervation in normal and aged bladder contraction we used guanethidine (1 μM), which prevents the synaptic release of noradrenaline. This treatment had no effects in strips from adult guinea pigs (Fig. 7A) but increased the contraction in strips from aged animals (Fig. 7B), indicative that in aged urinary bladder EFS releases noradrenaline which, probably through β-adrenergic receptors, leads to relaxation. This effect increased with the frequency of stimulation (6.9 ± 2.15 % increase at 0.5 Hz and 27.1 ± 3.39 % increase at 40 Hz) and was reversed by treatment with melatonin (Fig. 7C). Melatonin treatment in young adults did not modify the effects of L-NAME or guanethidine (data not shown).

The data presented above show that cholinergic neurons control purinergic and afferent fibres. To assess whether this control also occurred for the nitrergic and adrenergic innervation, we treated the strips with 100 μM L-NAME or 1 μM guanethidine in the presence of 1 μM atropine. Though L-NAME or guanethidine alone resulted ineffective in adult strips, in atropine-treated strips these inhibitors increased the EFS-induced response (Fig. 8A & 8B), indicating that in young adult guinea pig urinary bladder the neural release of NO and noradrenaline is inhibited by acetylcholine. The contraction in L-NAME and atropine treated strips was 160.3 ± 13.7 % respect to atropine treated strips at 0.5 Hz and 144.5 ± 12.16 % at 40 Hz. For guanethidine and atropine treated strips the response was 180.0 ± 10.30 % respect to atropine treated strips at 0.5 Hz and 152.8 ± 9.74 % at 40 Hz (Fig. 8). This cholinergic modulation of inhibitory innervation is exclusive of adult animals, since it was absent in both aged and melatonin-treated animals (Fig. 8).
Oxidative stress in aged guinea pig urinary bladder

MDA level, an index of lipidic peroxidation, was significantly higher (\(P < 0.05\)) in bladders from aged animals (0.83 ± 0.097 nmol/mg of protein, \(n=7\)) than in those from young adult animals (0.55 ± 0.043 nmol/mg of protein, \(n=8\)). In parallel, aging caused a significant (\(P < 0.01\)) reduction in GSH levels from 5.0 ± 0.20 to 3.8 ± 0.29 nmol/mg of protein (adult and aged groups, respectively). Melatonin treatment reversed the elevations in MDA levels and the changes in GSH levels evoked by ageing (MDA: 0.52 ± 0.084 nmol/mg of protein; GSH: 5.0 ± 0.40 nmol/mg of protein, \(n=6\)).

**DISCUSSION**

The results of the present study demonstrate that aging impairs EFS-evoked contractile response in guinea pig urinary bladder through a decrease in detrusor contractility together with the functional impairment of excitatory nerves and the sensitization of inhibitory fibres. The impaired neurogenic response correlated with an increase in markers of oxidative stress at the urinary bladder level. Melatonin treatment recovered the neurogenic contraction, reversed most of the age-induced neurotransmission alterations and restored the levels of oxidative stress markers to those of young adult individuals.

In our experimental conditions EFS of isolated detrusor induces a neurogenic contraction, as shown by the strong inhibition by TTX or by a combination of neurotransmission antagonists. This neurogenic contraction was lower in aged guinea pig detrusor, a finding in keeping with previous reports of age-associated loss of urinary bladder innervation (5, 13) and neuromuscular function (31), but disagrees with other findings (34, 46, 47). Similar to human and other species, the main excitatory neurotransmitter in guinea pig bladder was acetycholine (23, 46). Our data indicate that
the rest of the contraction is due to purinergic and afferent (capsaicin sensitive) fibres, in keeping with the reported presence of tachykinins (including SP and neurokinins) in sensory afferent nerves of the urinary bladder in several species (27). Although tachykinins have an obvious afferent functions, they may contract detrusor after peripherical release, as shown in other organs such as the gastrointestinal tract (26). In our model aging induced a functional loss of purinergic neurotransmission and reduced SP, the excitatory component of sensory fibers, similar to previous reports in rat bladder (7), but did not alter the cholinergic component of the contraction, while in human bladder it evokes a decrease in the cholinergic neurotransmission and an increase in the purinergic component (46).

In addition to the changes reported above, we describe here for the first time the presence of neuromodulation between different neural components in urinary bladder contraction. The combination of atropine and suramin shows that in aged animals acetylcholine binding to muscarinic receptors inhibits the release of excitatory ATP from purinergic fibres. Although the type of cholinergic receptor involved is out of the scope of our study, this mechanism can explain the loss of purinergic contraction in aged detrusor.

The present study shows that, in addition to the loss of excitatory neurotransmission, alterations in the inhibitory innervation also contribute to the impairment in contractility. Although NO is a well accepted non-adrenergic, non-cholinergic inhibitory neurotransmitter in other smooth muscles, such as vascular and gastrointestinal, its functional role on detrusor is controversial. NO synthase is present in detrusor muscle (11, this study) but the release of NO in response to EFS and its relaxing effects are deceiving (21, 24). Similar to NO, the role of catecholamines in the control of urinary bladder is controversial (for review see 3). Inhibitory (β) and
excitatory (α) (37) adrenergic receptors have been described, but the net effect of noradrenergic release on urinary bladder depends on multiple factors (species, age, sex, region of bladder, etc). We found that only in aged strips the blockade of nitricergic (L-NAME) or adrenergic nerves (guanethidine) enhanced the EFS-evoked contraction, indicating that in aged bladders the relevance of inhibitory innervation is increased. In the case of young adult animals the release of these relaxing neurotransmitters is inhibited by cholinergic modulation of nitricergic and adrenergic terminals, and the loss of this neuromodulation during aging contributes to the impaired contractile response present in aged individuals, which is supported by the absence of changes in NOS labelled neurons in aged bladder. Therefore, aging mainly induces changes in the neuromodulation of the intrinsic plexus leading to impairment of contraction. To some extent, this is reminiscent of the alterations of cortical synapses observed in aged brain (19).

Although our data show that the loss of neurogenic contraction is in part due to a specific pattern of alterations in the release of neurotransmitters and in the reciprocal neuromodulation, they also support the participation of myogenic mechanisms, as we have found that aging also decreases myogenic contractions in response to exogenous agonists (bethanechol and ATP) and depolarization. The hypocontractility of aged detrusor can be related to changes in the process of calcium sensitization in detrusor cells since there is not a decrease in Ca\(^{2+}\) mobilization in response to agonists in aged cells (Gomez-Pinilla, Camello and Pozo, unpublished data).

Regarding the subcellular mechanisms leading to the observed effects of aging in bladder contraction, our finding that aging is associated with an increase in levels of oxidative stress markers (high lipidic peroxidation and low GSH levels) suggests that this factor contributes to the process. In urinary bladder several conditions associated to
high levels of free radicals lead to altered detrusor responses (29, 30, 36, 41), and oxidative stress impairs the contraction in response to muscarinic activation (9). Moreover, this link is further supported by the normalization of the oxidative parameters in melatonin-treated animals. Melatonin is a potent scavenger and antioxidant agent (43) and the clear beneficial effects presented here is likely to be related to mitigation of oxidative stress. Beneficial effects of melatonin have been described in conditions of oxidative stress associated with cystitis (36) and in patients with nycturia (10) where melatonin normalizes the altered state of bladder. It is noteworthy that recent evidence shows that melatonin restores mitochondrial function and preserves this organelle from the oxidative damage associated to aging and inflammatory diseases (reviewed in 28), in keeping with the central role of mitochondria in the oxidative-related mechanisms of aging. In addition, melatonin prevents lipid peroxidation by scavenging peroxyl radical which has an important role in the propagation of the chain reaction driving to extensive cellular damage (38).

Regarding the targets of melatonin, our results indicate that they can be the nerves, since melatonin-induced improvement of neuromuscular function is associated to the recovery of the sensitiveness to capsaicin, suramin, L-Name and guanethidine, but the effects of melatonin also take place in the muscle cells, since melatonin improves the contraction of guinea pig detrusor muscle. These effects of melatonin are specific for aged tissue, since melatonin treatment of adult animals did not increase the contractility, as in aged detrusor, but even caused a slightly decrease in the KCl-induced response. Similarly, it did not cause much change in the intrinsic plexus and when there was a small effect (such as the increase in CGRP content) this was to the contrary to that induced in aged bladder. By now, there is no explanation for these selective effects of melatonin in aged tissue, but the indolamine has also differential effects in apoptosis
and cell death depending on the condition of the tissue. Thus, in immune cells (39) and other tissues (35) melatonin reduces apoptosis, but it is proapoptotic in several cancer cell types (28).

All together, it is clear that melatonin can target several mechanisms compromised by the aging process in urinary bladder, explaining the improvement of in vivo urinary bladder function recently reported by our group in this model (16). This conclusion is also supported by related results in aged guinea pig gallbladder, another smooth muscle rich reservoir organ where melatonin treatment normalizes the age-induced altered neural function in a similar way to that described in this report (15). In view of the clearly advantageous effects of melatonin in age-associated alterations, the use of this hormone as a protector of lower urinary tract is a promising alternative to less efficient actual treatments.

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LEGENDS TO FIGURES.

Fig. 1. Aging impaired EFS-elicited contractile response in guinea pig urinary bladder. A: Original recording showing urinary bladder contraction elicited by EFS (0.3 ms duration, 0.5-40 Hz, 350 mA, for 10 s every 3) applied to untreated and melatonin-treated adult and aged guinea pigs. Traces are typical of 15 to 17 strips. The response was smaller in strips from aged animals (** P<0.01, Bonferroni post-ANOVA test), and melatonin restored it to normal values. B: Summary of peak amplitudes of the EFS-induced contraction in the three groups. (n= 15-17, ** P < 0.01 by ANOVA). The neural origin of EFS-evoked responses was demonstrated with the use of 1 µM tetrodotoxin (TTX) (C) and the inhibitors guanethidine (1 µM) plus atropine (1µM) plus suramin (100 µM) (GAS) (D) (n= 7-10). There are not significant differences between animals groups in C and D.

Fig. 2. Aging did not alter the cholinergic component of the contraction in the guinea pig urinary bladder. Effect of 1 µM of atropine on EFS-elicited contractile response in guinea pig urinary bladder from adult (A), aged (B), adult treated with melatonin (C) and aged treated with melatonin (D) guinea pigs. After EFS was performed in control conditions, strips were incubated for 20 min with atropine and EFS was repeated. Atropine reduced the contraction in a frequency-dependent manner with maximal inhibition (50 %) at 40 Hz. Inset shows corresponding original recording of 25 Hz-evoked responses from each animal group in the absence and presence of atropine. E: Summary of atropine effect on EFS response in the three guinea pig groups. No significant differences between groups were found at any of the frequencies tested. Data
are from 25-13 urinary bladder strips. (** \( P < 0.01 \) by ANOVA). F: Original western blots using anti-Choline acetyl transferase (ChAT) and anti-\( \alpha \) tubulin antibodies on bladder smooth muscle strips from adult (A), aged (Ag), adult treated with melatonin (A+ML) and aged treated with melatonin (Ag+ML). The blots are representative of four others. The histogram summarizes levels (mean ± sem) of ChAT protein expression normalized to \( \alpha \)-tubulin content for each western analysis, expressed as fold increase respect to adult values.

**Fig. 3. Aging reduced purinergic component of guinea pig urinary bladder contraction and melatonin restored it.** A: Effect of 100 \( \mu \)M suramin on EFS-elicited contractile response in bladder strips from adult guinea pigs. After suramin treatment the neurogenic contractile response is reduced, indicating that ATP released from purinergic fibers contributes to contraction. In aged strips suramin resulted ineffective (B) but melatonin treatment reestablished the suramin sensitivity (C). Insets show corresponding original recording of 25 Hz responses from each animal group in the absence and presence of suramin. D: Summary of 100 \( \mu \)M suramin-induced reduction on EFS response in the three guinea pig groups and in adult guinea pigs treated with melatonin (open triangles). The effect of suramin diminished with the frequency of stimulation. Data are from 5-6 strips. (* \( P < 0.05 \), ** \( P < 0.01 \) by ANOVA).

**Fig. 4. Aging reduced the role of excitatory sensory afferent fibers in the guinea pig urinary bladder.** Effect of capsaicin on EFS-elicited contractile response in urinary bladder strips from adult (A), aged (B) and melatonin treated aged guinea pigs (C). After EFS was performed in control conditions, strips were incubated for 20 min with 10 \( \mu \)M capsaicin to promote afferent sensory denervation. Note that capsaicin reduced the EFS-
induced contraction indicating that neural activation releases an excitatory component from sensory fibers. Insets show corresponding original recording of 25 Hz evoked contraction from each animal group in the absence and presence of 10 μM of capsaicin. (D) Summary of the reduction evoked by capsaicin treatment on EFS response in the three guinea pig groups and in adult treated with melatonin. The sensory participation is smaller in bladder from aged animals. Data are from 5-9 urinary bladder strips. (* P < 0.05 and ** P < 0.01 by ANOVA). Original western blot analysis of the expression of CGRP (E) and SP (F) in adult (A), adult treated with melatonin (A+ML), aged (Ag) and aged treated with melatonin (Ag+ML). Anti-α tubulin antibody was used as loading control. The blots are representative of other 4 experiments. The histograms summarize the level of expression (mean ± sem) of CGRP and SP, expressed as fold increase respect to adult values after normalization to tubulin content.

Fig. 5. Effects of cholinergic nerves on purinergic and sensory fibers in the urinary bladder. Effect of 100 μM suramin after atropine (1 μM) on EFS-elicited contractile response in urinary bladder from adult (A), aged (B) and melatonin treated aged animals (C). Atropine does not block the inhibitory effect of suramin. Data are from 5-6 urinary bladder strips. D, E and F: in strips pretreated with atropine (1 μM) capsaicin (10 μM) resulted ineffective, indicating that cholinergic activation is necessary to release excitatory neurotransmitters from sensory nerves. Data are from 5-6 urinary bladder strips. (** P < 0.01 by ANOVA).

Fig. 6. Aging reveals inhibitory nitrergic innervation in guinea pig urinary bladder. Original recordings of bladder strips contraction elicited by EFS before and after application of 100 μM L-NAME in adult (A), aged (B) and melatonin-treated aged
guinea pigs (C). L-NAME treatment increased the response to EFS only in aged strips. Traces are typical of 5 to 8 strips. D: Summary data of the increase induced by 100 µM L-NAME on EFS-evoked contraction in the three experimental groups. (n=5-8, * P < 0.05 by ANOVA). E: Original western blot analysis of the expression of NO synthase (NOS1) in adult (A), adult treated with melatonin (A+ML), aged (Ag) and aged treated with melatonin (Ag+ML). Anti-α tubulin antibody was used as loading control. He blots are representative of other 4 experiments. The panel on the right summarizes the level of expression (mean ± sem) of NOS1, expressed as percentage of adult values after normalization to tubulin content.

Fig. 7. Aging evokes a hipereactivity of inhibitory adrenergic innervation in guinea pig urinary bladder. Original recordings of urinary bladder contraction elicited by EFS in the presence and absence of 1 µM guanethidine in adult (A), aged (B) and melatonin-treated aged guinea pigs (C). Adrenergic depletion evoked by guanethidine increased the response to EFS indicative of noradrenaline-induced relaxation. Traces are typical of 6 to 11 strips. D: Summary data of guanethidine-induced increase on EFS-induced contraction. (n=6-11, * P < 0.05 by ANOVA).

Fig. 8. Aging suppresses cholinergic inhibition of relaxing innervation in the guinea pig urinary bladder. Effect of 100 µM L-NAME (A) or 1 µM guanethidine (B) after atropine (1 µM) pretreatment on EFS-elicited contraction in young, aged and melatonin-treated aged guinea pig urinary bladder. Note that L-NAME or guanethidine enhanced the effects of neural response only in young adult animals, indicating that in aged individuals cholinergic inhibition of relaxing nitrergic and adrenergic nerves is lost. Data are from 5-10 urinary bladder strips. (**) P < 0.01 by ANOVA).
REFERENCES


Figure 1

A

Adult

2 mN/mg

90s

Aged

Adult + Melatonin

Aged + Melatonin

0.5 Hz 1 Hz 2 Hz 3 Hz 4 Hz 5 Hz 10 Hz 15 Hz 25 Hz 40 Hz

B

Contraction (mN/mg)

GAS

TTX

Adult

Aged

Adult ML

Aged ML

0.5 Hz 1 Hz 2 Hz 3 Hz 4 Hz 5 Hz 10 Hz 15 Hz 25 Hz 40 Hz

C

Inhibition (%)

TTX

Adult

Aged

Adult ML

Aged ML

0.5 Hz 1 Hz 2 Hz 3 Hz 4 Hz 5 Hz 10 Hz 15 Hz 25 Hz 40 Hz

D

Inhibition (%)

GAS

Adult

Aged

Adult ML

Aged ML

0.5 Hz 1 Hz 2 Hz 3 Hz 4 Hz 5 Hz 10 Hz 15 Hz 25 Hz 40 Hz
Figure 2

A

B

C

D

E

F

- ChAT expression (fold increase respect to adult)

- α-Tubulin

- 75 kDa

- 55 kDa
Figure 3

A

- Control
- Suramin

B

- Control
- Suramin

C

- Control
- Suramin

D

- Adult
- Aged
- Adult + Mel
- Aged + Mel

Inhibition (%)

Contraction (mN/mg)

Frequency (Hz)
Figure 5

A  
Adult

B  
Aged

C  
Melatonin

D  
Adult

E  
Aged

F  
Melatonin

- Control
- Atropine
- Atropine + Suramin
- Atropine + Capsaicin
Figure 6

A

Adult

100 µM L-NAME

2 mN/mg

15 Hz 25 Hz 40 Hz

B

Aged

15 Hz 25 Hz 40 Hz

C

Aged + Melatonin

15 Hz 25 Hz 40 Hz

180s

D

Adult

Increase (%)

Frequency (Hz)

E

NOS1

150 kDa

α-Tubulin

55 kDa

NOS1 expression

(fold increase respect to adult)

A+ML Ag Ag+ML
Figure 7

A. Adult

B. Aged

C. Melatonin

D. Adult

E. Aged

F. Melatonin

G. 1 µM Guanethidine

H. 180s

2 mN/mg

15 Hz 25 Hz 40 Hz

Adult

Aged

Melatonin

Increase (%)
Figure 8

A

Adult

Aged

Melatonin

B

Adult

Aged

Melatonin

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