ACS Chemical Neuroscience

Research Article

pubs.acs.org/chemneuro

Age-Related Changes in Melatonin Release in the Murine Distal Colon

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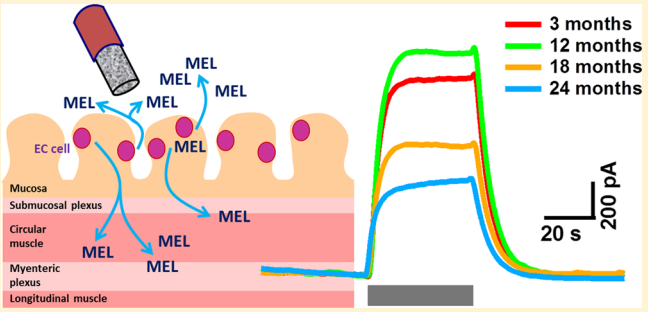
ABSTRACT: Constipation and fecal impaction are conditions of the bowel whose prevalence increases with age. Limited information is known about how these conditions manifest; however, functional deficits are likely to be due to changes in signaling within the bowel. This study investigated the effects of age on colonic mucosal melatonin (MEL) release and the consequences this had on colonic motility. Electrochemical measurements of MEL overflow demonstrated that both basal and mechanically stimulated MEL release decreased with age. The MEL/serotonin also decreased with increasing age, and the trend was similar to that of MEL overflow, suggestive that age-related changes were primarily due to a reduction in MEL levels. Levels of *N*-acetylserotonin and the *N*-acetylserotonin/serotonin ratio were reduced with age, providing an explanation for the reduction in MEL release. Decreases in colonic motility were observed in animals between 3 and 24 months old. Exogenous application of MEL could reverse this deficit in aged colon. In summary, we propose that the age-related decline in MEL release may be due to either decreases or alterations in mechanosensory channels and/or a loss in levels/activity of the *N*-acetyltransferase enzyme responsible for the synthesis of *N*-acetylserotonin. Decreases in MEL release may explain the decreases in colonic motility observed in 24 month old animals and could offer a new potential therapeutic treatment for age-related constipation.



KEYWORDS: *Melatonin, distal colon, aging, enterochromaffin cell, constipation*

Constipation and fecal impaction are disorders that are prevalent with aging.[1,2] These conditions are often associated with a decrease in the patient's quality of life and also result in increased healthcare costs.[3,4] The causes of chronic constipation in the elderly are likely to be multifactorial and include the effects of age on gastrointestinal (GI) tract physiology, comorbidities, increase in medication use, loss of mobility, reduced caloric intake, and ano-rectal sensory changes.[5] One factor proposed to contribute to age-related constipation is a decrease in the number of myenteric neurons.[6] However, a reduction in myenteric neuronal number is also observed in humans with no detectable gastrointestinal disorders, and therefore, their involvement in age-related constipation is questionable.[7] The most likely factor is an alteration in signaling evoked by the neurotransmitters or neurochemicals that regulate the bowel; however, limited studies have been conducted to understand the functional changes associated with aging.

Aged rodent models have also been shown to have symptoms similar to constipation suggesting that they may be a good model to study this age-related disorder.[8] Additionally, aged rodents also demonstrate a loss of myenteric neurons consistent with the human data.[6,9] We have however observed that myenteric neuron numbers are maintained in the aging mouse distal colon.[10]

Melatonin (MEL) is well-known as a paracrine hormone that is secreted in a cyclic manner by the pineal gland.[11] MEL controls biological rhythms, pigment metabolism, immune response, metabolism of free radicals, monitoring of mood and sleep, and cell proliferation and differentiation.[12,13] MEL is also present in the GI tract, and the enzymes and receptors that are necessary for its biosynthesis and activity have been observed in humans, rats, and rabbits.[14,15] MEL is synthesized by the enzymes hydroxyindole-*O*-methyltransferase (HIOMT)[16] and *N*-acetyltransferase (NAT), and both enzymes have been detected in the GI mucosa.[17,18] Studies have also localized the enzymes to the enterochromaffin (EC) cells in the mucosa.[19,20] Many studies have identified that NAT is the rate-limiting enzyme in the biosynthesis process. MEL is made on demand and can pass across cell membranes to affect its targets in the GI tract.[14] Many of these effects appear to be via MT_2 receptors, although some of its antioxidant properties may be receptor independent.[14]

MEL has been shown to have a wide range of effects on GI function. Most often, MEL is believed to function as a physiological antagonist of the actions of serotonin in the GI tract.[19,21,22] However, studies have also shown that MEL can influence intestinal muscles either directly[23] or via the

Special Issue: Monitoring Molecules in Neuroscience

Received: January 15, 2013
Accepted: April 30, 2013
Published: April 30, 2013

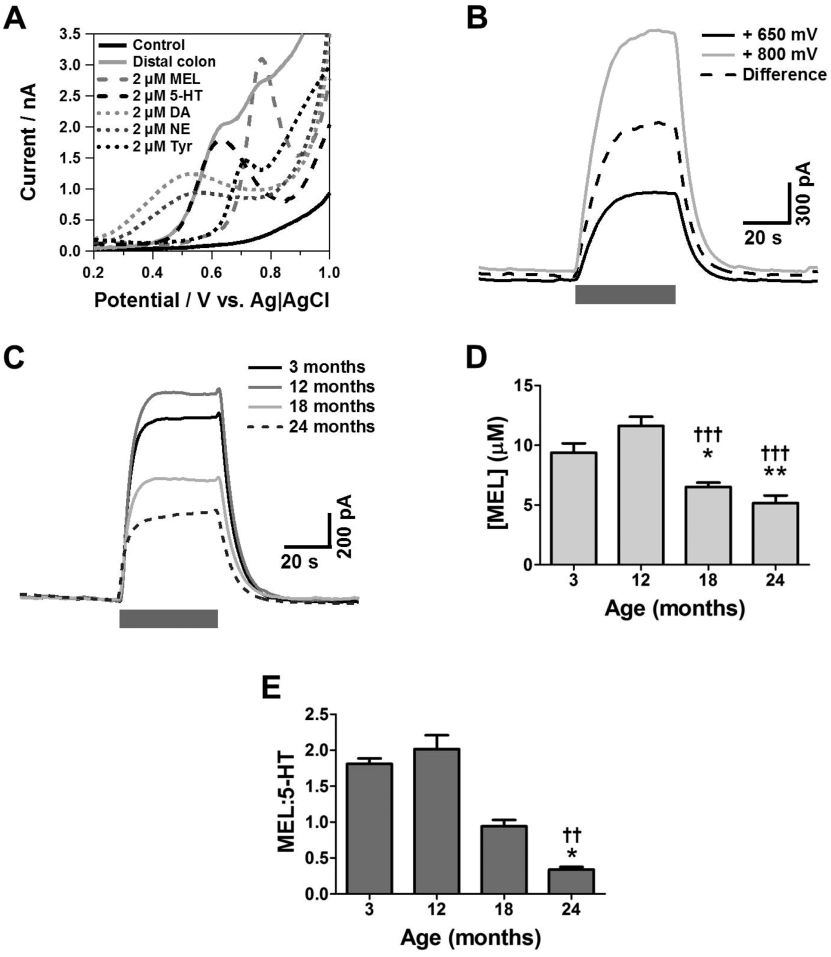


Figure 1. Electrochemical determination of melatonin release from distal colon mucosa. Monitoring of all electroactive substances released using differential pulse voltammetry from the mucosa (A) showed the presence of serotonin and melatonin, when compared to standards. Melatonin was monitored amperometrically by obtaining the current difference between +650 and +800 mV vs Ag|AgCl, where DA is dopamine, NE is norepinephrine, and Try is tryamine. (B) Gray bar indicates the duration the BDD electrode was positioned 0.1 mm over the mucosa. Current responses from 3, 12, 18, and 24 month old animals are shown in (C). The population data for the melatonin is shown in (D). The melatonin to serotonin ratio is shown in (E). Data shown as mean ± SEM, $n = 6$, *$p < 0.05$ and **$p < 0.01$ vs 3 month old animals and $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$ vs 12 month old animals.

myenteric plexus.[24] Other studies have also claimed that MEL inhibits the contraction of smooth muscles in the ileum.[25] Due to the variety of actions MEL can have on intestinal function, understanding how MEL signaling changes with age is important. MEL treatment has been shown to extend lifespan in C57/balbc mice and caloric restriction, a well-recognized mechanism for extending lifespan in a wide variety of organisms, has been shown to increase MEL levels in rats.[26] MEL treatment can also reverse age-related increases in mucosal 5-HT availability[27] and markers of inflammation in mouse colon.[28] Taken together, these studies suggest that MEL may be antiaging both at the organismal level and also at the level of individual tissues. Therefore, understanding the changes in MEL levels with age may provide insight into the decline in colonic function.

The aim of this study is to understand the age-related changes in MEL release in the mouse colon and to determine if these changes correlate with altered motility. The basal levels of MEL will be monitored in 3, 12, 18, and 24 month old animals. The age-related alterations in mechanically stimulated MEL release will be investigated, along with changes in the level of the MEL precursor, *N*-acetylserotonin. These data will be compared to age-related changes in colonic motility. The influence of MEL and the MT_1 and MT_2 receptor antagonist luzindole on fecal pellet transit in both 3 and 24 month animals will be investigated. The role and potential therapeutic use of MEL for age-related constipation will be discussed.

■ RESULTS AND DISCUSSION

Age-Related Changes in Basal Melatonin Release. Figure 1A shows a differential pulse voltammogram obtained 0.1 mm over the mucosal surface using a boron-doped diamond (BDD) electrode, where two oxidation peaks were observed. When compared to standards, the first oxidation peak at +625 mV was comparable to that of serotonin. The second oxidation peak was at +770 mV and was matched with the oxidation peak potential of MEL. Other electroactive compounds such as dopamine, tryamine, and norephinephine did not have a response that matched the oxidative peaks observed from the mucosa. There were no other oxidation peaks observed over the potential window investigated. These two oxidation peaks have also be observed in other animal models, such as CD1 female mice, guinea pigs, and rabbits, where responses were attributed to serotonin and MEL.[27,29,30] Another electroactive

dx.doi.org/10.1021/cn4000617 | ACS Chem. Neurosci. 2013, 4, 879−887

substance that is either a precursor or metabolite of these two neurochemicals was not observed during chromatographic studies of the extracellular components released from the mucosa over a time frame similar to electrochemical recordings.[31] Overall, we can conclude that both MEL and serotonin are released from the mucosa into the lumen; however, the precise source of MEL release is still unknown. Many studies have indicated that the enzymes responsible for the synthesis of MEL are present in the EC cell.[15,32] However, MEL is released on demand, and thus, it has been difficult to identify its source to cellular locations using immunohistochemistry.

Due to the fact that both serotonin and MEL can be oxidized at a potential of +800 mV, the response to serotonin (obtained at +650 mV) is subtracted to solely provide the MEL response (Figure 1B). Background subtraction is often utilized to enhance the analytical signal, mainly during neurochemical analysis in the brain, where fast scan rates are employed (>100 V s^{-1}).[33,34] When signals are recorded in the brain, the dynamic features of the analytical response provide information regarding the neurotransmission process.[35] During our recordings, the dynamic features of the signal (rise time and decay of the current) are created by the movement of the electrode toward and away from the mucosal surface, and thus, only the current response at background and while over the tissue can be utilized when interpreting the analytical signal. From the responses observed in Figure 1B, when the sensor is located over the tissue, steady-state current responses are observed. This is mainly felt to be due to the constant low-level mechanical stimulation of the villi from the shear-flow of the buffer flowing through the tissue bath. Due to the fact that this physical parameter remains constant during the analytical recordings, the estimation of the MEL level can be determined accurately and the background subtraction approach provides an effective current response. However, this approach is limited by the fact that the recordings are made sequentially at two potentials over the same tissue location and thus estimations of the signal may be altered over the time frame investigated.

Figure 1C shows responses of MEL obtained from distal colon mucosa from animals aged 3, 12, 18, and 24 months old. A clear plateau in the analytical current is observed when the sensor is held above the mucosa. The population data for each age group is shown in Figure 1D. There is a significant age-related decrease in MEL levels ($p < 0.001$, $n = 6$, one-way ANOVA). Further analysis of this data showed that MEL levels decreased significantly between 3 and 18 months ($p < 0.05$, $n = 6$, Tukey test) and also decreased between 3 and 24 months old animals ($p < 0.01$, $n = 6$). There was also a significant decrease in MEL release between the 12 month tissue and both the 18 and 24 month old animals ($p < 0.001$, $n = 6$). The increase in the current response between 3 and 12 months was not significant. Another study that investigated the changes in MEL overflow with age in mice; however, no differences were observed in the distal colon.[27] These differences may be due to the fact that the strain of mouse varied between the two studies (CD-1 vs C57BL/6) and also this study was conducted in male rather than female mice.

Importantly serotonin is known to be present in the EC cell and known to drive colonic motility. We investigated if the MEL/serotonin altered with age as alterations in serotonin availability could influence the role of melatonin (Figure 1E). There was a significant age-related decrease in the MEL/serotonin ratio ($p < 0.01$, $n = 6$ Kruskall-Wallis). Further analysis showed that MEL/serotonin decreased between 3 and 24 month old animals ($p < 0.05$, $n = 6$, Dunn's) and also between 12 and 24 month old animals ($p < 0.01$, $n = 6$). Such similarities in the MEL/serotonin and the MEL overflow (Figure 1D) are suggestive that MEL may be the mucosal signaling molecule that alters with increasing age. In our study, the age-related decrease in MEL overflow could be attributed to a decrease in the levels of the precursor *N*-acetylserotonin or an alteration/expression of the enzymes responsible for the synthesis of MEL itself.

Alterations in Mechanical Driven MEL Release. The influence of mechanical force on the release of MEL was investigated with age. Figure 2 shows the response to mechanical force, which was provided by stimulating the villi adjacent to the BDD electrode with a glass capillary. Figure 2A shows the protocol utilized for recording, where the current response was monitored before (zone 1), during (zone 2), and after (zone 3) mechanical stimulation with a glass capillary. Figure 2B shows the response following mechanical stimulation in all age groups, where the gray bar indicates the duration of stimulation. There is an increase in the current observed in 3, 12, and 18 month old animals following mechanical stimulation. The rise time was identical in these age groups. It took ∼12 s to achieve maximum current response in all animals that elicited a response. The population data is shown in Figure 2C with the stars indicating the time points used for statistical comparison of the various phases of the recording. In 3, 12, and 18 month tissue, there is a clear and significant increase in the MEL current following a mechanical stimulus ($p < 0.001$, $n = 5$). Increases in both 3 and 12 month tissues were not significantly different from each other. However, the increase observed in the 18 month tissue was significantly lower than that observed in both 3 month ($p < 0.01$) and 12 month ($p < 0.05$) tissue. There was no significant difference in the current recorded in the 24 month tissue following a mechanical stimulus when compared to the baseline current. These changes are similar to the trend observed for the basal current in Figure 1D. This is suggestive that either age related changes in mechanosensory receptors or alterations in the activity of MEL synthesis enzymes may explain the deficits in MEL release with age. There are many studies that have indicated that NAT is the rate-limiting enzyme for MEL synthesis.[16,36] It is responsible for the production of the *N*-acteylserotonin which in turn is the main precursor for MEL. Studies in the retina have shown that altering the intracellular cyclic AMP (cAMP) content can regulate NAT activity, which is, in turn, critical in the regulation of MEL biosynthesis.[37] Many studies on isolated EC cells or EC cell mimics, the BON cell, have shown that mechanical stimulation increases intracellular Ca^{2+}, which in turn could potentially affect cAMP levels as adenylate cyclase enzymes can be Ca^{2+} dependent. Thus, mechanosensory channels could be directly linked to the output of NAT.

In 3 month tissue, comparison of the 20 s period postmechanical stimulation (zone 3) with the values recorded during the stimulus (zone 2) failed to show a significant difference, demonstrating that the response to mechanical stimulation was sustained for this period. In 12 and 18 month tissue, the same comparison showed a significant decline in the poststimulus current ($p < 0.01$ for both age groups). In 24 month old animals, no differences in the current were observed during and post mechanical stimulation. Overall, this data is suggestive that there is an age-related decline in the sustainment of the MEL current post mechanical stimulation.

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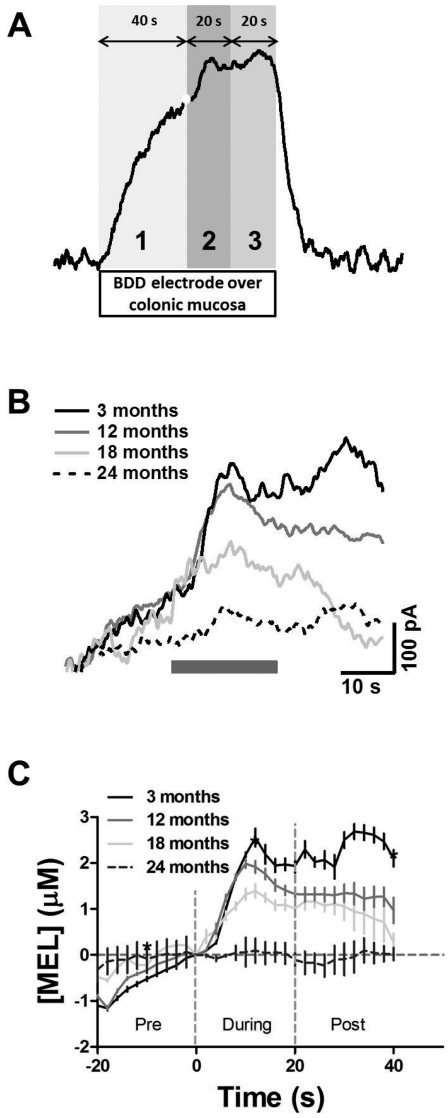

Figure 2. Alterations in mechanically driven melatonin release with age. The protocol utilized for recordings is shown in (A). Recordings are taken before (zone 1), during (zone 2), and after (zone 3) mechanical stimulation. The white dot indicates the point against which responses are normalized and also indicates the initiation of mechanical stimulation. Current responses from 3, 12, 18, and 24 month old animals are shown in (B). The gray bar indicates the time frame when the glass capillary was utilized to mechanically stimulate villi located adjacent to the BDD electrode. The overall response from multiple animals of all age groups is shown in (C), where the mechanically stimulated melatonin release is shown between 0 and 20 s, and the post mechanically stimulated response shown between 20 and 40 s. The stars indicate the points on the trace utilized for statistical comparison. Data shown as mean ± SEM, $n = 5$.

The sustained current is a marker of the activity in the mechanotransduction pathway. The results therefore indicate that the mechanotransduction pathway that drives the release of MEL is impaired with age and that this impairment could be due to either an impairment or loss of NAT with age and/or a loss or alteration in mechanosensory ion channels. In order to further examine the mechanism by which this process is impaired with age, we decided to examine whether NAT function was impaired with age.

Changes in *N*-Acetylserotonin Levels during Aging. Chromatographic analysis was carried out to monitor the levels of *N*-acetylserotonin as a marker of the function of NAT. Levels of this key neurochemical would provide an insight into the biosynthesis of MEL and could help to understand the age-related decrease in MEL release observed. Figure 3A and B shows sample chromatographs obtained from mucosa tissue. Initially, chromatographic analysis was carried out to monitor for MEL; however, the levels of MEL were below the detection limits of our system in any mucosal samples, further supporting the claim that the neurochemical is released on demand. Based on the response in Figure 3B, there is a clear peak separation between all the components. A decrease in *N*-acetylserotonin levels (peak 5) was observed between 3 and 24 month old tissue. Other changes in the level of the signaling molecules associated with the serotonergic pathway are also observed.

The level of *N*-acetylserotonin was monitored over the age groups, and the overall data is shown in Figure 3B. There is a significant age-related decrease in the amount of *N*-acetylserotonin observed in the mucosa. There was a significant reduction in the amount of *N*-acetylserotonin observed in 18 and 24 month old animals when compared to 3 month old animals ($p < 0.001$, $n = 6$). In a similar fashion, there was a significant reduction in the amount of *N*-acetylserotonin observed in 18 ($p < 0.01$, $n = 6$) and 24 month old animals ($p < 0.001$, $n = 6$) when compared to 12 month old animals. There was also a reduction in the concentration of *N*-acetylserotonin between 18 and 24 month old animals ($p < 0.01$, $n = 6$).

Following this, we investigated the *N*-acetylserotonin/serotonin ratio to understand if the levels or activity of NAT were altered with age (Figure 3D). There was a significant reduction in the *N*-acetylserotonin:serotonin ratio in 18 and 24 month old animals when compared to 3 month old animals ($p < 0.05$, $n = 6$, Dunn's)

Overall, the trend observed for the reduction of *N*-acetylserotonin levels is very similar to that observed for basal MEL levels and of the levels of MEL release evoked by the mechanical stimulus. Overall, this is highly suggestive that the age-related alteration in MEL biosynthesis is due to an impairment of NAT activity or expression with age and also a reduction in mechanical stimulation. However, these two factors may be linked.

Age-Related Alterations in Fecal Pellet Motility. The distal colon is responsible for fecal pellet motility and helps in regulating defecation. Fecal pellet motility was investigated in animals between 3 and 24 months old, ages where the most significant changes in MEL release were observed. Figure 4 shows the motility of an artificial fecal pellet through the isolated but intact colon. Representative traces for both 3 and 24 month old animals can be observed in Figure 4A. The traces show the movement of the pellet through the colon, for a maximum duration of 30 min. In both the 3 and 24 month old animals, the movement of the pellet occurred in a stepwise fashion. Interestingly, the typical trace for a 24 month colon shows that the pellet movement stalls after 10 min. In some cases, the pellet shows small oral and aboral movements in the colon denoted by the oscillating trace (Figure 5B). There was a significant decrease in the migration of the artificial pellet in 24 month old animals compared to 3 month old animals ($p < 0.001$, $n = 5$, 2-way ANOVA, Figure 4B). After 14 min, a significant difference in the migration of the pellet was observed between the two age groups ($p < 0.05$, $n = 6$, post hoc Tukey

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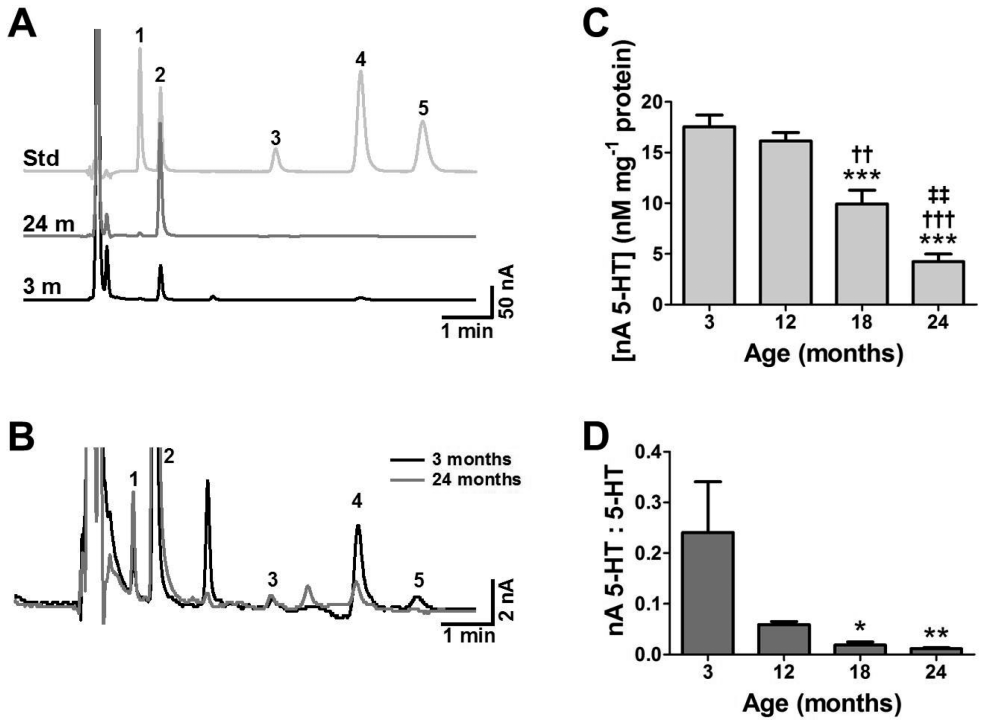

Figure 3. Levels of *N*-acetylserotonin monitored using high performance liquid chromatography. In (A), chromatographic responses of the standards (top trace) and a sample trace from the colonic mucosa of a 3 month old (bottom trace) and 24 month old animal (middle trace) are shown. In (B), the responses from 3 and 24 month old animals are shown at a higher resolution. The amount of *N*-acetylserotonin found within the mucosa tissue from 3, 12, 18, and 24 month old animals is shown in (C). The ratio of *N*-acetylserotonin:serotonin is shown for all age groups in (D). Data shown as mean ± SEM, $n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs 3 month old animals, $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$ vs 12 month old animals, and $^{\ddagger\ddagger}p < 0.01$ vs 18 month old animals. Solutes: (1) 5-hydroxytryptophan, (2) serotonin, (3) tryptophan, (4) 5-hydroxyl indole acetic acid, and (5) *N*-acetylserotonin.

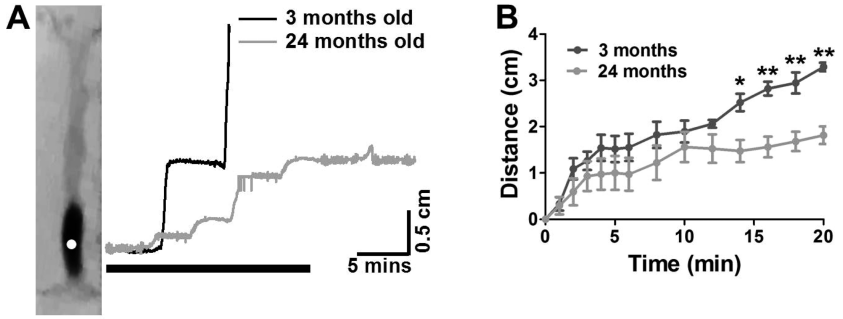

Figure 4. Alterations in fecal motility with age. Representative traces of fecal pellet motility are shown for 3 and 24 month old animals in (A). The white dot on the pellet indicates the point tracked during recordings, and the linear trace shown indicates the movement of the fecal pellet from the oral to the anal end of the colon. The black bar indicates the duration for which the population data was obtained between multiple animals. The overall data for 3 and 24 month old animals is shown in (B), where the movement of the pellet over 20 min at 2 min intervals is shown. Data shown as mean ± SEM, $n = 5$, $^{*}p < 0.05$ and $^{**}p < 0.001$ 3 month old vs 24 month old animals.

test). In three of the six 24 month old animals investigated, the fecal pellet failed to completely evacuate the colon within the 30 min utilized for the trial conditions. Alterations in signaling within the colon are most likely to contribute to this altered motility and may explain the age-related increase in colonic transit time. These changes in colonic motility also mimic those observed in constipation, which is well-known to be prevalent in the elderly population,[2,38] and those observed in aged guinea pigs.[39]

Role of Melatonin in Driving Fecal Pellet Motility. We investigated if decreases in MEL could explain the deficits in colonic motility observed in the 24 month old animals. Figure 5 shows the responses of a single trial in which an artificial fecal pellet is monitored as it moves through the colon in 3 and 24 month old animals (Figure 5A and B, respectively). MEL decreases the colonic transit time of the fecal pellet in both young and old animals and appears to alter the motility pattern from a stepwise movement to a pattern where the pellet is observed to move linearly through the colon. To investigate if the effects of MEL were receptor mediated, luzindole a nonselective $MT_{1/2}$ receptor antagonist was perfused across the colon. The traces clearly show that colonic transit time was increased in the presence of luzindole in both age groups. These data are suggestive that MEL receptors are functional in both 3 and 24 month old animals and that MEL acts as a prokinetic agent in the mouse colon.

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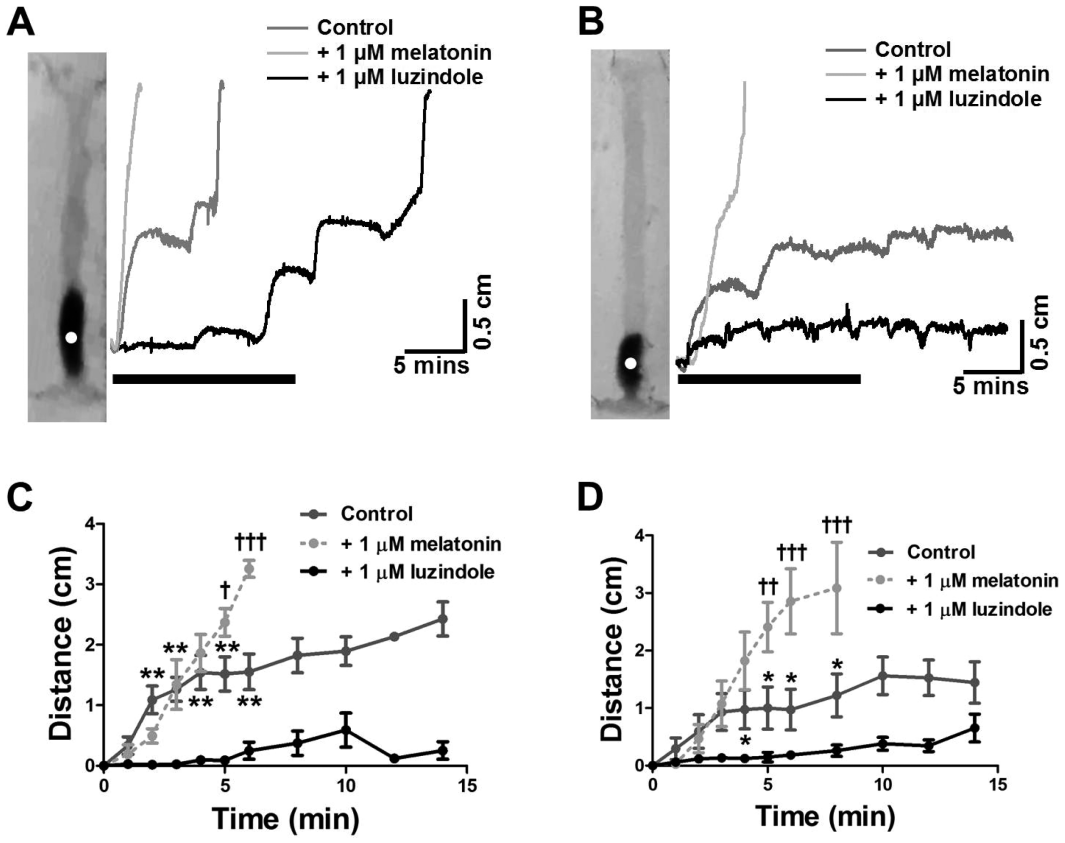

Figure 5. Influence of endogenous melatonin on fecal motility with age. Representative traces of fecal pellet motility are shown for 3 and 24 month old animals in (A) and (B), respectively. The white dot on the pellet indicates the point tracked during recordings, and the linear trace shown indicates the movement of the fecal pellet from the oral to the anal end of the colon. The black bar indicates the duration for which the population data was obtained between multiple animals. The overall data for 3 month old animals is shown in (C) and for 24 month old animals in (D), where the movement of the pellet over 14 min at 2 min intervals is shown. Data shown as mean ± SEM, $n = 5$, $^{*}p < 0.05$ and $^{**}p < 0.001$ control vs 1 μM luzindole, $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, and $^{\dagger\dagger\dagger}p < 0.001$ control vs 1 μM melatonin.

The overall population data is shown in Figure 5C and D. Two-way ANOVA, with time and drug treatment as the variables, showed a significant difference in the movement of the fecal pellet in 3 month old animals ($p < 0.001$, $n = 5$). Drug treatment also significantly altered pellet motility ($p < 0.001$, $n = 5$). Specifically, the pellet moved further in MEL after 6 ($p < 0.05$, $n = 5$) and 8 min ($p < 0.001$, $n = 5$) when compared to the control conditions in 3 month old animals. Luzindole significantly reduced the distance moved by the fecal pellet over 14 min in 3 month old animals when compared to both MEL and control conditions ($p < 0.01$, $n = 5$). Interestingly, MEL increases motility and thus acts as a pro-kinetic agent in the colon. Many studies have argued that MEL's role in the intestinal tract is to act as an antagonist of serotonin, and thus, it was anticipated that MEL should reduce motility.[22,40,41] This hypothesis is mainly due to the fact that serotonin is a well-established pro-kinetic modulator of colonic motility.[42,43] In our study, the concentration of MEL utilized showed pro-kinetic effects and importantly seems to change the stepwise movement into a more fluidlike transit throughout the bowel. Whether MEL has direct effects on the colon or acts to modulate the response of the tissue to serotonin is currently unclear. These findings are partially supported by dose-dependent functional bioassays, where the concentration of MEL utilized varied the muscle tone.[41,44]

Two-way ANOVA comparing time and drug treatment showed a significant change in fecal pellet motility with time under all conditions in 24 month old colons ($p < 0.001$, $n = 5$). Drug treatment also significantly influenced motility of the pellet ($p < 0.001$, $n = 5$). There was a significant increase in the distance moved by the fecal pellet in MEL after 5 ($p < 0.01$, $n = 5$), 6, and 8 min ($p < 0.001$, $n = 5$) compared to the control conditions in 24 month old colons. There is far greater variance in the response to MEL in 24 month old animals, which may reflect variance in the tissue morphology with age due to individual variability in the tissue susceptibility to the aging process. As MEL freely diffuses across cell membranes, altered tissue structure can influence the pathways for MEL to reach its receptor pathway. There was a significant increase in transit time of the fecal pellet under luzindole in 24 month old colons when compared to both MEL and control conditions ($p < 0.01$, $n = 5$).

Importantly, MEL reverses the age-related decline in colonic motility. MEL was able to reduce colonic transit times in both 3 and 24 month old colons, and luzindole, a MEL antagonist, increased transit times in colons from both age groups. The observation that the effects of a fixed concentration of luzindole were more marked on the 24 month colon when compared to 3 month colon was consistent with our observations that the release of MEL from aged colons is reduced. However, we cannot exclude the possibility that luzindole blocks the effects of MEL equally in both age groups and that the remaining differences in motility reflect the loss of another pro-kinetic pathway in the aged tissue. No other studies have been

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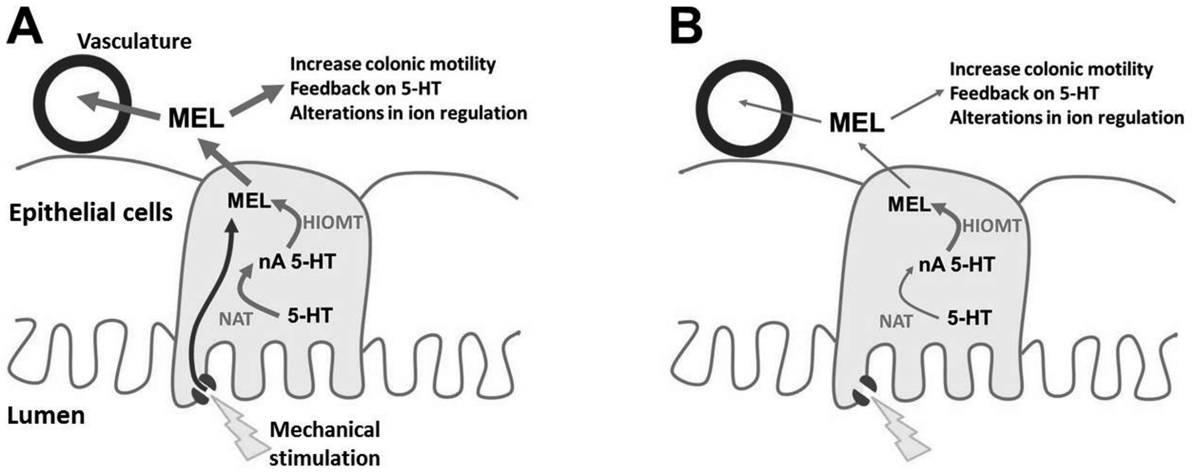

Figure 6. Alterations in melatonin signaling with aging. (A) Proposed signaling mechanism in 3 month animals and how this alters in 24 month old animals (B). Decreases in melatonin release are due to a reduction in the turnover of *N*-acetylserotonin, and there is a loss in mechanically evoked MEL release. Such changes may explain the age-related reductions in motility observed with age.

conducted to investigate the role of MEL on colonic motility with age. Another study investigated the role MEL supplementation would play on the biosynthesis of MEL and serotonin in aging mice. This study found that MEL reduced levels of serotonin and MEL, which would be expected to lead to an increase in transit time.[27] However, in studies on patients suffering irritable bowel syndrome with constipation, MEL treatment provided mixed results in reducing constipation.[45,46]

The data above suggest that MEL treatment may be useful for treating age-related constipation. Currently, only one trial has examined the effects of MEL on colonic transit time (CTT) in healthy humans.[46] In this study, CTT was increased in healthy patients, contradicting the data seen in our current study and contradicting the use of MEL in humans with constipation. It is interesting to note that orally administered MEL also would be predicted to increase CTT in mice through a reduction in luminal serotonin and MEL overflow.[27] Both these studies differ from the current study in which exogenous MEL was applied directly to the isolated but intact colon. This therefore suggests that following oral administration MEL may either have an alternative effect in other regions of the GI tract or target sites outside the GI tract that could lead to the increased CTT in both mice and humans. Therefore, for MEL to be useful in treating age-related constipation, a more detailed knowledge of how it targets the colon is required so that selective agents can be developed that can directly stimulate the colon.

In conclusion, our study has demonstrated that MEL/5-HT levels are reduced with age, which matches the reduction in MEL overflow observed. This loss in MEL overflow is due to a loss or alteration to either mechanosensory receptors and/or the levels/activity of the NAT enzyme. Both these factors may be linked in reducing the overflow of MEL, which in turn may decrease the amount of luminal MEL able to reach $MT_{1/2}$ receptors in the muscle. Such effects may explain the decrease in the colonic motility observed with age (Figure 6).

METHODS

Animals. All procedures were carried out according to U.K. Home Office regulations and were approved by the University of Brighton Ethics Committee. Male C57BL/6J mice were obtained from Harlan UK at 8 weeks of age and housed in individual ventilated cages under barrier-reared conditions until required. Animals were maintained at 19.0 ± 1 °C, 55% humidity and fed on a maintenance diet (RM1 (E) 801002 (Special Diet Services) chow).

Intestinal Preparation. Animals aged 3, 12, 18, and 24 months were stunned and exsanguinated following cervical decapitation. The whole colon was harvested 2 cm proximal to the anus and placed in ice cold oxygenated (95% O_2 and 5% CO_2) Krebs' buffer solution, pH 7.4 (117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose).

Electrochemical Monitoring of Basal Melatonin Release. Electrochemical measurements were made using a boron-doped diamond (BDD) microelectrode as previously described.[47] A 2 cm segment of the distal colon was opened along its mesenteric border and pinned in a Sylgard-lined Teflon (Dow Corning, UK) recording chamber and superfused with warm (37 °C) Krebs solution at a flow rate of 2 mL/min. Tissues were perfused for 30 min prior to commencing a series of measurements.

For the identification of MEL release from the distal colon, differential pulse voltammetry was carried out using CHI630B potentiostat (CH Instruments, Austin, TX). Recordings were carried out in a potential window between +0.2 and +1.0 V with a pulse amplitude of 50 mV and pulse duration of 200 ms. Measurements were made using a stainless steel auxiliary electrode and Ag|AgCl (3 M KCl) reference electrode.

For continuous amperometric recordings of MEL overflow, measurements were carried out using a BioStat multichannel potentiostat (ESA Biosciences, Inc.). The BDD electrode potential was held over the tissue at +650 mV vs Ag|AgCl which was sufficient to oxidize serotonin at a mass transfer limited rate. Using a micromanipulator, the BDD electrode was positioned several centimeters away from the mucosa for several seconds. For recordings, the electrode was positioned 0.1 mm over the tissue for 40 s, where reproducible oxidation currents were recorded. Following this, the potential was increased to +800 mV vs Ag|AgCl, at which both serotonin and MEL were oxidized and the whole protocol was repeated. This procedure was repeated five times for each tissue.

Detection of Mechanical Stimulated Melatonin. For the detection of mechanical stimulated MEL release, the same recording protocol as above was utilized, with recordings conducted at +650 and +800 mV vs Ag|AgCl. During amperometric recordings, the BDD microelectrode was positioned in the bulk media away from the tissue for several seconds. For tissue recordings, the electrode was held 0.1 mm over the tissue for 80 s, as shown in Figure 2A. At 40 s into the recording, the villi located within 100 μm of the BDD electrode were mechanically stimulated with a glass capillary for 20 s. The glass capillary was utilized to distort the villi, and the force applied to the tissue was kept constant by utilizing a fixed distance moved by the micromanipulator (precision of ∼5%). Therefore, as shown in Figure

dx.doi.org/10.1021/cn4000617 | ACS Chem. Neurosci. 2013, 4, 879−887

2A, the current was monitored prior, during, and after mechanical stimulation for a maximum of 20 s. The recording was repeated five times over a single tissue area.

HPLC Measurements. High performance liquid chromatography was utilized to monitor the levels of *N*-acetylserotonin. A 1 cm^2 segment of the distal colon was isolated, and using a sharp scalpel the mucosal layer of the tissue was scraped from the tissue of 3, 12, 18, and 24 month old animals. The mucosal tissue was placed in 500 μL of ice cold 0.1 M perchloric acid. Samples were homogenized and centrifuged at 20 000*g* for 5 min prior to chromatographic analysis.

The HPLC apparatus consisted of a Jasco HPLC pump (model PU-980) and Rheodyne manual injector equipped with a 20 μL loop. A Kinetic ODS 2.6 μm 150 mm × 4.6 mm i.d. analytical column with a guard column (Phenomenex, Macclesfield, U.K.) was employed. The HPLC system was run at a flow rate of 1.0 mL min^{-1}. A CHI630B potentiostat (CH Instruments, Austin, TX) was used to control the detector voltage and record the current. A 3 mm glassy carbon electrode (flow cell, BAS) served as the working electrode and was used with a Ag|AgCl reference electrode and a stainless steel block as the auxiliary electrode. Amperometric recordings were carried out, where the working electrode was set at a potential of +850 mV vs Ag|AgCl reference electrode. Control and data collection/processing were handled through the CHI1001A software.

The stock buffer for the mobile phase comprised the following: 0.1 M sodium acetate, 0.1 M citric acid, and 27 μM disodium ethylenediaminetetraacetate (EDTA). This was then buffered to pH 3.0. The mobile phase was prepared with the stock buffer mixed with methanol in the ratio of 8:2 (v/v) and degassed after mixing.

Standard solutions were prepared from 1 mM stock standards of each analyte and were made up in 0.1 M perchloric acid (BDH). Each of the standard solutions was prepared on the day of analysis and stored at 4 °C prior to injection. A calibration plot was obtained by running *N*-acteylserotonin at a concentration range of 0.02−10 μM. The peak areas obtained from chromatographic analysis of all injected samples were converted to concentrations utilizing the calibration responses of the neurochemicals. The concentration of *N*-acetylserotonin was normalized by the protein content using the Bradford method.

Fecal Pellet Motility Assays. The whole colon was harvested from 3, 12, 18, and 24 month old animals and loosely pinned in a Sylgard-lined flow bath. The isolated colon was constantly perfused in oxygenated Krebs' buffer solution at 37 ± 1 °C at a flow rate of 8 mL min^{-1}.

The spontaneous evacuation of the natural fecal matter was allowed for up to 30 min. However, if this was not achieved during this time frame, the fecal pellets were gently removed from the isolated colon by perfusing the lumen of the colon with warmed Krebs' ringer. The colon was then left to stabilize for 15 min, prior to recordings. Measurements were carried out using an epoxy-coated artificial fecal pellet. For each age group, a fecal pellet that was the average size/shape for each age group was utilized. The artificial fecal pellet was inserted into the proximal end of the bowel using a glass capillary. The pellet was monitored using a video camera and tracked using Ethovision tracking software. Following successful completion of a trial, the experiment was repeated two further times and the average response was utilized. Measurements were conducted on all age groups, and the maximum time that a trial was conducted was 30 min. This experiment was repeated in the presence of 1 μM MEL and the $MT_{1/2}$ receptor antagonist 1 μM luzindole.

Data Analysis. Measurements conducted using amperometric detection were carried out using two different potentials, at +650 and +800 mV. The potential set at +650 mV was utilized for the sole detection of serotonin as published elsewhere; however, both serotonin and MEL were detected at +800 mV. The difference in the current obtained at these two potentials provides the response of MEL alone. The current responses at +650 and +800 mV and the differences are shown from a 3 month animal in Figure 1B.

For electrochemical data, the population means were compared using one-way ANOVA (GraphPad Prism) and significant differences were determined using posthoc Tukey tests. HPLC peaks for the analytes of interest were calculated using CHI potentiostat 1050 software and were analyzed for normality. The concentration of *N*-acetylserotonin was normalized by the total amount of protein. Age-related differences were determined using one-way ANOVA (GraphPad Prism). Fecal pellet motility was measured using the Ethovision tracking software (Ethovision XT vs7), where the distance moved every 2 min over 14 min was recording for each trial. The population means were analyzed using two-way ANOVA (GraphPad Prism) and significant differences were determined using posthoc Tukey tests. All data were presented as mean ± SEM, and *n* represents the number of animals used for each experiment

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Author Contributions

M.S.Y. and B.A.P conceived and directed the study. All authors conducted experiments included in the manuscript. B.A.P. drafted the manuscript. S.F., M.S.Y., and B.A.P edited the manuscript. All authors read and agreed on the final draft of the manuscript.

Funding

S.F., B.A.P., and M.S.Y. are supported by BBSRC "Aging bladder and bowel" Grant BB/G015147/1. B.A.P. was supported by University of Brighton sabbatical grant.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We would like to thank Prof. Greg Swain for kindly donating the boron-doped diamond microelectrodes utilized within this study. We are grateful to the staff of the University of Brighton Bioresource Centre for assistance with animal handling.

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