Effect of Melatonin and 5-Methoxycarbonylamino-N-Acetyltryptamine on the Intraocular Pressure of Normal and Glaucomatous Mice

Alejandro Martínez-Águila, Begoña Fonseca, María J. Pérez de Lara, and Jesús Pintor

Departmento de Bioquímica y Biología Molecular IV, Facultad de Óptica y Optometría, Universidad Complutense de Madrid, Madrid, Spain

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ABSTRACT

Melatonin is a neurohormone that is produced not only by the pineal gland but also by several ocular structures. One of the main physiologic roles of melatonin is the reduction of intraocular pressure (IOP). Using both control C57BL/6J and glaucomatous DBA/2J mice as well as TonoLab tonometry, this study evaluated the effect of melatonin and 5-methoxycarbonylamino-N-acetyltryptamine (5-MCA-NAT) when glaucomatous pathology was fully established and compared pharmacological behavior in treated mice versus control mice. In addition, 5-MCA-NAT was tested to determine its effects on ameliorating increased IOP in a glaucoma model. The results demonstrate that melatonin and 5-MCA-NAT can reduce IOP in a concentration-dependent manner. The EC$_{50}$ values for melatonin in control and glaucomatous animals were 34 µM and 50 µM, respectively. Interestingly, melatonin decreased IOP in 19.4% ± 3.7% and 32.6% ± 6.0% of control and glaucomatous mice, respectively, when the animals were studied at age 12 months. 5-MCA-NAT reduced IOP in the same manner and was able to stop IOP progression in glaucomatous mice. Use of melatonin receptor antagonists showed that hypotensive effects were blocked by the MT$_2$ receptor antagonists luzindole and 4-phenyl-2-propionamidotetralin in the case of melatonin and by only 4-phenyl-2-propionamidotetralin in the case of 5-MCA-NAT. In conclusion, melatonin and 5-MCA-NAT can effectively reduce IOP in a glaucoma model, and their hypotensive effects are more profound in the glaucoma model than in control animals.

Introduction

Glaucoma is an optic neuropathy in which progressive death of the retinal ganglion cells and loss of their axons in the optic nerve leads to blindness (Quigley, 2011). Elevated intraocular pressure (IOP) is one of the main risk factors for the development of glaucoma. If the pressure is high or remains slightly higher for a long period of time, this can lead damage in the ciliary arteries and mechanical damage to the optic nerve head, which can also contribute to blindness or impairment of the visual field (Brusini and Johnson, 2007).

In the search of new compounds for the treatment of glaucoma, melatonin has been tested as a natural compound with ocular hypotensive effects and interesting neuroprotective actions (Rowland et al., 1981). This molecule and some of its analogs, such as 5-methoxycarbonylamino-N-acetyltryptamine (5-MCA-NAT), produce a marked reduction in IOP in normotensive and hypertensive conditions (Serle et al., 2004; Crooke et al., 2013; Martínez-Águila et al., 2013; Pescosolido et al., 2015).

Melatonin and its analogs exert their actions by three types of receptors: namely, MT$_1$, MT$_2$, and the putative MT$_3$ melatonin receptors (Dubocovich et al., 1998). The first two are negatively coupled to adenylate cyclase, whereas MT$_3$ seems to be positively linked to the same signal transduction mechanism (Huete-Toral et al., 2015). There is controversy regarding these receptors, since some authors claim that MT$_3$ receptors are quinone reductase 2 (Nosjean et al., 2000), but this identity has not been demonstrated in ocular tissues. Indeed, the silencing of quinone reductase 2 by small interfering RNA did not modify the activity of the MT$_3$ agonist 5-MCA-NAT or of the antagonist prazosin (Alarma-Estrany et al., 2009).

Although the effect of melatonin agents on the modulation of IOP has been described in the scientific literature, there are currently no conclusive results regarding their effects in a glaucoma model. Approaches with some melatonin analogs, such as agomelatine, using a hypertensive condition (Trendelenburg position) clearly indicated the effects of these indoles on the reduction of IOP when the pressure was high (Martínez-Águila et al., 2013), but more precise experiments in a real glaucoma condition are required.

In the search for new glaucoma treatments, several animal models have been developed, most of which are based on elevating IOP in animals. Generating an artificial increase in
IOP with biochemical agents (e.g., chymotrypsin) and surgical procedures (e.g., episcleral vein cauterization or laser trabeculoplasty) are efficient protocols for elevating IOP, but these approaches damage several tissues that might be targets for reducing ocular hypertension and glaucoma. Therefore, in addition to the need for elevation of IOP, a glaucoma animal model must be minimally invasive to guarantee that most of the ocular structures will be intact and suitable for drug treatment (Crooke et al., 2012a).

One interesting animal model with increased eye pressure is the DBA/2J mouse strain, which spontaneously develops pseudoxfoliative glaucoma (Anderson et al., 2002). Progression of the disease in DBA/2J mice occurs as a result of changes in the anterior chamber. These mice develop glaucoma as a result of iris pigment dispersion and atrophy of the iris stroma caused by mutations in Glycoprotein non-metastatic b and Tyrosinase-related protein 1 (John et al., 1998). Pigment accumulation in the trabecular meshwork causes clogging and reduced flow of aqueous humor, causing a moderate increase in IOP between 2 and 6 months and a strong increase between 8 and 12 months (Saleh et al., 2007; Harazny et al., 2009). The increase in IOP produces changes in the retina and optic nerve (Libby et al., 2005; Pérez de Lara et al., 2014).

Most of the experiments performed to date with melatonin and 5-MCA-NAT have been carried out in either normotensive animals (Pintor et al., 2001) or in hypertensive/glaucoma conditions (Serle et al., 2004; Martínez-Águila et al., 2013), but not in an animal model that develops the disease spontaneously. In addition, 5-MCA-NAT has not yet been tested as a possible preventive treatment for glaucoma. Therefore, this study demonstrates that melatonin and 5-MCA-NAT actively reduce IOP in a real model of glaucoma, and the latter can reduce the increased IOP observed in nontreated animals.

Materials and Methods

Mice. Experiments were performed on adult female C57BL/6J mice (n = 15; control animals) and DBA/2J mice (n = 15; glaucomatous animals) obtained from Charles River Laboratories (Saint-Germain-NUelles, France). All animal maintenance and experimental procedures followed Spanish and European guidelines for animal care in the laboratory and animal research (Guide for the Care and Use of Laboratory Animals) and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were housed (n = 1–4 mice per cage) in a 12-hour light/dark cycle; all animals were fed ad libitum. DBA/2J and C57BL/6J mice were studied at age 3, 6, 9, and 12 months.

IOP Measurements. Melatonin (Sigma, St. Louis, MO) and 5-MCA-NAT (Tocris, Bristol, UK) were formulated in isotonic saline containing 1% dimethylsulfoxide and tested at different concentrations from 10−8 M to 10−6 M. These substances were applied in drops to the corneas at a fixed volume of 2 μl in both eyes. Control animals received the same volume of saline plus 1% dimethylsulfoxide. IOP was measured with a TonoLab noninvasive rebound tonometer supplied by Tiotat Oy (Icare, Finland). To avoid the putative effect of the circadian rhythm, the IOP was always tested at the same time of day. Six consecutive measurements were taken for each animal, and these readings were obtained on each eye.

To study the effect of melatonin and 5-MCA-NAT, two IOP measurements were taken before melatonin and 5-MCA-NAT were instilled and once every hour for 6 hours. Luzindole (a nonselective melatonin antagonist), 4-phenyl-2-propionamidotetralin (4-PPDOT) (an MT2 melatonin antagonist), and prazosin (an MT3 melatonin antagonist) were used as antagonists of melatonin receptors (all were tested at 100 μM).

To study the effect of the different antagonists tested, 2 μl was instilled 30 minutes before the agonist, at a concentration of 100 μM, and IOP was measured in the same fashion as previously described.

Control IOP measurements were performed in DBA/2J and C57BL/6J mice at age 3, 6, 9, and 12 months to determine the variation of IOP. The mentioned compounds were tested when animals were aged 10–12 months, with a minimum of 3 days between experiments.

Another group of 18 animals was selected to test the preventive long-time effect of 5-MCA-NAT. In this experiment, basal IOP was measured from age 5 to 6 months and then animals were randomized into two groups: control or 5-MCA-NAT-treated animals. To test the hypotensive effect of 5-MCA-NAT, the compound was applied three times a week from 6 to 9 months. The IOP in treated animals was measured once a week and values were compared with those of the nontreated control group.

Anesthesia. Mice were anesthetized by inhalation of isoflurane using a Matrix VIP 3000 Calibrated Vaporizer (Midmark, Versailles, OH). This instrument supplies oxygen from an attached tank at 50 × 55 p.s.i. Oxygen is mixed with isoflurane and sent to two outflows at 500 ml per minute, delivering 2.5% of isoflurane in oxygen to the animal. One outflow entered a box where mice were placed for initial sedation. After approximately 2 minutes, the sedated animal was positioned for IOP measurement and clinical examination and a nose cone delivered the isoflurane gas/oxygen mixture. The nose cone permitted access to the eyes.

Statistical Analysis. All data are presented as means ± S.E.M. Statistical differences between treatments were calculated using analysis of variance (ANOVA) and the t test. Plotting and fitting were carried out with GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA).

Results

IOP Changes Related to Age and Development of Glaucomatous Pathology. To see the expected differences in IOP between control C57BL/6J mice and glaucomatous DBA/2J mice, IOP was measured from age 3 to 12 months every 3 months.

IOP was stable along the time under study in the control C57BL/6J mice, while in the DBA/2J mice there was an increase starting at 9 months of age. At this time, IOP was significantly higher (25 % of increase) compared to 3-months-old DBA/2J mice. (P < 0.001, two-way ANOVA with Tukey post-test, n = 14). This increase reached 153% over basal IOP at 12 months old (P < 0.001, two-way ANOVA with Tukey post-test, n = 14), as shown in Fig. 1.

Effect of Melatonin and 5-MCA-NAT on Normal and Glaucomatous Animals. To examine the effect of both melatonin and its 5-MCA-NAT analog in both control and glaucomatous mice, time course studies were carried out by applying single 100-μM (2 μl) doses of each melatoninergic agent. Changes in IOP were followed for 6 hours. Drug treatments were applied in DBA/2J and C57BL/6J mice when the animals were aged 10–12 months.

Melatonin decreased IOP in 19.4% ± 3.7% and 32.6% ± 6.0% of C57BL/6J and DBA/2J mice, respectively, with a maximum effect at 4 hours in both animals when the animals were studied at age 12 months (Fig. 2B).

Interestingly, application of 5-MCA-NAT produced a reduction in IOP of 20.7% ± 2.8% and 29.3% ± 4.3% in C57BL/6J and DBA/2J mice, respectively (Fig. 2A). The maximal reduction in IOP was obtained 3 hours after application of the agent. Both strains of animals were challenged at age 12 months.
Concentration-Response Curves for Melatonin and 5-MCA-NAT. Melatonin and 5-MCA-NAT were tested in a broad range of concentrations starting from $10^{-8}$ M to $10^{-3}$ M to study their effect on IOP. The melatonin concentration-response curve depicted a sigmoidal behavior presenting pD2 values of $10^{-4.3}$ M and $10^{-4.5}$ M for C57BL/6J and DBA/2J, respectively, which were equivalent to EC50 values of 34 $\mu$M and 50 $\mu$M (Fig. 3B) (two-way ANOVA test with Tukey post-tests, $n = 4$). When 5-MCA-NAT was assayed, this compound also presented a sigmoidal curve showing pD2 values of $10^{-5}$ M and $10^{-5.2}$ M for C57BL/6J and DBA/2J, respectively, which were equivalent to EC50 values of 6.8 $\mu$M and 10.3 $\mu$M (Fig. 3A, two-way ANOVA test with Tukey post-tests, $n = 4$).

Antagonist Studies. IOP was previously demonstrated to be controlled by melatonin receptors in a normotensive model (Alarma-Estrany et al., 2011). To investigate whether there is a difference in the antagonism of the receptors in both types of animals, different antagonists were tested at a concentration of 100 $\mu$M as indicated in the Materials and Methods.

For melatonin, luzindole and 4-PPDOT were able to reverse the hypotensive effect in DBA/2J mice, whereas the MT3 antagonist parazosin was unable to do so. In C57BL/6J mice, all antagonists returned IOP to control values (Fig. 4).

For 5-MCA-NAT, the only antagonist that was able to reverse the effect of this compound in the DBA/2J mice was 4-PPDOT, an MT2 melatonin receptor antagonist (Fig. 4). In C57BL/6J mice, all antagonists returned IOP to control values.

Long-Time Effect of 5-MCA-NAT. 5-MCA-NAT is able to produce a reduction in IOP that is measurable over a few hours (Fig. 2B). To fully understand the effect of 5-MCA-NAT during the development of the glaucomatous process, this compound was applied three times a week for 3 consecutive months (from age 6 to 9 months in both control and glaucomatous animals). As shown in Fig. 5, there was a clear trend of a reduction in the IOP increase for animals treated with 100 $\mu$M 5-MCA-NAT compared with glaucomatous animals treated with saline. Although the trend was visible from the second month of treatment (eighth week), it was only possible to see statistically significant differences in the last 2 weeks of measurements (Fig. 5, $P < 0.01$). At this point, the differences in IOP were approximately 13% of reduction in the IOP compared with the nontreated animals.

Discussion

This study describes the effects of the neurohormone melatonin and its analog 5-MCA-NAT in their ability to reduce IOP in both glaucomatous and nonglaucomatous murine models. These results indicate that both compounds can significantly reduce IOP in a glaucomatous (DBA/2J) model when the animals are challenged with this compound at the moment when IOP is higher. A similar effect can be observed in the control animals (C57BL/6J mice), although there was not an increase in IOP with aging in this model as occurs in the glaucomatous model. It is important to note that the hypotensive melatonin effect was more profound in the glaucomatous model than in the control, and this effect was also present when 5-MCA-NAT was used instead (Figs. 2 and 3). This was especially evident when comparing the corresponding concentration-response curves. The reductions in glaucomatous mice were more marked than those obtained in control mice.

There is an interesting point that requires some consideration. When concentration-response curves for melatonin or 5-MCA-NAT were studied in DBA/2J glaucomatous animals, it was not possible to reach the IOP values these animals had before the pathology started, not even the values of the normotensive model (C57BL/6J mice) present at age 12 months. This indicates that although the maximal doses...
applied reduce IOP, they are not efficient enough to return pressure to the prepathology values. This finding suggests that although melatoninergic mechanisms to reduce IOP exist (Pintor et al., 2001; Ismail and Mowafi, 2009), as happens in some other models, the system cannot return to those pressure conditions that occurred before the pathology started. It is not clear why IOP does not reach lower values, but indeed, it has been reported that glaucomatous conditions, apart from causing retinal damage, can affect those structures (ciliary body and trabecular meshwork) that produce and drain

**Fig. 3.** Concentration-response curves for melatonin and 5-MCA-NAT. (A) Melatonin concentration-response curves in the glaucomatous model (DBA/2J, upper traces) and control (C57BL/6J, lower traces). (B) 5-MCA-NAT concentration-response curves in the glaucomatous model (DBA/2J, upper traces) and control (C57BL/6J, lower traces). \( *P < 0.05; **P < 0.01 \) versus control (two-way ANOVA with Tukey post-test, \( n = 4 \)).

**Fig. 4.** Effect of melatonin receptor antagonists on melatonin and 5-MCA-NAT in both C57BL/6J and DBA/2J mice models. The effect of melatonin was antagonized by luzindole and prazosin in DBA/2J mice and by all compounds in C57BL/6J mice (upper panels). The effect of 5-MCA-NAT was blocked by 4-PPDOT, an MT2 receptor antagonist in DBA/2J mice, and by all of the compounds in C57BL/6J mice (lower panels). \( *P < 0.05; **P < 0.01; ***P < 0.001 \) versus control (two-way ANOVA with Tukey post-test, \( n = 4 \)).
aqueous humor. Environmental factors such as hypoxia or an acute increase in induced hydrostatic pressure lead to inflammation processes and alterations in apoptotic signaling components, triggering cell death in anterior chamber structures (Zhou et al., 2005). This could be why melatonin and analogs are unable to fully return IOP to prepathology values in such advanced stages of glaucomatous disease. As indicated, all of the pharmacological experiments were performed at the peak of pressure, although changes in IOP were already detected at age 9 months (Pérez de Lara et al., 2014).

An interesting experiment was performed by applying the melatonin analog in a long-term fashion. In this sense, experiments were performed by applying 5-MCA-NAT three times a week for 3 months. These experiments demonstrated a clear tendency of the melatonin analog to counteract the increase in IOP observed in nontreated animals. Although an apparent effect starting from the eighth week of treatment seems to exist, this was not statistically significant until the last two measurements. It is likely that by applying the melatonin analog daily and/or by using a bigger number of animals, the errors would be smaller and thus the differences would be statistically significantly closer to the moment 5-MCA-NAT is applied. If this occurs, the pressure would be better controlled and the evolution of the pathology would be stopped, as happens for other pharmacological treatments (Bhowmik et al., 2012).

The importance of melatonin and its receptors in the regulation of IOP and presumably participation in glaucoma pathophysiology was previously described (Alcantara-Contreras et al., 2011; Tosini et al., 2013). In this context, our results contribute to the former idea, showing that melatonin and 5-MCA-NAT applied to a glaucoma model that develops the pathology without surgical, physical, or chemical modifications can reduce IOP in the hypertensive model.

Prior studies reported on the action of these two compounds in normotensive animals, such as rabbits (Pintor et al., 2001, 2003), or in hypertensive/glaucoma conditions (Serle et al., 2004; Martinez-Aguila et al., 2013). The reduction in IOP in models of hypertension (e.g., monkeys) presented a reduction pattern that was different from that observed in our exfoliative model. The monkey model needs up to 3 days to present reductions in IOP that can be statistically significant (Serle et al., 2004). This delay, compared with the effect we report in this study, may be attributable to a long-term effect of melatonin and its analog on IOP, apart from differences in the species and in the hypertension model itself. Long-term effects of melatonin and 5-MCA-NAT have been described in the eye. These effects are mainly due to the modulation of carbonic anhydrase and regulation of adrenergic receptor gene expression (Crooke et al., 2011, 2012b, 2013). Altogether, we have demonstrated not only the acute and long-term effect of 5-MCA-NAT on IOP but also that 3-month treatment in a glaucoma situation helps ameliorate the elevation of IOP in our mouse model.

It is important to compare our results with previous works described in the literature regarding the dosage and observed effects. For instance, the reduction in IOP detected in cataract patients treated with melatonin showed that oral doses of 10 mg can reduce IOP by 23% (Ismail and Mowafi, 2009). These authors only used melatonin once; moreover, their patients were normotensive and yet IOP was still significantly reduced. In our study, melatonin and 5-MCA-NAT were topically applied and we performed both acute and long-term experiments. In absolute values, the reduction in IOP when we applied melatonin on the ocular surface was 13% more effective than the human experiments (a 23% reduction in normotensive humans versus 32% in glaucomatous mice). It is necessary to be aware that both mechanisms of administration will provide different local concentrations of melatonin, although the amount that we topically applied was approximately 50 ng. Agomelatine treatment in glaucomatous humans is apparently closer to our idea of using melatonin to reduce IOP in glaucomatous mice. Nevertheless, it is important to note that agomelatine not only stimulates melatonin receptors but is also a 5-HT2c serotonin receptor antagonist (You et al., 1992). Pescosolido et al. (2015) provided a daily dose of 25 mg; the reduction in IOP was observed 15 days after starting the treatment and was stable for the other 15 days, with an average reduction of IOP of 30%. In our case, chronic treatment with 5-MCA-NAT reduced IOP only 10% compared with nontreated glaucomatous animals, which is not as strong as that obtained with agomelatine for our study period. The difference, apart from the mechanism of administration, may be that we applied 5-MCA-NAT three times a week and not daily. It could be the case that if we apply the melatonergic agent more often, the results of chronic treatment would appear earlier and the IOP reduction may be stronger. Nevertheless, the closest model using 5-MCA-NAT was performed by Serle et al. (2004). In this model, the compound was applied topically in a volume of 25 μl at 2% (roughly 0.5 mg, equivalent to 73 mM) twice a day for 5 days. In this case, the maximal reduction in IOP was 20%. Comparatively speaking, our results were obtained with a significantly lower concentration of melatonergic agents than the ones applied in glaucomatous monkeys. It seems clear that it is necessary to study both the most effective dose and also the posology to obtain the best results.

According to the pharmacological profile of the melatonin antagonists some comments can be indicated, but it is necessary to be aware that the antagonists have been tested at a single dose. Therefore, some changes might be found if a full characterization with the antagonists were performed. Our experience in other species (e.g., rabbits) demonstrated that we did not observe changes in the receptors involved in melatonin and 5-MCA-NAT actions when using single doses of antagonists or when they were characterized using pA2 values.

![Figure 5](image-url)
(Pintor et al., 2003). Nonetheless, a full characterization is necessary to evaluate these effects in more detail. Concerning the profile observed using single concentrations of melatonin receptor blockers, it seems that the effects of the tested antagonists lumelodine (nonselective), 4-PPDOT (MT2), and prazosin (MT2) show that the blockade of melatonin and 5-MCA-NAT actions are not identical in both mice strains. According to our results, it was possible to observe some differences only in glaucomatous DBA/2J mice. The effect of melatonin was reversed by any of the tested antagonists in C57BL/6J mice and only prazosin was unable to fully reverse the effect of melatonin in DBA/2J mice. This suggests that in this model, the presence of a putative MT3 receptor is unlikely. On the other hand, it was possible to see more differences in the effect of 5-MCA-NAT in the two models studied. Although all of the tested melatonin receptor antagonists reversed the effect of 5-MCA-NAT in C57BL/6J mice, lumelodine slightly and MT3 antagonist 4-PPDOT in particular achieved this in DBA/2J mice. 5-MCA-NAT is reported to be an agonist of the putative MT3 melatonin receptor (Molinari et al., 1996; Pintor et al., 2001; Serle et al., 2004). Our results indicate that this substance acts on MT2 receptors. This is not the first time this action has been reported to occur via MT2 melatonin receptors. Indeed, it has been suggested that this compound can act via MT2 receptors in some models, thus becoming not so selective (Vincent et al., 2010). In addition, and in relation to IOP changes, it has been observed that 5-MCA-NAT may produce its hypotensive action by activating an MT2 receptor apart from the previously suggested MT2 melatonin receptor (Alarma-Estrany et al., 2011).

One interesting aspect to take into account concerns the apparent high concentrations used to treat the animals for both agonists and antagonists. Limiting steps for ocular drug delivery including the ocular physiology regarding tear drainage, together with the ability of the molecules to pass through the cornea (depending on their chemical properties). It has been demonstrated that to obtain pharmacologically active concentrations of melatonin agonists and antagonists in the aqueous humor, it is necessary to topically apply them at micromolar concentrations (Alarma-Estrany et al., 2009).

Therefore, the concentrations used are enough to stimulate melatonin receptors within the eye. This aspect is also very relevant when dealing with antagonists, especially prazosin, which has been claimed to be a MT3 receptor antagonist as well as an α1 adrenergic receptor antagonist (Stokes and Weber, 1974). Since the mentioned adrenergic receptor may contribute to the regulation of IOP as previously described (Pintor, 2009), it is possible that the application of prazosin may reflect an action on α1 adrenergic receptors rather than MT3 melatonin receptors. To clarify this point, we recently described that when prazosin is topically applied at micromolar concentrations, the intraocular concentration of this antagonist acts mainly on the MT3 melatonin receptor rather than on the α1 adrenergic receptor, thus discarding the possible interaction with the adrenergic receptor (Huette-Toral et al., 2015).

In summary, the administration of melatonin or 5-MCA-NAT to an exfoliative model of glaucoma has an acute hypotensive effect. Moreover, this study provides evidence showing that the application of 5-MCA-NAT regularly along the development of glaucoma reduces the progressive elevation of IOP observed in this glaucoma model. All of these positive effects will protect the retina from further development of the pathology.

Authorship Contributions

Participated in research design: Pintor.
Conducted experiments: Martinez-Aguila, Fonseca.
Performed data analysis: Martinez-Aguila.
Wrote or contributed to the writing of the manuscript: Martinez-Aguila, Fonseca, Perez de Lara, Pintor.

References


Address correspondence to: Dr. Jesús Pintor, Departamento de Bioquímica y Biología Molecular IV, Facultad de Óptica y Optometría, Universidad Complutense de Madrid, C/ Arcos de Jalón, 118, E-28037 Madrid, Spain.
E-mail: jpintor@vet.ucm.es