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Retinal Cell Biology

Retinal control of scleral precursor synthesis

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ABSTRACT

There is a light-dependent diurnal rhythm in scleral precursor synthesis (SPS). In form-deprivation myopia (FDM) there is an increase in SPS. Daily periods of normal vision prevent FDM, but light intensities that maintain the retinal dark–light switch in its dark state do not, implicating melatonin, dopamine, enkephalin, neurotensin and somatostatin in the control of SPS. FDM is also prevented by pirenzepine, a cholinergic muscarinic antagonist. To test if these compounds control the rate of SPS, agonists and antagonists were applied directly to the sclera, but the only effects detected were at very high concentrations, making them physiologically insignificant.

Key words: acetylcholine, dark–light switch, dopamine, enkephalin, form-deprivation myopia, melatonin, retina, sclera.

Form-deprivation myopia (FDM) in chickens results in an increase in axial elongation[1] and an accompanying increase in scleral precursor synthesis (SPS) as measured by rates of sulfate incorporation into proteoglycans.[2] Normally axial elongation varies diurnally with growth during the day but not at night. However, during FDM there is also growth at night, resulting in a larger eye overall.[3] From these findings we expected that scleral precursor (such as proteoglycan) synthesis would also have a diurnal rhythm, and we have shown that there is such a rhythm in SPS, but the highest activity is seen at night and the lowest seen during the day.[4] This rhythm is controlled by the external lighting conditions not by a circadian rhythm.

An important aspect of this light control is that there is a delay of around 4 h between the lights going off and the subsequent rise in SPS rates. Similarly there is a gradual decrease in SPS over 6 h after the lights come on. So what is controlling this rhythm? Retinal neurotransmitters such as dopamine[5], enkephalins[6] and acetylcholine[7] have all been implicated in FDM and hence scleral growth. In addition to this, dopamine and enkephalins are part of the retinal dark–light switch.[8] This switch flips from its dark to light state over a narrow range of light intensities. This is the same range that appears to control SPS during both normal eye growth and during FDM.[9]

If this switch is involved then it would be expected that either dopamine acts as an inhibitor (as it is released during the day when SPS decreases) or melatonin and enkephalins (or neurotensin or somatostatin) are stimulants (as they are released at night when SPS reaches high levels). But how do they effect the sclera? There are two possibilities. Either these neurotransmitters have receptors on the sclera and act on it directly, or they are part of a flow of messages to the pigment epithelium, the choroid and ultimately the sclera. We have therefore tested whether any of these neurotransmitters have direct effects on rates of sulfate incorporation in scleral tissue *in vitro*.

METHODS

Chickens were kept on a 12 : 12 h light–dark cycle with the lights on at 6 am and off at 6 pm. At midday, the chickens were anaesthetized with isoflurane and decapitated, and the eyes were removed and hemisected. The posterior parts of the eyecups were cleaned of retina, pigment epithelium, vitreous and other tissue, and scleral buttons of a fixed size were trephined.

These scleral buttons were pre-incubated for 30 min in a buffer (120 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 25 mmol/L D-glucose, 5 mmol/L KCl, 3 mmol/L $MgCl_2$ and 1.8 mmol/L $CaCl_2.2H_2O$) containing the agonist/antagonist being tested. The tissue was then incubated for 1 h with $^{35}SO_4$ added to the medium. Both incubations were carried out at 37°C in the light. As the retina had been removed, the light-insensitive sclera should continue growing according to

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Table 1. Amount of sulfate incorporation into sclera after incubation with various compounds (% of control)

	Concentration				
	1 μmol/L	10 μmol/L	100 μmol/L	1 mmol/L	K_d
Leu-enkephalin	97.8	101.9	101.3	—	Low nmol/L
Met-enkephalin	94.2	91.8	113.8	—	Low nmol/L
Melatonin	109.9	61.4 **	65 **	—	Low pmol/L
ADTN (DA agonist)	—	152 **	148 **	48.5 ***	Low nmol/L
Clozapine (DA antagonist)	—	99	67.5 **	32.5 ***	Low nmol/L
Carbachol (non-specific ACh agonist)	—	105.3	95.8	78.6 ***	Low nmol/L
Pilocarpine (M1 muscarinic ACh agonist)	—	134 *	144.2 **	143.2 ***	Low nmol/L
Atropine (muscarinic ACh antagonist)	96.7	71.3 **	19.6 ***	2.45 ***	Low nmol/L
Pirenzepine (M1 muscarinic ACh antagonist)	—	99.8	90.4	97.7	Low nmol/L

Degrees of significant difference compared to control (n=8, P<0.05): *significant, **very significant, ***extremely significant.

whether it had come from light-adapted or dark-adapted birds. After incubation the glycosaminoglycans (GAG) were cleaved from the proteoglycan backbone by extraction in 0.5 mmol/L NaOH overnight. All the label in the NaOH extract was ethanol precipitable, and was measured to provide an index of incorporation of label into GAG.

Results

The effects of the agonists and antagonists on rates of sulfate incorporation can be seen in Table 1. The enkephalins had no effect. Melatonin had an inhibitory effect, although only at 10 μmol/L or greater. The dopamine agonist ADTN stimulated rates of sulfate incorporation at 10–100 μmol/L, but inhibited at 1 mmol/L. Interestingly, clozapine, which is a dopaminergic antagonist, also inhibited but this effect was obvious at slightly lower concentrations (100 μmol/L) and was much more striking. The non-specific cholinergic agonist carbachol had no effect until 1 mmol/L where it inhibited. However, the M1 muscarinic receptor agonist (pilocarpine) had a stimulatory effect. The non-specific muscarinic antagonist (atropine) inhibited quite substantially at 10 mmol/L or greater, but the M1 antagonist pirenzipine had no effect.

Discussion

As all the experiments were done at midday, the choice of agonist and antagonist depended on when the various neurotransmitters in question were released. For example, enkephalins and melatonin are not released in the light, so it was expected that adding them (or an agonist) to the incubation medium would cause an increase in rates of sulfate incorporation, if there were receptors on the sclera to accept them. The fact that enkephalin had no effect indicates that there probably are no such receptors or that they are not linked. Melatonin had an effect although it was the opposite of what was expected, being inhibitory. However, it is important to note that this effect was not seen until a concentration of 1 mmol/L was reached. Given that the dissociation constant (K_d) for melatonin is in the picomolar range (Table 1), it seems reasonable to assume that the effect seen was not physiologically significant. In fact, this argument can be used for the dopaminergic and cholinergic results also, as they were all at much higher concentrations than the corresponding dissociation constants for relevant receptors.

Interestingly, the inhibitory effects of atropine and lack of effect of pirenzepine agree with effects obtained on scleral cartilage and chondrocytes.[10] However, antagonists act to block the effects of the neurotransmitter by competing at receptor binding sites. Therefore, when there is no neurotransmitter present, it is difficult to understand how the antagonists could have an effect.

It seems that none of the compounds tested have physiologically significant effects on rates of sulfate incorporation and hence SPS when tested directly on the sclera. What remains to be tested is whether they have any effect indirectly, via the pigment epithelium and choroid. This can be done by applying the compounds *in vivo*.

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