Title of Thesis

Pharmacological exploration of anticipatory anxiety through the fear-inhibited light reflex in man

Φαρμακολογική διερεύνηση του άγχους αναμονής μέσω της εκ φόβου αναστολής του φωτοανακλαστικού στον άνθρωπο

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Σύντομο Βιογραφικό

Γεννήθηκε στη Θεσσαλονίκη το 1973. Το 1998 αποφοίτησε από την ιατρική ακαδημία της Σόφιας και ολοκλήρωσε τη ψυχιατρική ειδικότητα το 2009 στο Ψυχιατρικό Νοσοκομείο Θεσσαλονίκης. Ενδιάμεσα είχε εργασθεί ως αγροτικός γιατρός στο νομό Ρεθύμνου (2001-2002) και πραγματοποίησε ένα κομμάτι της ειδικότητας στο Θεραπευτήριο Ψυχικών Παθήσεων Χανίων (2003-2005).

Το 2004 αποφοίτησε από το μεταπτυχιακό πρόγραμμα «Νευροεπιστημών» του Πανεπιστημίου Κρήτης. Η πτυχιακή έρευνα αυτού του προγράμματος έγινε στο εργαστήριο μελέτης συμπεριφοράς, με επικεφαλής τον τακτικό καθηγητή ψυχιατρικής κ. Παναγιώτη Μπίτσιο και η παρούσα εργασία διδακτορικής διατριβής αποτελεί τη συνέχεια εκείνης της πρώτης μελέτης.

Από το 2010 εργάζεται ως γιατρός του Ε.Σ.Υ στη ψυχιατρική κλινική του Πανεπιστημιακού Νοσοκομείου Ηρακλείου, προσπαθώντας ν' ανταπεξέλθει – μαζί με τους άλλους συναδέλφους - στο τεράστιο φόρτο που καλούνται να αντιμετωπίσουν οι δημόσιες δομές ψυχικής υγείας στις μέρες μας.

Δημοσιεύσεις

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Ευχαριστίες

Με αφορμή τη δημοσίευση αυτής της εργασίας θα ήθελα να ευχαριστήσω θερμά κάποιους ανθρώπους χωρίς τη βοήθεια των οποίων δε θα ήταν δυνατό να τα καταφέρω. Πρώτα απ' όλους θα ήθελα να ευχαριστήσω τον επιβλέποντά μου, τακτικό καθηγητή ψυχιατρικής κ Παναγιώτη Μπίτσιο, ο οποίος όχι μόνο με συμβούλευε και με καθοδηγούσε με τις γνώσεις και την εμπειρία του αλλά και με βοήθησε πολύ παραπάνω από ό, τι είχε υποχρέωση να κάνει σε κάθε στάδιο αυτής της έρευνας. Το ίδιο ισχύει και για την κ. Στέλλα Γιακουμάκη, επίκουρη καθηγήτρια στο τμήμα ψυχολογίας, η οποία ήταν πάντα πρόθυμη να με βοηθήσει ακόμα και στις πιο μικρές λεπτομέρειες.

Θα ήθελα να ευχαριστήσω ακόμη όλα τα υπόλοιπα μέλη της επταμελούς επιτροπής για τη συμβολή τους, τις διορθώσεις και το χρόνο που αφιέρωσαν για την ολοκλήρωση της εργασίας. Παράλληλα, οφείλω ένα μεγάλο ευχαριστώ και στους δεκάδες εθελοντές, συναδέλφους και μη, που συμμετείχαν ενεργά, με όρεξη και φιλότιμο στη πειραματική διαδικασία χωρίς τη βοήθεια των οποίων θα ήταν αδύνατη η διεξαγωγή αυτής της μελέτης.

Τέλος, ευχαριστώ όλους τους δικούς μου ανθρώπους που με αγαπούν και με στηρίζουν όλα αυτά τα χρόνια και που αποτελούν βασικό κίνητρο στην προσπάθειά μου να εξελίσσομαι σε όλους τους τομείς της ζωής μου.

Επταμελής επιτροπή:

- Μπίτσιος Παναγιώτης, Αναπληρωτής Καθηγητής
- Θερμού Κυριακή, Καθηγήτρια
- Καστελλάκης Ανδρέας, Αναπληρωτής Καθηγητής
- Παναγής Γεώργιος, Καθηγητής
- Σίμος Παναγιώτης, Καθηγητής
- Γιακουμάκη Στυλιανή, Επίκουρη Καθηγήτρια
- Σπανάκη Κλειώ, Επίκουρη Καθηγήτρια

Περίληψη

Η ανακάλυψη της κορομετρίας υπερύθρων στην δεκαετία του 50, έδωσε μεγάλη ώθηση στην έρευνα που στις δεκαετίες 60 έως 80 καθιέρωσε τις γνώσεις μας γύρω απο την φυσιολογία, την φαρμακολογία και την ψυχοφυσιολογία του κορικού συστήματος ως πειραματικού μοντέλου. Η ερευνα στη δεκαετία του 90 εκμεταλεύθηκε την πρόοδο στα ζωικά μοντέλα εξαρτημένου φόβου και καθιέρωσε την στενή σχέση μεταξύ της «εκ φόβου αναστολής του κορικού φωτοανακλαστικού» και της «εκ φόβου ενίσχυσης του ανακλαστικού αιφνιδιασμού», δύο παραδείγματα κατάλληλα για μεταφραστική έρευνα του φόβου και άγχους. Ενα πρόσθετο πλεονέκτημα του κορικού πειραματικού συστήματος είναι οτι διαχωρίζει μεταξύ διεργασιών προσοχής/διέγερσης που αντανακλώνται στην τονική ή φασική αύξηση της κορικής διαμέτρου και διεργασιών φόβου ή άγχους που αντανακλώνται στην αναστολή του φωτοανακλαστικού. Η πρώτη διαμεσολαβείται απο αύξηση του τόνου του συμπαθητικού συστήματος και του διαστολέα μύ της ίριδας, ενώ η δεύτερη απο αύξηση της κεντρικής αναστολής του παρασυμπαθητικού πυρήνα Edinger-Westphal και μείωση του παρασυμπαθητικού τόνου στον σφιγκτήρα μύ της ίριδας. Στην παρούσα διατριβή, εκμεταλευθήκαμε αυτές τις ιδιότητες του κορικού πειραματικού συστήματος για να μελετήσουμε την δράση σεροτονεργικών φαρμάκων (κετανσερίνη – πείραμα 1) και μή βενζοδιαζεπινικών αγχολυτικών (βουσπιρόνη και προπρανολόλη στα πειράματα 2 και 3 αντιστοίχως) στο επίπεδο εγρήγορσης όπως μετράται μέσα απο την διάμετρο της εν ηρεμία κόρης στο σκοτάδι. Βρήκαμε οτι η κετανσερίνη αλλά όχι η βουσπιρόνη ή η προπρανολόλη είχε ένα πλήρως υπνηλικό προφίλ στο κορικό σύστημα αλλά και σε άλλες αντικειμενικές και υποκειμενικές μετρήσεις της εγρήγορσης. Στα πειράματα 4 και 5 μελετήσαμε το προφίλ της κετανσερίνης και της βουσπιρόνης αντιστοίχως στο μοντέλο της εκ-φόβου αναστολής του φωτοανακλαστικού. Βρήκαμε οτι η βουσπιρόνη αλλά όχι η κετανσερίνη

εξομάλυναν την εκ-φόβου αναστολή του φωτοανακλαστικού, σύμφωνα με προηγούμενες μελέτες σε ζώα με το εννοιολογικά παρόμοιο παράδειγμα της εκ-φόβου ενίσχυσης του ανακλαστικού αιφνιδιασμού και σύμφωνα με την αγχολυτική δράση της βουσπιρόνης αλλά όχι της κετανσερίνης στην κλινική. Οι μελέτες αυτές βοηθούν στην κατανόηση τόσο της εκ φόβου αναστολής του φωτοανακλαστικού ως μοντέλου εγρήγορσης και άγχους, όσο και στην κατανόηση της νευροδιαβίβασης της εγρήγορσης και του άγχους στον άνθρωπο. Επειδή το τρέχον παράδειγμα της εκ-φόβου αναστολής του φωτοανακλαστικού μιμείται φασικό φόβο/άγχος που είναι η βάση του φοβιών/πανικού, στο πείραμα 6, αναπτύξαμε ένα εναλλακτικό πρωτόκολλο που επιτρέπει την καταγραφή της έναρξης, πορείας και τερματισμού της φοβικής αναμονής, που είναι στη βάση του παρατεταμένου άγχους που συναντούμε στην γενικευμένη αγχώδη διαταραχή. Η εγκυρότητα της μεθόδου επιβεβαιώθηκε με την παράλληλη (εντός της ίδιας συνεδρίας) εφαρμογή του πρωτοκόλλου στο συγγενές και έγκυρο παράδειγμα της εκκ φόβου ενίσχυσης του ανακλαστικού αιφνιδιασμού.

Abstract

The discovery of the infrared pupillometer in the 50s gave impetus to research which in the 60s through to the 80s established a great deal of knowledge on the physiology, pharmacology and psychophysiology of the pupillary test system. Pupillary research in the 90s took advantage of progress made in animal models of conditioned fear and established the close relationship of the fear-inhibited pupillary light reflex and the fear-potentiated startle reflex, two paradigms that are suitable for translational research in fear and anxiety. An additional advantage of the pupillary test system is that it can separate between attentional/general arousal processes reflected in tonic or phasic increases in pupil diameter (a response mediated by increase in sympathetic output to the iris dilator muscle) and fear- or anxiety-related processes that are reflected in the inhibition of the light reflex (mediated by central, supranuclear inhibition of the parasympathetic Edinger-Westphal nucleus and reduced output to the iris constrictor muscle). In this thesis, we exploited these properties of the pupillary test system and studied the effects of serotonergically acting drugs (ketanserin in experiment 1) and non-benzodiazepine anxiolytic drugs (buspirone, propranolol in experiments 2 and 3) on physiological arousal as measured by resting pupil diameter in the dark. Ketanserin, but not buspirone or propranolol had a fully sedative profile in the pupillary test system and other objective and subjective measures of arousal. In experiments 4 and 5 we examined the profiles of ketanserin and buspirone respectively on the fear-inhibited light reflex. We found that buspirone but not ketanserin normalized fear-inhibiton of the light reflex consistent with animal studies on the conceptually similar startle-potentiated startle reflex paradigm and in agreement with the clinical utility of buspirone but not ketanserin in human anxiety disorders. The studies of this thesis advance both the understanding of the pupillary test system as a model of arousal and anxiety as well as the pharmacological mediation of

human arousal and anxiety. Because the current fear-inhibited light reflex models phasic fear/anxiety, in experiment 6 we developed an alternative light reflex protocol that allows for the study of the onset, time-course and offset of anticipatory fear, which is more relevant to sustained rather than phasic anxiety disorders in humans i.e. generalized anxiety rather than phobic/panic anxiety disorders. We validated this protocol with the established fear-potentiated startle paradigm in humans.

CONTENTS

1 GENERAL BACKGROUND

1.1 FUNCTIONAL ANATOMY, INNERVATION AND NEURO-CHEMISTRY OF THE IRIS

Anatomy of the human iris

The iris can be conceived as a diaphragm interposed between the anterior and the posterior chambers of the eye, and is thus surrounded by aqueous fluid. Because the posteriorly located lens pushes the central part of the iris forward, the iris has a three-dimensional shape of a shallow, truncated cone. Its root is very thin and posteriorly it merges with the ciliary body, which surrounds the lens and controls accommodation. The axial border, which is unattached, rests lightly on the lens and forms a central aperture termed the pupil. From embryonic and functional viewpoints the iris consists of two main parts: the posterior leaf, containing the iris muscles and posterior pigment epithelium, all derived from the neuroepithelium of the optic cup, and the mesodermal anterior stroma, a much thicker bulk of loose but orderly three-dimensional meshwork of collagen fibres which carries the iris vessels, nerves, and chromatophores. The anterior border layer of the iris stroma (towards the cornea) is made up of denser tissue than its middle portion and contains more pigmented cells especially in darker irides. The iris muscles are derived from neuroectoderm of the optic cup. The iris sphincter muscle is a thin (0.7 - 1 mm) band of smooth muscle cells, forming a continuous ring around the pupillary margin; the dilator muscle is a radially arranged thin layer of primitive myoepithelial cells. The dilator stretches from the peripheral iris root to the area beneath the sphincter ring, within 0.3 mm of the pupillary margin.

Parasympathetic innervation of the iris

The receptors for the pupillary light reflex are identical to those for vision, namely the rods and cones of the retina (Loewenfeld, 1966; Hultborn *et al.*, 1978, Thompson, 1987). The phylogenetically oldest "W" or gamma retinal ganglion cells (40% of the total population of retinal ganglion cells), which are located in all areas of the retina, transmit information about incident light brightness. The projections of the "W" (or gamma) retinal ganglion cells travel in the optic nerves and the larger part of them, like the visual fibres, crosses in the optic chiasm (peripheral decussation), whereas some remain uncrossed, resulting in numerous ganglion cells in the temporal retina to send axons to the ipsilateral optic tract. The pupillary afferents constitute almost 10% of all fibres in the optic tracts, and before the tract reaches the lateral geniculate nucleus they separate from the optic tract and run by way of the branchia of the superior colliculi to the rostrodorsal midbrain (Figure 1). They do not enter the colliculi, but pass mediorostrally to the pretectal area at the mid brain-diencephalon junction. There they synapse on the pretectal neurones of the pretectal nuclei (Magoun and Ranson, 1935; Sillito and Szbrozyna, 1970; Hultborn *et al.*, 1978, England and Wakly, 1991). The pretectal neurones redistribute the afferent imputs to the occulomotor nuclei, projecting bilaterally to the occulomotor complex. Anatomical studies by Hultborn *et al.* (1978) suggested that particularly the olivary pretectal nucleus and the nucleus of the posterior commissure both project to the Edinger-Westphal complex. Indeed, recent studies in rats have shown that the retina projects bilaterally to the olivary pretectal nucleus (Clarke and Ikeda, 1985; Trejo and Cicerone, 1984), the primary retinorecipient nucleus in the pathway of the pupillary light reflex. The olivary pretectal nucleus then projects bilaterally to the Edinger-Westphal nucleus as well as to the nucleus of the posterior commissure, which itself

does not receive retinal input but projects bilaterally to the Edinger-Westphal nucleus (Young *et al.,* 1994). While the projections from the olivary pretectal nucleus to the Edinger-Westphal nucleus contribute to the consensual pupillary light reflex, the bilateral retinal projection to the olivary pretectal nucleus is the main determinant component of the consensual light reflex (Young *et al.,* 1994). Beyond this central hemidecussation both crossed and uncrossed pretectal fibres skirt around the Sylvian aqueduct and central grey matter to reach the rostral third of the oculomotor nuclei complex, namely the small-celled autonomic anteromedian and Edinger-Westphal groups, whose role is to serve the constriction of the pupil and the accommodation of the lens. In fact, these nuclei constitute the centre of the reflex and it is here where the afferent path reaches its end. It is also at the Edinger-Westphal nucleus (and the pretectal nuclei described before) where central inhibition occurs (see below), while the efferent pathways convey excitatory activity only.

Efferent preganglionic pupilloconstrictor fibres from the motor nucleus enter the orbit with the inferior division of the 3rd nerve, after a long and superficial course in the occulomotor nerve. They then pass to the 3rd nerve branch that supplies the interior oblique muscle, and from there reach the ciliary ganglion, by way of its short motor root. All of the parasympathetic pupillary fibres synapse with ciliary ganglion neurones. The post-ganglionic pupilloconstrictor fibres travel to the eye as groups of fine nerve bundles that surround the optic nerve and pierce the sclera near its entrance into the globe. These short ciliary fibres pass along the episcleral space to the anterior segment of the eye. Pupillary and accommodation fibres appear intermingled in this course.

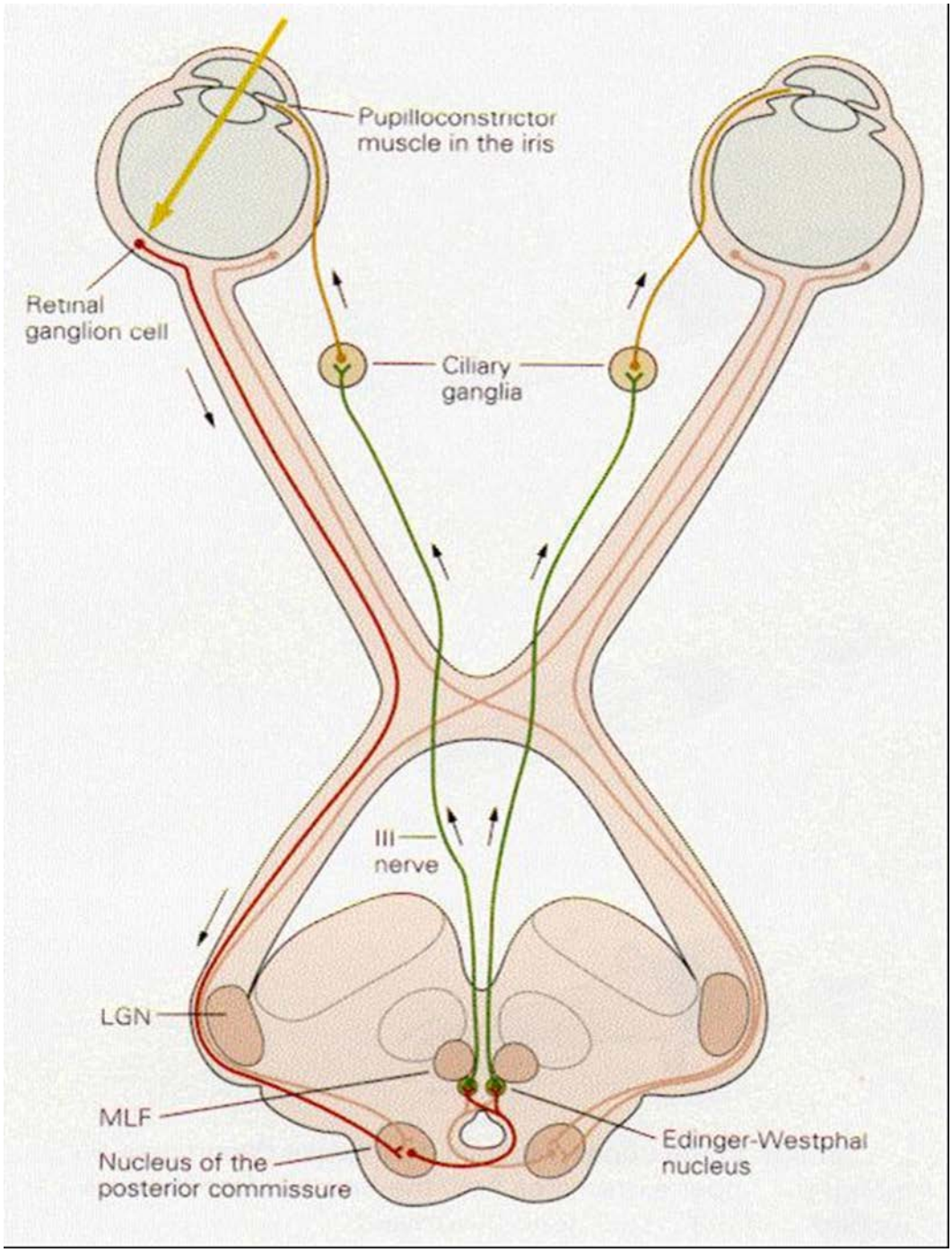


Figure 1. Parasympathetic innervation of the iris.

Sympathetic innervation of the iris

From the hypothalamus in the diencephalon arises the great efferent sympathetic fibre system which innervates all visceral organs of the body, a small part of which is the sympathetic pathway to the iris dilator muscle of the eye (Figure 2). In its lengthy course, it descends through the entire brainstem and cervical spinal cord, and then again ascends in the neck, in the cervical sympathetic chain. It re-enters the skull, and associated with the fifth nerve, passes quite close to its hypothalamic origin toward the orbit and so to the eye. Three segments make up this path, namely, the "central (first) neurone" from the hypothalamus to the cervicothoracic cord; the "preganglionic (second) neurone" from the cord to the superior cervical ganglion; and the "postganglionic (third) neurone" from there to the iris.

From the hypothalamus, the central pupillodilator tract runs caudally via the subthalamic-prerubral area. At the level of the mammillary bodies it begins to shift laterally, just dorsal to the substantia nigra. At the level of the superior colliculi it is concentrated in the ventrolateral tegmentum; and it then continues ventrolaterally throughout the remaining brainstem. In the human brain the sympathetic fibres are located more rostrally in the medulla compared to cat's brain, because of the relatively well developed inferior olivary nucleus which occupies the ventral area. There is good agreement about the course of the central pupillodilator tract as described above, between four recent detailed investigations (Kerr and Brown, 1964; Koss and Wang, 1972; Lowey *et al.,* 1973; Saper *et al.,* 1976). This descending brainstem path used to be considered diffuse, but lately it has been found to be quite discrete (Kerr and Brown, 1964; Koss and Wang, 1972). It is possible however that this pathway is under control of the noradrenergic nuclei of the lower brainstem (both areas A1/A5 and the locus coeruleus) on the hypothalamus (see section 2.5.4). In the

cord, the fibres travel superficially in the lateral funicilus until, at the C8 to T2 level, they turn mesially and synapse with the preganglionic sympathetic neurones in the intermediolateral cell column ("Budge's centre"). Although it is generally believed that the descending sympathetic fibre tract just described has one or several relays between the hypothalamus and spinal cord, Saper *et al.* (1976) were able to demonstrate that some direct fibres exist, which run this entire course from the hypothalamus to the intermediolateral cell column of the spinal cord without interruption. It is not known however, whether these neurones with the long axis serve pupillary dilatation or different sympathetic functions.

The preganglionic pupillary sympathetic fibres arise from the lateral horn cells of the cervicothoracic spinal cord. They leave the cord by its ventral roots and after they join the sympathetic chain, they turn upward toward the head. They traverse the first thoracic and inferior cervical ganglia and then pass without interruption via the ansa of Vieussens, the middle cervical ganglion, and the cervical sympathetic nerve to the superior cervical ganglion, near the bifurcation of the internal and external carotid arteries, where they synapse with the postganglionic neurones.

Postganglionic fibres for the pupil leave the superior cervical ganglion at its rostral pole and join the carotid plexus, which is adjacent to the internal carotid artery, into its canal in the temporal bone. They approach the Gasserian ganglion and enter the ophthalmic branch of the trigeminal nerve just distal to its origin from that ganglion. After they run in the middle fossa they then pass into the nasociliary branch of the fifth nerve and, after bypassing the ciliary ganglion, they reach the iris dilator muscle via the long ciliary fibres.

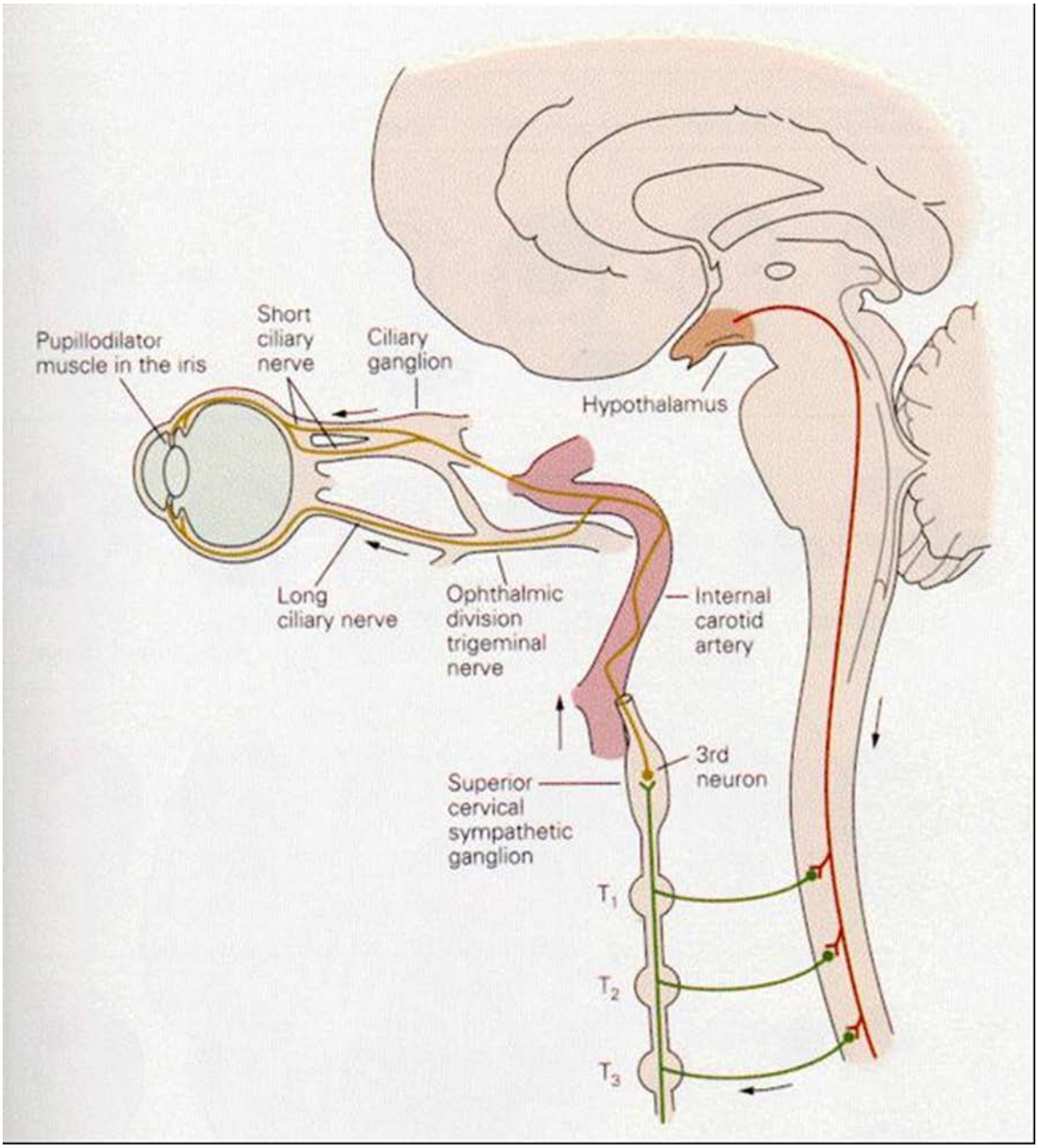


Figure 2. Sympathetic innervation of the iris

Neurotransmitter receptors of the iris

Classically, the two main controlling and best studied systems of the iris are the sympathetic and parasympathetic innervations, whose influences on the iris muscles can be modulated at their synaptic and effector sites. Increased parasympathetic activity results in miosis due to the release of acetylcholine at the neuroeffector sites on the iris sphincter muscle and increased sympathetic activity results in mydriasis which is due to the release of noradrenaline to the neuroeffector sites on the dilator muscle.

The iris sphincter muscle contains cholinoceptors of the muscarinic type (Sitaram *et al.,* 1983; Hutchins and Hollyfield, 1984). Of the three subtypes of muscarinic receptors (M_1, M_2, M_3), which have been found in isolated irides (Bognar *et al.,* 1989), the M_1 and M_3 subtypes are located at post-junctional sites, inducing the contraction of the sphincter muscle (Bognar *et al.,* 1989, 1990), whereas the M_2 muscarinic receptors are probably located pre-junctionally. The iris sphincter muscle also contains small numbers of α- and β- adrenoceptors (van Alpen, 1976; Sitaram *et al.,* 1983). These probably serve for the relaxation of the sphincter during noradrenaline release from the noradrenergic terminals of the dilator muscle, resulting in dilatation (Jaanus *et al.,* 1989, Loewenfeld, 1993a).

In man, the iris dilator muscle contains predominantly α- and very few β-adrenoceptors (Jaanus *et al.,* 1989) and the main subtype responsible for mydriasis is the α_1 adrenoceptor (Sitaram *et al.,* 1983; Lefkowitz *et al.,* 1990). There is also a parasympathetic inhibitory innervation of the dilator muscle, which is probably partially mediated via M_2 muscarinic receptors (Fuder *et al.,* 1986; Bognar *et al.,* 1989) located pre-junctionally, on the iris noradrenergic nerves to mediate inhibition of noradrenaline release, and thus relaxation of the dilator muscle during miosis.

Recent research investigates the mediation of pupillary responses to light by other transmitters other than the acetylcholine and noradrenaline. Serotonin (5-hydroxytryptamine, 5-HT), is considered as a putative modulator of the neurotransmission in the iris (Moro *et al.*, 1981). Indeed, ICI 169,369, a $5HT_2$ receptor antagonist, has been found to cause miosis (Millson *et al.*, 1991), thus suggesting that $5HT_2$ receptors may play a role in the control of pupillary responses, either via a direct postsynaptic $5HT_2$ receptor mediated dilatation, which when antagonised results in miosis, or via pre-synaptic inhibitory $5HT_2$ receptors modulating parasympathetic tone, which when antagonised increases parasympathetic tone and results in miosis, or finally, via a central effect on $5HT_2$ receptors, on efferents from the third nerve nucleus.

A number of studies deal with the effects of morphine and opioids on pupillary kinetics, raising questions about a peripheral and/or a central action of morphine on the iris (Fanciullacci *et al.*, 1984; Rabinowitz and Kozczyn, 1987; Klemfuss and Adler, 1986). Some authors suggest the existence of specific opiate receptors in the iris (Fanciullacci *et al.*, 1984) and others have suggested that the pupillary dilatation to naloxone in heroin users could be used as a clinically practical and objective method to determine the level of opiate tolerance (Higgins *et al.*, 1985; Ghodse *et al.*, 1986, 1995).

Another important area of research is the miosis induced by peptides (tachykinines) especially substance P and neurotensin. Animal studies of irides in vitro have shown that substance P is a potent miotic, involved mainly in maintaining the constriction of the pupil after ocular irritation, rather than inducing rapid changes during the light reflex (Bito *et al.*, 1982). However, there is evidence that neurotensin and substance P may contribute to the neural control of iris motility (Hernandez *et al.*,

1985). Instillation of substance P in humans in vivo has been found to produce a dose-dependent miosis, suggesting a specific receptor for substance P (Alessandrini *et al.*, 1991). The miosis was fast, thus suggesting sufficient bioavailability of substance P, although weaker than the miosis produced by cholinomimetics or α_1 adrenoceptor blockers and could not counteract the homatropine-induced mydriasis. These data suggest that substance P is a low potency peptide and is perhaps linked to the release of acetylholine from the cholinergic nerve terminals of the iris (neuromodulator). It has been suggested, that sensory substance P- containing fibres from the iris play a role in the miosis observed after the antidromic stimulation of the trigeminal nerve resulting in the release of substance P.

The whole issue of the neurotransmitter receptors of the iris is debated at great length and certainly, more post-mortem studies are needed with irides from donors using highly potent and specific agonists or antagonists, to classify in detail the nature of iris receptors and their functional role.

1.2 PHYSIOLOGY OF THE IRIS

1.2.1 The pupillary light reflex

The "shape" of the reflex response

The pupil's reaction to a weak flash of light has a long latency, in a slow, brief and incomplete constriction. The higher the stimulus intensity, the more forceful the pupil reaction becomes. The light reflex response then appears with shorter latency, faster contraction velocity and more extensive contraction amplitude, which are maintained for a longer time. Short flashes of very bright light suffice to drive the pupil into tight miosis that may last for several seconds, since excessively bright light interferes with the pupillary redilatation process (Loewenfeld, 1993b).

With infrared binocular pupillometers, which allow the study of the pupils' reaction to light flashes in complete darkness, and with the assistance of computerized averaging techniques, one can obtain smooth light reflex profiles as illustrated in FIGURE 1. This typical waveform of a pupillographic trace of light reflex represents the change in pupil diameter plotted against time demonstrating all the parameters currently studied, and illustrates the presumed activity of the autonomic nervous system in shaping the response.

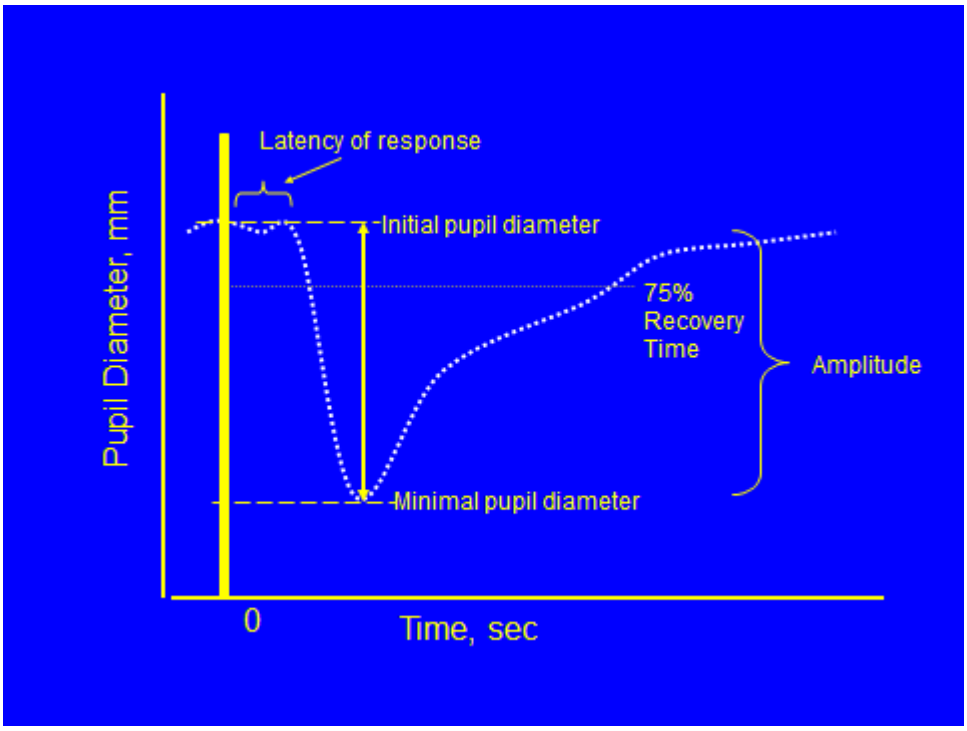


Figure 1. The shape of the light reflex response to a light stimulus

Onset latency

The time that elapses between the onset of light stimulus and the beginning of pupillary contraction is called the latent period of the reflex response. The latency depends firstly on the quickness of the iris sphincter muscle, which sets the minimal latency, and secondly on the intensity of the light stimulus, which determines the duration of an additional time delay, consumed by the retinal receptors and the neural reflex arc; the dimmer the light or the smaller the retinal area stimulated or the less complete the previous dark adaptation, the longer the latency of the light reflex response will be. In mammals, the minimal latency is in the 180 to 200 ms range and the additional delay for weak light is about 250 ms. Latencies almost exclusively depend upon retinal output, unlike the amplitude of the light reflex, which is modified mainly by central “supranuclear” inhibition (see below). They are thus very interesting in that they can furnish insight into how the retina and the central nervous

system process sensory information. Clinically, they are useful indicators of retinal function (Loewenfeld, 1993b).

Amplitude

The amplitude and constriction velocity of the light reflex depend on the activity of neurones in the parasympathetic Edinger-Westphal nucleus. This activity is regulated by two factors: a) stimulus intensity and b) supranuclear inhibition of the Edinger-Westphal nucleus. Thus for subjects with intact afferent and efferent branches of the reflex arc and with equally adapted retinas to darkness, the brighter is the light stimulus, the larger will be the number of neurones recruited in the Edinger-Westphal nucleus and the more vigorous will be their firing, and thus the greater will be the depth of the pupillary constriction and the more extended the amplitude of the response. However, the number of recruited neurones as well as their firing rates can be attenuated proportionally to the amount of central supranuclear inhibition of the Edinger-Westphal nuclei. One major source of central supranuclear inhibition at the level of Edinger-Westphal nucleus arrives from the hypothalamus (Smith, 1992; Loewenfeld 1993b).

Recovery (redilatation) time

The decrease in pupil size in response to the onset of a light stimulus, is followed by a redilatation to initial levels in response to the offset of the stimulus. Electrophysiological studies in animals ~~indicate~~ have shown that a “light-off” reaction involves an excitatory effect on sympathetic activity recorded from the superior cervical ganglion, previously inhibited by light (Nisida *et al.*, 1960; Passatore *et al.*, 1977; see also next section on “Darkness Reflex”). Thus, the redilatation in response to light

stimulus offset is largely dependent on intact sympathetic function of the hypothalamus and the peripheral sympathetic chain (Loewenfeld, 1993a, 1993h, Smith, 1992). Indeed, recovery time is found prolonged by sympatholytic drugs (Morley *et al.*, 1991) which makes it a valid index of central and peripheral sympathetic activity. It provides important information about the state of the sympathetic nervous system which in fact does not appear elsewhere so directly in the time-course of the pupillary light reflex. In practice, the 50% or 75% of the recovery time is measured. Redilatation is faster at the beginning of the recovery phase when the firing of Edinger-Westphal neurones decreases, until they stop (peak aptitude) and the central sympathetic system takes over (Smith, 1992). Thereafter, the peripheral sympathetic input to the iris widens the pupil more slowly and the redilatation velocity decreases. There are indications that the fast redilatation phase solely reflects withdrawal of the parasympathetic tone that determines the rate of recovery of pupil size after a light stimulus (Smith, 1992; Loewenfeld, 1993a; Heller *et al.*, 1990), whereas the slow redilatation phase is a pure measure of peripheral sympathetic activity (Smith, 1992; Loewenfeld, 1993a, 1993h). Thus, only the slow redilatation phase is affected in subjects with peripheral sympathetic lesions (i.e. Horners' syndrome or drug-induced peripheral sympathectomy) (Loewenfeld, 1993a, 1993h; see also section "Overview and integration of pupillary reflexes").

1.2.2 The pupillary darkness reflex

The pupillary reaction to a short dark interval in eyes previously adapted to ambient light, is called "darkness reflex". The dark period elicits pupillary dilatation, preceded by a latent period slightly longer than that of a light reflex; after the light reappears, the pupils contract below the previous light-adapted diameter and then

redilate to baseline (triphasic response) (see FIGURE 2). In a sympathectomised iris, it can be seen that the dilatation in response to the dark pause is reduced compared to the reaction of the intact pupil but it is not abolished (Lowenstein and Loewenfeld, 1969). Thus, the darkness-evoked dilatation is due to: a) reduction of impulse traffic to the parasympathetically innervated pupillary sphincter, which affects both pupils and b) increased sympathetic discharges to the pupillary dilator, which can reach the intact iris only.

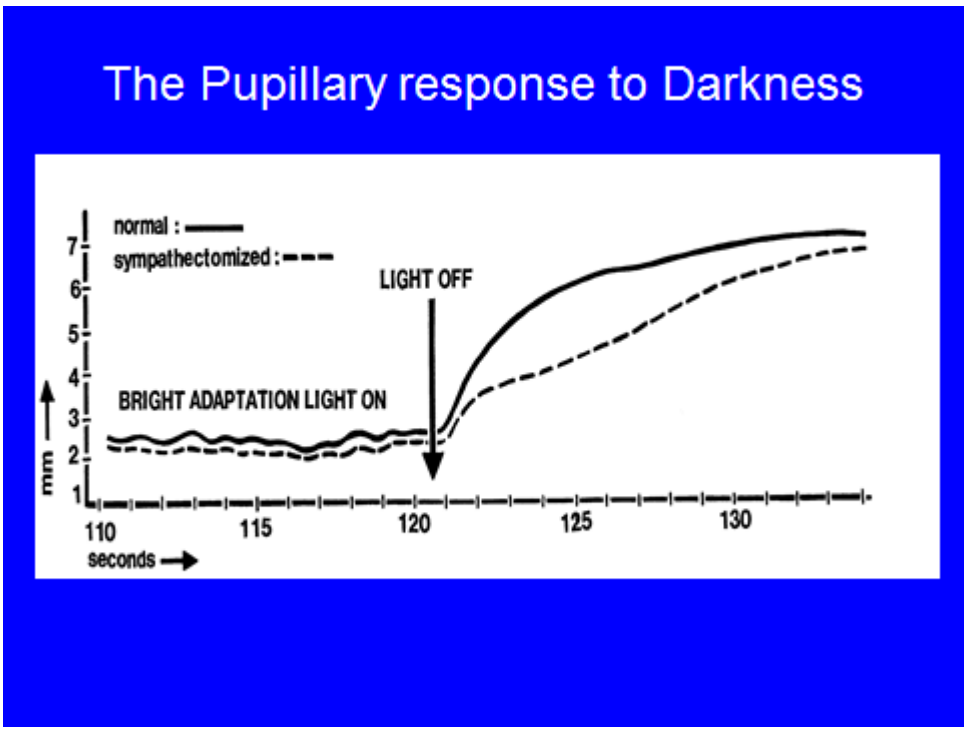


Figure 2. The pupillary "darkness" reflex

Darkness stimuli elicit retinal "off" discharges which are probably partly responsible for the dilatation phase of the response, and reappearance of the light elicits retinal "on" discharges (because during the dark period the retina has gained sufficient sensitivity to the reappearing light) which are responsible for the secondary contraction-redilatation movement (Loewenfeld, 1993c); thus the secondary contraction-redilatation movement of the darkness reflex has essentially the same

mechanism as the light reflex. Both “off” and “on” retinal discharges are visible in the Electroretinogram (Kawabata, cited in Loewenfeld, 1993c).

Indeed, single units in the pretectal region in cats, which fire continuously only in darkness (“off” discharges) and are tonically inhibited in the presence of ambient light, have been isolated. Electrical stimulation of these units was followed by pupillary dilatation, even in cats whose brainstem had been transected in the midpontine region so that the descending sympathetic pupillary dilator pathway had been interrupted (Nisida *et al.,* 1959; Cavaggioni *et al.,* 1968; Smith *et al.,* 1968). In agreement with these results, Sillito and Zbrozyna (1970) noted that cessation of retinal illumination was followed by postexcitatory depression of activity in the pupilloconstrictor interneurones, identified by antidromic responses to electrical stimulation of the fibres converging upon the pupilloconstrictor nucleus. On the basis of those results it is thought that the reduction in impulse traffic to the parasympathetically innervated pupillary sphincter during a dark period is caused by inhibitory retinal discharges which reach the oculomotor neurones via the light reflex pathway and the intercalated midbrain neurones.

The sympathetic component of the darkness reflex is also well established by electrophysiological studies; sympathetic nerve impulses recorded from the central stump of the divided cervical chain (Passatore, 1976a, 1976b, 1977; Nishino *et al.*, 1976) or of the long ciliary nerves (Nisida *et al.,* 1960, Okada, 1960) are inhibited when lights are turned on and are released from inhibition when the light is turned off, followed by pupillary dilatation. The accessory optic system (developed especially in rodents) and in particular retino-suprachiasmatic connections are considered to play a role in the mediation of this response. Indeed, retinal input has an excitatory effect on

the suprachiasmatic nucleus of the hypothalamus, and during electrical stimulation of this nucleus the cervical sympathetic nerves cease firing (Nishino *et al.,* 1976).

The extensiveness of the darkness-evoked dilatation is directly related to the intensity of the previously adapting light; when this light's intensity is reduced (or when the dark period is very short) the dilatation has smaller amplitude, or it may be missing altogether, while the secondary contraction and redilatation upon readmission of light are relatively well preserved (Lowenstein and Loewenfeld, 1969). This is because the retinal "off" discharge is much weaker than the retinal "on" wave; in the case of low intensity adapting light, no effective "off" signal would be generated, whereas, in the case of a very short dark period, the pupillary dilatation elicited by the "off" discharge would be overpowered by the contraction evoked by the more intense "on" discharge that follows it before the pupil has had a chance to dilate (Lowenstein and Loewenfeld, 1969; Loewenfeld 1993c). Clinically, the darkness reflex is useful for the diagnosis of Horner's syndrome (reduced amplitude of dilatation) and in physiological/pharmacological experiments for the monitoring of sympathetic function. The darkness reflex can be quite stable even when elicited hundreds of times, in contrast to the psychosensory dilatation (see below) which quickly habituates. It can be enhanced by ocular instillation of sympathomimetics, or reduced by sympatholytic agents (Loewenfeld and Newsome, 1971).

1.2.3 Psychosensory (reflex) dilatation

In response to sensory or central nervous stimulation of intact, conscious subjects and animals, the subjects' pupils dilate rapidly and may reach maximal size. There is no specific afferent path; any sensory stimulus (except for light) as well as spontaneous thoughts or emotions have the same effect and serve as a non-specific

afferent path for pupillary reflex dilatation. After the repetitive administration of the same stimulus this response quickly disappears (habituation).

With regard to the known neurophysiology of pupillary dilatation, it does not seem likely that this response is under subcortical initiation and control. Although the hypothalamus provides the definitive final point for propagation of autonomic nervous system activity, the normal time course for psychosensory dilatation is obtained only when cortical influences (either direct to the hypothalamus or mediated through thalamic pathways) remain intact; under early stages of anaesthesia for instance, when cortical activity is suppressed but no changes are detected in the electrical excitability of the hypothalamus, pupillary dilatation after sensory and cortical stimulation is slower and does not achieve maximal amplitude although electrical stimulation of the hypothalamus in the same condition produces a full response (Loewenfeld, 1958). In addition, weak cortical stimulation provides strong sympathetic discharges in normal, awake animals. In her detailed experimental analysis, Loewenfeld (1958) concludes only that the centre for initiation of the normal dilatation reflex must lie above the midbrain, but does not invoke the activity of any specific cortical region, since stimulation of most cortical areas results in pupillary dilatation. Impulses from the cortex, through cortico-limbic connections (cortico-thalamo-hypothalamic pathway) arrive at the hypothalamus and bring it into play as the major motor centre for the active sympathetic component of the pupillary reflex dilatation. From the hypothalamus efferent discharges travel along the sympathetic pathway to the iris dilator muscle of the eye. When this efferent path is cut, the pupil is still able to dilate in response to psycho-sensory stimuli, although the reactions are much more reduced in extent and speed. This "residual" psychosensory dilatation is supposed to be due to simultaneous inhibition of parasympathetic outflow at the level

of the Edinger-Westphal nucleus after a psychosensory stimulus. Control over the parasympathetic component of pupillary movements can also be influenced by cortical mechanisms. An indirect pathway, also inhibitory, has been found via the aforementioned cortico-thalamic-hypothalamic pathway, with hypothalamic fibres projecting to the Edinger-Westphal nucleus (Lowenstein, 1955; Saper *et al.*, 1976, Koss and Wang, 1972; Koss *et al.*, 1984; Koss, 1986). It has also been suggested that direct cortical pathways, possibly originating in area 8, act to inhibit the Edinger-Westphal nucleus (Lowenstein, 1955).

The simultaneous sympathetic activation and parasympathetic inhibition during a reflex dilatation are the neurological mechanism for the reflex dilatation in response to brief and weak stimuli. However, under the influence of more intense or longer stimuli, a humoral mechanism comes into play and modifies the time-course of pupillary reflex dilatation. In that case the response is bi- or triphasic; after the first (described above), there is a second dilatation (onset latency: 2-3 s in the cat, longer in man, duration: up to 10 s). The latter is due to the arrival of bloodstream noradrenaline to the iris, released from noradrenergic nerve terminals in the periphery. This humoral dilatation may be fused with a third phase (onset latency: 12-15 s) as a result of the arrival of adrenaline from the adrenal glands, in case of unusually powerful and prolonged stimuli (Loewenfeld, 1993d).

1.2.4 The reaction to near vision

When the focus of the eyes is moved from a far to a near point, three independent movements occur simultaneously: a) contraction of the internal rectus muscle which results in convergence of the visual axes in order to keep the image placed upon corresponding retinal areas of the two eyes, b) contraction of the ciliary

muscle which results in accommodation of the lens in order to correct the focus of the eye, and c) contraction of the pupils in order to increase the depth of field (called near-vision pupillary reflex) (Loewenfeld, 1993e). This near-vision reaction of the eye is the most highly developed of all eye movements (complete only in primates and man) requiring exquisite co-ordination of the striated extraocular and smooth intraoccular muscles. The stimulus for the reaction is the perception of the unsharp image of an approaching object. This afferent message activates specific areas in the occipital cortex, which then elicits the appropriate motor responses co-ordinating the internal recti and the intraocular muscles. The descending fibre paths that connect the cortical sites with the oculomotor nucleus have not yet been identified (see Jampel, 1959). The motor centre and efferent path are the same as for the light reflex, namely the third nerve nucleus (Edinger-Westphal nucleus) and its axons which run along with the oculomotor nerve, with a synapse in the ciliary ganglion.

In normal young adults the pupillary near-vision reflex to a rigorous near-vision effort is as extensive as the contractions to bright light. The amplitude of the response depends on the effort expended, not only upon the distance of the target from the eye. The reaction is the same in the two eyes even when one eye does not converge because of asymmetric placement of the target or when one eye is covered. In many subjects the near-vision reflex is less extensive than the light reflex. This is not a clinically significant condition because of the subjective features of the reflex (depends upon co-operation and effort expended) unless it can be established that an adequate near-vision effort was made. However, when the near-vision reaction is more extensive than the subjects' best light reflex a "light-near" dissociation exists, which is always part of a pathologic pupillary syndrome (see below).

1.2.5 Pupillary oscillations in light (hippus)

In steady ambient illumination the pupils oscillate continuously in irregular rhythm. The brighter the light, the smaller and more frequent are these movements, while they slow down and become more extensive in dimmer illumination. They continue as long as the light is on and tend to be somewhat more vigorous in young, excitable subjects than in older people. These light induced pupillary oscillations have been named “pupillary unrest” (Loewenfeld, 1993b, 1993f) and are not pathological (Figure 3). They vary among individuals and they are remarkably similar in identical twins. Their time-amplitude characteristics (duration: 1/3 to 2 s, extent: up to 1.5 mm) must therefore be part of each person’s genetic make-up. Their mechanism is still unknown. They are absent only in dim light or darkness, when the light reflex pathway is interrupted, or when the iris is damaged and in patients with spastic miosis (Loewenfeld, 1993b, 1993f).

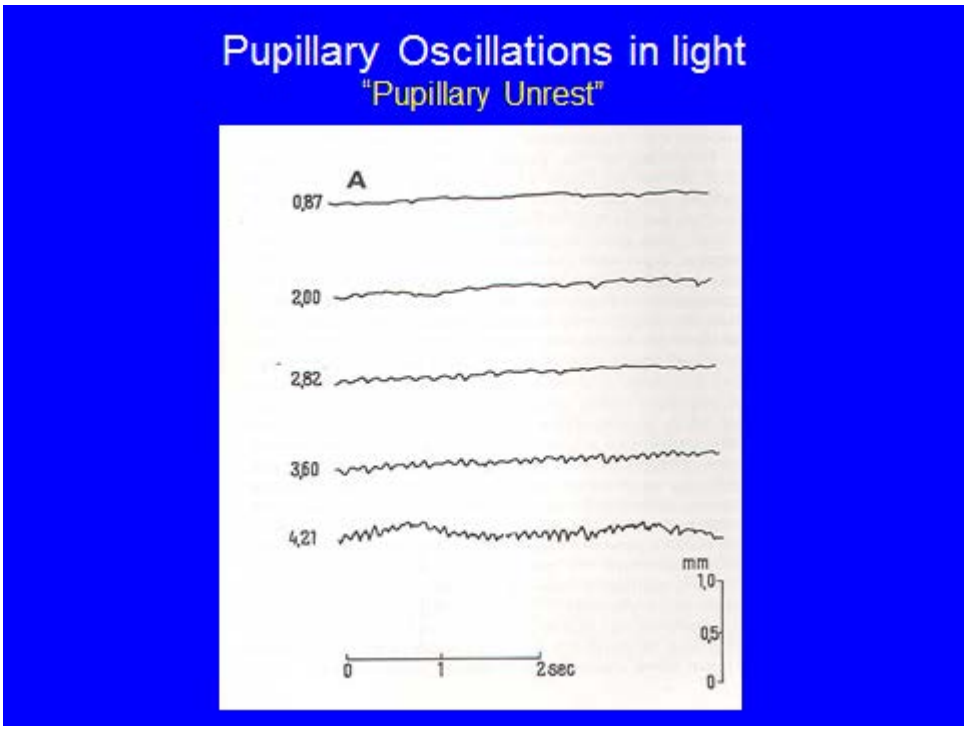


Figure 3. Hippus. Numbers on the left represent ambient light intensity expressed in log-Trolands.

1.2.6 Pupillary oscillations in darkness

The pupils of healthy well-rested subjects in darkness are usually quite stable for several minutes at a time and the normal "pupillary unrest" that occurs under diffused light ceases promptly as soon as the light is turned off. The pupils then remain large and steady as long as the subject stays fully alert; but sooner or later, under the influence of boredom or / and fatigue, the pupils begin to oscillate and also to decrease in size [fatigue waves (Loewenfeld, 1993i, 1993g, 1993f)] (Figure 4). This is encountered when the subjects remain quietly in darkness for lengthy periods without anything to do, and this phenomenon should not be attributed to the experimental procedure or the drug used.

Fatigue waves differ in timing and extent from pupillary unrest induced by steady light. They are rather slow (three seconds or longer) and vary in depth from just perceptible wavering to huge ups and downs that cover a great part of the range of pupillary mobility, while light-induced "unrest" consists of small wavelets that increase in frequency with the intensity of illumination. Fatigue waves are of great research interest and have not been systematically studied. There are only qualitative reports which relate them to subjects' level of arousal (decline of pupil size as the subject becomes more tired and sleepy and increase in pupil size in response to the intrusion of thoughts and emotions as the subject tries to stay awake). Thus, there are wide differences between subjects reflecting the operation of different aspects of arousal such as the subjects' general state of health and degree of tiredness at the time of the experiment, as well as their emotional state, motivation and willingness to participate in the experiment. The neural correlates for these conditions and their role in fatigue waves are not known. It is known, however, that each individual has a characteristic pattern of responding, repeated more or less regularly in every

experiment. Since this pattern is the same in identical twins, it must be anchored in the genetic make-up of the individual (Loewenfeld, 1993g, 1993i). Age must also be considered, since with advancing decades, there is a steady loss of central inhibition and thus of pupil size in darkness (Loewenfeld, 1993i).

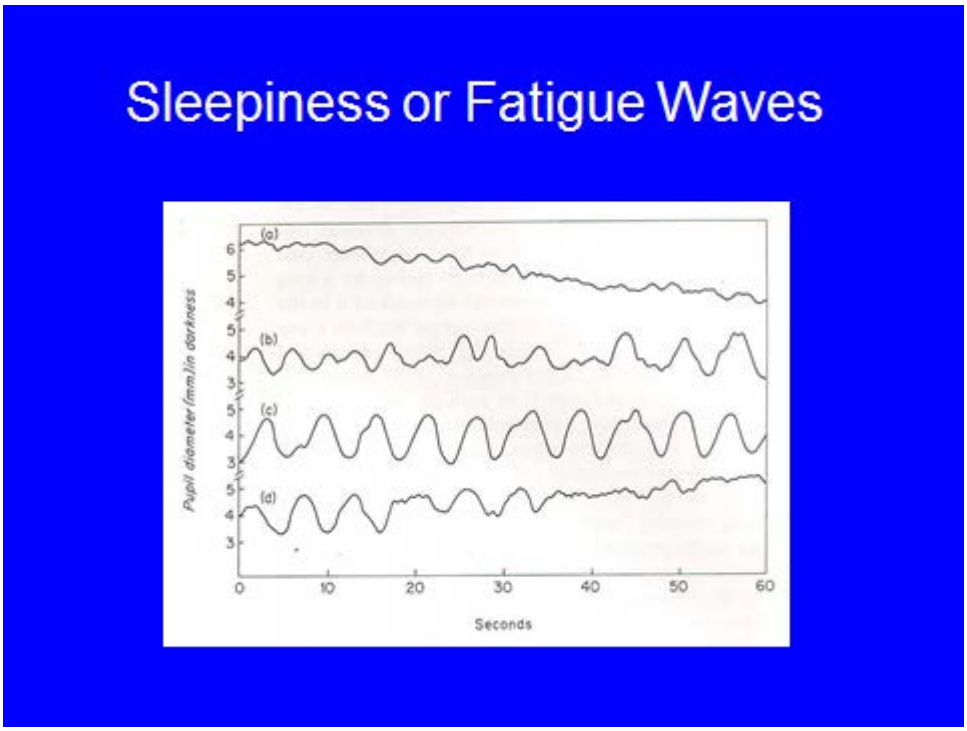


Figure 4. Pupillary oscillations in darkness – fatigue waves

1.2.7 Overview and integration of pupillary reflexes

The mechanisms that determine amplitude and form of normal pupillary reflexes (light and darkness reflex) are first iris mechanics, second reciprocal innervation with activation of the agonistic muscle and inhibition of the antagonist, and third central nervous interactions that facilitate or suppress one or the other reflex component. These mechanisms are discussed below.

A) Iris mechanics. The iris muscles have an extraordinarily wide range of movement. In human eyes the pupil can vary from more than 9 mm to less than 1 mm. Other factors being equal, it is easier for a large pupil to contract than it is for a pupil

that is already small; and conversely, an already large pupil cannot dilate as freely to a given stimulus as can a smaller pupil (baseline effect or law of initial values). The normal pupil has a linear range of movement within which both contractions and dilatations evoked by physiological stimuli move freely. Above and below this range the movements begin to lose in speed and amplitude. Corresponding to the linear and alinear ranges of pupillary movement, there are linear and alinear morphological alterations in the iris. These probably are based, to a large degree, on the geometric conditions in the iris that require compression of circularly oriented tissues around the pupil in miosis, and compression of radially oriented structures in the ciliary portion of the iris in mydriasis. The time-amplitude pattern of pupillary reflexes is therefore influenced considerably as the limits of contractility or of enlargement of the pupil are approached. The critical diameters beyond which these alinearities become significant and distort pupillary reflexes have been determined by Loewenfeld and Newsome (1971) with physiological-pharmacological studies in animals and humans. For most human subjects the lower end for linear pupillary contraction (floor) is 3-3.5 mm and the upper end for linear pupillary dilatation (ceiling) around 6 mm. These are slightly different among different individuals but always the same for a given subject at all times, and should be taken into consideration in studies of pupillary reactivity.

B) Reciprocal innervation and inhibition. Reciprocal innervation and inhibition first described by Sherrington (1911), is an important mechanism for all movements of the body. Combined innervation of the agonist muscle and inhibition of the antagonist one produce much more sensitive and rapid response than the contraction of the agonist muscle itself. The two iris muscles, sphincter and dilator, can be viewed as an agonist-antagonist pair, ruled by the law of reciprocal inhibition,

and pupillary movements with their fine gradations and accuracy, are especially well suited to demonstrate this interplay (Loewenfeld, 1993a).

When the pupil enlarges due to psychosensory stimulation, the dilator muscle is the agonist. It is activated by sympathetic impulses and, simultaneously, the parasympathetic outflow to the antagonistic sphincter muscle is reduced (Loewenfeld, 1993a). The movements called forth by these two mechanisms differ: stimulation of the cervical sympathetic nerve elicits fast, extensive enlargement of the pupil, while parasympathetic inhibition results in sluggish dilatation of much lesser amplitude; furthermore, the parasympathetic-inhibitory mechanism outlasts the stimulus for some time, while the active-sympathetic mechanism subsides promptly. In the natural reflex the fast rise of the sympathetic and the long duration of the inhibitory components are combined and produce a peak speed of the reflex which is almost twice as high than the dilatation speed produced by supramaximal stimulation of the sympathetic nerve to the dilator muscle.

There is also evidence that during the light reflex, contraction of the sphincter is accompanied by relaxation of the dilator muscle. This is supported by the behaviour of the sympathetically denervated pupil either after local instillation of sympatholytics (Loewenfeld, 1993a), in Horner's pupil (Lowenstein, 1956; Loewenfeld 1993h), or in diabetes mellitus (Smith, 1992); the normal light reflex is followed by two distinct phases of pupillary redilatation. During the pupillary contraction and the first redilatation phase the sympathectomized pupil behaves exactly as the normal one, and the pupils remain equal. This indicates that during pupillary contraction and the early phase of redilatation sympathetic impulses are absent not only on the operated side but also on the normal side. The normal sympathetic output must be suppressed until

about a second after light stimuli, to be resumed during the later phase of pupillary redilatation when the normal pupil enlarges faster than the sympathectomized one.

C) Central nervous integration. As discussed previously, pre-junctional M_2 muscarinic receptors were isolated in the dilator muscle which may serve to mediate relaxation of the dilator muscle during pupillary constriction (Fuder *et al.,* 1986; Bognar *et al.,* 1989); pre-junctional α- and β- adrenoceptors have also been isolated in the sphincter muscle (van Alpen, 1976; Sitaram *et al.,* 1983) but their physiological role is unknown; however, it has been postulated (Jaanus *et al.,* 1989) that these may serve the relaxation of the sphincter muscle during pupillary dilatation. Thus, it is possible that reciprocal inhibition, at least partially, takes place in the periphery via inhibitory sympathetic and parasympathetic terminals within the sphincter and dilator muscles, respectively.

However, the bulk of inhibition of the antagonistic muscles described previously takes place within the central nervous system; in each case the preganglionic motor neurones to the antagonistic muscle are hindered from firing at a rapid rate, so that the efferent outflow of these neurones is reduced (Loewenfeld, 1993a). Demonstration of the above statement has been done directly by recording sympathetic action potentials that reach the eye via the long ciliary nerves, during activation of the parasympathetic outflow to the sphincter muscle under the influence of light (Nisida *et al.,* 1960). During retinal exposure to light, the activity of the sympathetic neurones is inhibited proportional to the intensity of light. As discussed in the Section 1.2.2, single units have been found in the pretectal region in cats which are tonically inhibited in the presence of ambient light but fire continuously only in darkness to directly inhibit the Edinger-Westphal nucleus (Nisida *et al.,* 1959; Cavaggioni *et al.,* 1968; Smith *et al.,* 1968; Sillito and Zbrozyna, 1970). Furthermore,

during sensory or central nervous stimulation, a prompt activation of the cortex occurs with rapid firing of the agonistic sympathetic nerves to the pupillary dilator muscle (reflex dilatation) and complete cessation of firing of the antagonistic fibres to the sphincter muscle, as recorded directly in the form of action potentials of neurones in the cortex, the sympathetic chain and also the parasympathetic short ciliary nerves (Bonvallet and Zbrozyna, 1963). Loewenfeld (1958) has shown that activation of the sympathetic system alone (supramaximal stimulation of the cervical sympathetic chain) is not enough to block the light reflex; and further, parasympathetic inhibition resulting from sensory (sciatic) stimulation persists even after the sympathetic chain is cut; she also showed that stimulation of different areas of the brain can affect differentially the central sympathetic and parasympathetic systems. Thus, stimulation of the hypothalamus results in fast and extensive dilatation and simultaneous inhibition of the light reflex, but low intensity thalamic stimulation results only in abolition of the light reflex, whereas no sympathetic responses are elicited from that site (Loewenfeld, 1958). Furthermore, during cortical anaesthesia there is a reduction in electrical excitability of the cortex for all functions, the pupillary movements included. However, some cortical areas are less sensitive to anaesthesia than others (i.e. supraorbital cortex, cingulate gyrus), and their stimulation elicits mydriasis (residual and inextensive) even under deep anaesthesia (Loewenfeld, 1993a). Moreover, these residual dilatations were not diminished when the cervical sympathetic nerves were cut but they were abolished after transection of the 3rd cranial nerve. Thus, only the active sympathetic component of the dilatation was selectively abolished in anaesthesia, whereas the parasympathetic-inhibitory component was preserved. The above studies have shown that the central nervous

system plays an important and complex role in the regulation and control of pupillary movements.

To use Loewenfeld' words (Loewenfeld, 1993a) *"..a healthy iris in an intact eye, with normal afferent and efferent paths leading to and from a sound central nervous system, can have a tightly constricted pupil, or a widely dilated pupil, or anything in between; and pupillary reactions can vary from large and swift to small and sluggish from one moment to the next. Mechanical factors, characteristics of the stimuli, sensory adaptation, and integrity of nerve paths play a role. But these factors alone do not determine the features of the pupillary movements. The effectiveness of stimuli fluctuates, depending upon their emotional connotation and upon the physical and mental condition of the individual at the particular time. Obviously, the mechanisms responsible for these variations cannot reside solely in the simple reflex arcs of the midbrain and the spinal cord, for they involve changes in the level and the content of consciousness"*.

The constant interplay between cortical, diencephalic, limbic and reticular, pharmacologically distinct subsystems which all participate to various degrees must be responsible for the regulation and co-ordination of the central inhibition and facilitation of the various components of the pupillary reflexes. The modification of the pupillary reflexes by these structures and the pharmacology involved is an interesting area for research with striking a lack of systematic studies. As indcated in the title, the regulation of the autonomic control of the pupillary system and its modification by the central nervous system, forms the leading idea underlying this thesis.

1.3 METHODS FOR MEASURING PUPIL DIAMETER

In the past many different ways have been used to measure pupil diameter which are only of historical importance today. As an example, one of the simplest and crudest ways to measure pupil diameter involved the use of a plain millimetre rule, held between the observer and the subject at the level of the subjects' pupils, whereas a slightly more sophisticated but still low accuracy technique (unable to detect changes of less than 0.3 mm) was to compare the aperture of the pupil with a series of circular disks of graduated size (Hebb's pupillometer in 1900). The techniques used today are:

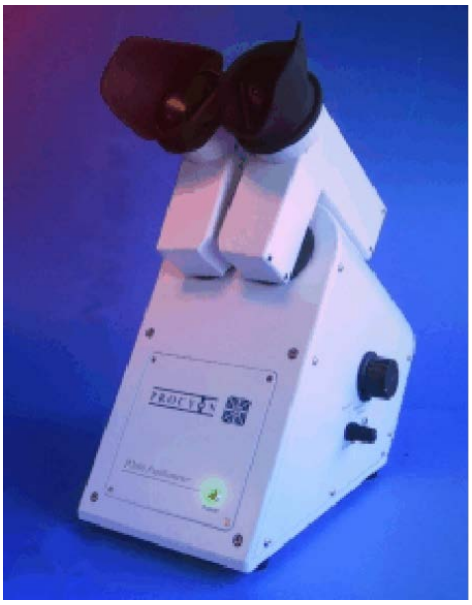
A) The photographic technique, which consists of taking photographs of the pupil using a camera that is mounted at a fixed distance from the subject's eyes. The illumination of the monochrome photographs is provided by a flash of less than 1 ms, so that the alteration in pupil size does not occur before the image has been stored on the film (Loewenfeld, 1963). Following development and fixing, the photographic negatives are projected onto a white screen and pupil diameter can be measured using a millimetre ruler. The sensitivity of this technique is quite high insofar as small changes in pupil diameter can be detected, the magnitude of the detectable change depending on the magnification factor of the photographic negatives. A further refinement in this technique is represented by the use of infra-red light source and infra-red sensitive film, which allow photographs of the pupils to be taken in darkness (Lowenstein and Loewenfeld, 1958).

B) Electronic infra-red binocular TV pupillometry which is by far the most sophisticated, accurate and sensitive technique (Loewenfeld, 1993k). The apparatus provides a direct and continuous recording of pupil size by means of infra-red

scanning at high frequency, the technique being dependent upon measurement of the amount of infra-red light reflected from the iris, which is a function of pupil size. For single measurements of pupil diameter, this technique offers little advantage over photography except for the fast data retrieval and analysis (see below). Even for such a purpose, however, due to its higher sensitivity, it allows assessment of pupil size more accurately in subjects with dark irides that cannot be photographed with precision. Furthermore, it provides instantaneous feedback of the quality of the measurement through a TV display (unlike all other techniques). Finally, and most importantly, its ability to provide a continuous running record of pupil size changes (in ambient illumination as well as in darkness) makes it an ideally suited technique for studying pupillary physiology [oscillations in light ("pupillary unrest") and darkness ("fatigue waves") as well as the kinetics of pupillary responses to sudden changes in illumination (darkness and light pupillary reflexes)]. The entire unit is housed in a specially designed quiet, insulated, dark room, with minimal light reflections during recording. The subject sits comfortably at a table upon which the cameras and the LEDs are mounted, and rests his head on an adjustable headrest. The pupil monitors and the recorders are ideally placed in a separate room or separated with a black cloth to minimize light and noise distraction. A spot of very dim light is positioned, directly opposite the subject's eyes, as a point of fixation, to prevent his eyes to wander out of camera focus. Although research with the pupil as a psychophysiological and psychopharmacological test-system in humans has been fruitful and active, it was limited to highly specialised laboratories. The bulk and the sophistication of the equipment, its prohibiting cost and the complex technique, make it impossible for use in smaller laboratories or in the clinical setting.

In the last two decades, infrared TV-based pupillometers, providing both analogue and digital outputs, have permitted direct interfacing with and control by laboratory microcomputers and even personal computers. Pupil diameter can now be measured with an accuracy of more than 0.025 mm at rates of up to 60 times per second. When data are averaged across multiple trials, background noise can be reduced and changes of less than 0.01 mm can be detected. As an example of the technological developments, it has been calculated that a research project investigating the reaction of the pupil to light, that required seven months of data collection and analysis in 1962, could be performed today within a single afternoon (Steinhauer, 1992).

In our laboratory we use a compact, desktop, binocular infrared television pupillometer (PROCYON 2000D). The entire system of light stimulation delivery and pupillary response recording is fully automated by means of a microcomputer. The stimulus intensity, duration and frequency are controlled by an appropriate and flexible "in-house" software package. The pupillary responses of both eyes are recorded on floppy disk and also plotted in real time on the computer screen.



The PROCYON 2000D pupillometer

1.4 PUPILLOMETRY AS A RESEARCH TOOL IN PSYCHO-PHYSIOLOGY

Compared to other autonomically innervated tissues, the human iris possesses particular characteristics, such as sharp structural delineation, precision of nervous control and thus responsiveness, which make it an ideal physiological system for basic and applied research. The pupil is the only autonomically innervated tissue which (by means of pupillometry) can be measured directly, without signal transformations, another important advantage that makes interpretation and scoring straightforward.

Interest in the pupil size as a psychophysiological variable was initiated by Hess (Hess and Polt, 1960) who claimed that positive emotions dilate and negative ones constrict the pupil. This notion soon was proven to be wrong but together with the polemic it also generated a great interest in pupillary studies which have centred largely on the pupillary dilatation known as “task-evoked” pupillary dilatation (TEPD). Changes in central nervous system activity that are systematically correlated to cognitive processing may be extracted from the raw pupillary record by performing time-locked averaging with respect to critical events in an information processing task. A TEPD bears the same relation to the pupillary record from which it is derived as does an event-related potential to spontaneous EEG activity. With averaging, short-latency (100-200 msec onset latency) phasic task-evoked dilatations appear, which terminate rapidly following the completion of processing (micropupillography). The magnitude of the TEPD appears to be a function of processing load or “mental effort” required to perform the cognitive task. The TEPD fulfils all the criteria proposed by Kahneman for any physiological parameter to be a reliable index of processing load

(Kahneman, 1973): a) it is sensitive to within-task variations in task demands produced by changes in task parameters, b) it reflects between-task differences in processing load elicited by qualitatively different cognitive operations, c) it captures between-individual differences in processing load as individuals of different abilities perform a fixed set of cognitive operations. Mental effort has been manipulated by a number of means, from arithmetic problems of varying difficulty (often a typical "mental stress" paradigm), to memory and language-based tasks (see Steinhauer and Hakerem, 1992; Beatty, 1982; Loewenfeld 1993m for reviews).

The conclusion from the very well controlled and meticulous work done on the TEPD so far is that these momentary pupillary dilatations are an index of overall processing load even when the composition of processing resources differs between tasks. The viewing of TEPD as a measure of the aggregate task-induced utilisation of multiple processing resources has been described as "analogue to an electric meter that measures the total amperage required at a given moment by the various electrical devices in a house", or "the use of a general physiological measure such as oxygen uptake as an indicator of the aggregate metabolic demands of a set of functionally distinct organs" (Beatty, 1982).

Pupillary dilatation can also be evoked by "guessing" or "uncertainty" tasks in which there is little effort employed in recognising a stimulus, but for which the "informational value" of the stimulus is high. Typically, simple click patterns show a quick habituation when the subject knows what each subsequent stimulus will be ("certainty" condition), but a clear dilatation occurs to the clicks when the subject is asked to guess ("uncertainty" condition) what stimulus pattern will occur (Hakerem *et al.*, 1974). The dilatation does not depend on stimulus designation, as it can occur in the absence of a stimulus if this absence conveys information, i.e. when a stimulus

was expected or if the absence signifies increased monetary payoff (emitted dilatation). The pupillary dilatation evoked by guessing, novelty and uncertainty is accompanied and correlated by increased amplitude of the P300 component of the event-related potentials (Tueting, *et al.*, 1970; Friedman *et al.*, 1973). All sorts of sensory stimuli can be employed in these tasks, even light flashes when their energy is so low (near visual threshold light flashes) that the normally induced excitation of the Edinger-Westphal nucleus is superseded by the supranuclear inhibition of this nucleus evoked by CNS structures presumably activated by the task (Hakerem and Sutton, 1966).

1.5 ANXIETY - PSYCHOLOGICAL STRESSORS AND THE PUPIL

When an organism perceives an external potentially tissue damaging or life threatening situation (threat) it reacts with a host of adaptive behavioural and physiological responses known as the "fight-flight" response (Cannon, 1929, 1931). The central role of the sympathetic nervous system in the formation of the fight-flight response was recognised as early as at the beginning of the century. Today there is a vast literature accumulated about the activation and the participation of the sympathetic nervous system in anxiety (Tyrer, 1976) which is basically centered around the galvanic skin reaction and cardiovascular parameters. Given that the pupil is a purely autonomically innervated tissue, and thus, expected to participate in any condition where the sympathetic nervous system is activated, it is surprising that it has received so little attention. Indeed, the TEPD (see previous section) has been viewed only in the context of purely cognitive processes and very little attention, if any, was given to emotional factors (see below).

Two examples of laboratory research in sympathetic activation are the exposure to high ambient temperature and the cold pressor test; these have been used extensively to elicit changes in autonomic functions (the pupil included) and will be discussed briefly.

Benjamin (1953) and McIntyre *et al.* (1968) found that high ambient temperature increased the sensitivity of the sweat glands to pilocarpine (a cholinoceptor stimulant), and more recently Van den Broek *et al.* (1984) and Banjar *et al.* (1987) replicated these results using carbachol. The pattern of effect produced by high ambient temperature was the same as that seen in anxious patients (Maple *et al.*, 1982; Buceta *et al.*, 1985). Furthermore, Banjar *et al.* (1987) found that the

hyperresponsiveness of the sweat glands to carbachol could be blocked by the classic anxiolytic drug diazepam. Other studies showed that physiological tremor (a sympathetically mediated physiological variable) was increased (Fleming *et al.,* 1991) and 75% recovery time of the pupillary light reflex (seen as an index of sympathetic activity-see section 1.2.1 "Recovery time" and Morley *et al.,* 1991) was reduced (Leung *et al.,* 1992) following exposure to a hot environment. These studies showed that exposure to high ambient temperature may mimic, at least in part, the autonomic changes seen in anxiety states.

The cold pressor test has been widely used, among others, to study the effects of acute stress (Mills and Farrow, 1981). The local application of a cold stimulus increases striated muscle sympathetic nerve activity, systolic blood pressure, and heart rate (Seals *et al.,* 1990), and has been conceptualized as an aversive stimulus that evokes the characteristic pattern of the alerting or defence response (Marriott *et al.,* 1990). This pattern of reactions, however, was not found to be different in patients with panic disorder compared to normal subjects (Grunhaus *et al.,* 1983). Furthermore, the cold pressor test failed to decrease the amplitude of the light reflex (Vidalaki, 1992) which was found to be reduced in a group of anxious patients (Bakes *et al.,* 1990). In the study of Vidalaki. (1992) however, the cold pressor test shortened the 75% recovery time of the light reflex (the length of which is inversely related to sympathetic activity), consistent with an enhancement of sympathetic activity (Morley *et al.,* 1991). Since 75% recovery time of the light reflex was also reduced (Leung *et al.,* 1992) following exposure to a hot environment, and in the absence of any evidence that exposure to high ambient temperature and the cold pressor test elicit subjective anxiety, it seems that both of them are not "realistic" models of clinical

anxiety states, but only sympathetic activators, thus modelling only some aspects of the physiological concomitants of anxiety.

Other models include psychological stressors such as the naturalistic exposure to phobic stimuli (i.e. spiders and snakes in phobic patients) (Fagestrom *et al.,* 1985), public speaking (McKinney *et al.,* 1983) etc. which have been used with success in eliciting genuine anxiety. These, however, are not practical from a methodological point of view and some may be particularly incompatible with pupillometry [e.g. viewing of aversive slides (Lang *et al.,* 1990) with inevitable differences in brightness].

In our attempt to investigate the role of an emotional, limbic contribution to the autonomic regulation of the pupil, we have chosen to use the threat of an electric shock and the actual electric shock delivery, as the main stressors. The reason for this choice is that this is a simple, practical, quantifiable and reproducible method, thus allowing maximum confidence in between-subject and between-experiment comparisons. The threat of a shock has much more face validity as an anxiety-provoking condition, and has been widely and effectively used over decades in animal models of anxiety (see section 1.6) and human psychophysiology (Deane, 1969; Reiman *et al.,* 1989). The face validity of the threat of a shock in producing conditioned fear and anticipatory anxiety has been effectively shown in the fear-potentiated startle paradigm in both animals and recently in humans (see Davis *et al.,* 1992, 1993 for reviews; see also section 1.7). In this paradigm the amygdala has been proven to play a critical role (Hitchkock and Davis, 1986, 1991; Davis *et al.,* 1992, 1993), consistent with this nucleus' central role in organising fear-related behaviours and physiological responses (Mishkin and Aggleton, 1981; Kapp *et al.,* 1984; Sarter and Markovwsitsch, 1985; Davis, 1992, 1993). One of the target areas of amygdala

activation is the hypothalamus (Davis, 1992) which elicits changes in the autonomic nervous system consistent with the fight-flight response. Reflex pupillary dilatation has been demonstrated in animals by stimulation of the amygdala (Koikegami and Yoshiba, 1953). Stimulation of the dorsomedial amygdala in cats results in pupillary dilatation, and lesions in the central grey matter markedly reduced pupil dilatation produced by such stimulation (de Molina and Hunsberger, 1962), suggesting that the central grey matter (another limbic structure involved in fear and anxiety) provides a contributory pathway for the pupillary dilatation.

In the experiments described in this thesis subjects' experience of the threat of an electric shock (cognitive and emotional) was recorded with appropriate standardized visual analogue scales (Bond and Lader, 1974) and was correlated with objective pupillary changes. The effects of threat were tested on baseline pupil diameter, and the pupillary light reflex, as opposed to the near-vision reaction (see section 1.2.4) or the reflex dilatation (see section 1.2.3). The pupillary light reflex is an easily and reliably elicited, recorded, quantified, and reproduced simple reflex, occurring in a highly controlled situation, thus allowing for between subjects and experiments comparisons.

It is an intriguing possibility that the TEPD discussed in the previous section can be attributed, at least partly, to the operation of structures mediating anxiety. There is some debate as to whether the TEPD can be viewed only in the context of purely cognitive processes. Normal subjects had increased TEPD when they were threatened with a shock (Polt *et al.*, 1970), however, this was interpreted only in terms of increased recruitment of "mental effort" and a more direct terminology involving the operation of CNS structures mediating anxiety was avoided. This could be less surprising if we consider the historical context of the times, when the pupil was

viewed as an accurate index of cognitive processing, per se. Whereas most authors insisted on a pure "cognitive" explanation of TEPD, others have argued that a more "emotional" explanation was needed. Thus, the possibility was then considered that subjects had always some anxiety associated with apprehension about evaluation on the part of the experimenter, which contributed in some of the amplitude of the TEPD, at least in tasks where an overt response about the completion of the task was needed (Bernick *et al.,* 1968). However, only three experiments so far have dealt with the involvement of "nonprocessing" factors such as motivational or other emotional factors in the amplitude and shape of the TEPD.

In this context, it was found that differences in monetary payoff improved the performance in a learning task as well as the amplitude of the TEPD (Kahneman *et al.,* 1968), and a more recent well controlled study (Steinhauer, 1982) showed that the manipulation of motivational factors influences the characteristics of pupillary motility, especially the shape of the recovery slope of the TEPD, and simultaneously recorded event-related potentials during guessing and betting tasks, indicating that the pupillary TEPD reflects cognitive and *affective* processes. There was only one experiment that examined the involvement of emotional factors in the most straightforward way, the study of Simpson *et al.* (1971) who indeed found increased amplitude of TEPD in patients with "audience anxiety" (today diagnosed as "social phobia"). This reflects both the lack and the value of pupillary experiments in psychiatric populations.

The debate for a purely cognitive vs. purely emotional-motivational explanation of the amplitude and shape of the TEPD is unproductive, especially if it does not lead to the design of experiments to test these hypotheses. Clearly, direct

connections among limbic regions, thalamus, and hypothalamus are well established, and the TEPD may well involve a limbic component.

1.6 BEHAVIOURAL MODELS OF ANXIETY IN ANIMALS

Since their introduction in the early 1960s, the benzodiazepine drugs, such as chlorodiazepoxide (the first clinically available benzodiazepine) and diazepam (the best studied and most representative benzodiazepine) have come to dominate the drug treatment of anxiety states, being the most widely prescribed of all psychoactive agents during the past 35 years. They also have a broad range of actions besides anxiolysis, such as sedation, muscle relaxation, and anticonvulsant properties and they are used to treat insomnia, epilepsy and disorders of muscle tone (Tyrer and Murphy, 1987).

Being the only drug treatment specifically for anxiety states for years, the pharmacology of benzodiazepines and their behavioural profiles in animal models of anxiety became the standard against which putative anxiolytics are tested. Furthermore, benzodiazepines became the standard drug to test potential new models of anxiety. Behavioural studies in animals have also contributed to the current knowledge of the therapeutic mechanisms underlying the therapeutic effects of benzodiazepines (File and Pellow, 1987). The earliest studies were carried out using the "conflict" test of anxiety (Geller and Seifter, 1960). In this test operant lever pressing is reinforced with food on an intermittent schedule (usually a variable-interval schedule) of reinforcement. Occasionally a discriminative stimulus (e.g. a light) is presented for periods of one or two minutes; during these periods responding is not reinforced with food but instead it is punished with electric shock. Benzodiazepines have been found to "release" responding from punishment-induced suppression, without affecting unpunished performance (Geller and Seifter, 1960). In the more recent paradigm of "elevated plus maze" (Pellow *et al.*, 1985) it was shown that rats avoid entering the open arms of a four-arm maze raised above floor level. In

the maze two arms are simple planks (open, “fear-provoking” environment) and two are enclosed with walls (protected, “safe” environment). Benzodiazepines increase the total amount of time spent on the open arms and reduce freezing behaviour. In the social interaction paradigm, the amount of time spent in sniffing, grooming and other interactive behaviours in which two rats engage when they are in the same chamber (File and Hide, 1978, 1979). The amount of interaction is reduced in anxiety-provoking situations (i.e. unfamiliar test chamber, bright light etc.) Benzodiazepines increase social interaction under these “anxiogenic” conditions (File and Hide, 1978, 1979; File, 1980). In the light-dark crossing paradigm, bright light (a naturally aversive condition for a rat) will prevent rats from crossing from a dark area to brightly lit area of a two compartment box (Green and Hodges, 1991). Benzodiazepines increase crossings into, and time spent in, the lit compartment. In the potentiated startle reflex paradigm (Davis, 1992) the rat’s natural startle response to a sudden loud noise is increased by the simultaneous presentation of a light flash which the rat has previously learnt to be associated with mild electric footshock. Benzodiazepines block this potentiation of the startle reflex.

All animal models of anxiety have in common the use of induced fear as an analogy of human anxiety. Models that respond to benzodiazepines by an appropriate behavioural change are said to be more valid than those that do not. However, direct comparisons with human anxiety with similar methods in humans are not possible, and these models’ relationship to human psychopathology of anxiety is not obvious. All these models suffer from anthropomorphic assumptions inherent to their interpretation which can limit the utility of this approach for studying the neural substrates of human psychopathology. Moreover, they all involve spontaneous behaviour in a relatively uncontrolled environment, making it impossible to

disentangle, for instance, perceptual and motivational contributions to observed drug effects. The types of behaviours involved in these paradigms are subject to a host of variables such as baseline activity, age, gender, strain, handling, apparatus dimensions, measurement technique, lighting. An exception to most of the above confounding factors is the fear-potentiated startle reflex, which presents with important methodological advantages. Firstly, the study of fear or anxiety is reduced to the study of a simple reflex, elicited in a highly controlled situation, with fewer opportunities for confounding variables to influence the results. Secondly, it is known that when a drug is administered for evaluation of its effects on any behavioural parameter, problems may arise, caused by between subject variability in this behaviour. With fear-potentiated startle reflex, one measures the within-subjects difference in reflex patterns in the presence versus the absence of the fear-provoking situation, circumventing the above problem. Thirdly, modification of the startle reflex by fear does not involve any obvious operant, thus drug-induced effects that might be expected to alter operant performance are circumvented. Fourthly, there are no shocks delivered in the testing sessions thus individual differences in shock sensitivity are overcome and what is more, effects of drugs cannot be attributed to drug-induced changes in the sensitivity to the shock. Fifthly, it is the only model to respond reliably to both benzodiazepines and buspirone, which is a clinically effective anxiolytic but does not have a consistent anxiolytic profile in conventional animal models of anxiety. Finally, drugs with no real clinical anxiolysis (e.g. a variety of treatments that alter serotonin transmission like cinanserin, cyproheptadine, ipsapirone, 8-OHDPAT) present with an anxiolytic profile in most conventional animal models probably as a result of response disinhibition (Shopsin *et al.*, 1976), whereas they have no

anxiolytic effect in the fear-potentiated startle paradigm, consistent with their clinical profile (Davis, 1988).

1.7 BEHAVIOURAL MODELS OF ANXIETY IN HUMANS

The fear-potentiated startle reflex

The human eyeblink component of the startle response is found elevated when subjects anticipate a shock (Grillon *et al.,* 1991; Hamm *et al.,* 1993), and the anxiogenic yochimbine has been found to potentiate the acoustic startle in humans (Morgan *et al.,* 1993), whereas the anxiolytic substance alcohol was found to decrease it (Grillon *et al.,* 1994). The fear-potentiated startle reflex is the only paradigm so far, that can be applied to humans as well as to animals, thus allowing direct comparisons with data obtained in experimental animals. After exclusion of prepulse inhibition studies of the startle reflex (a schizophrenia model), we found over 4250 studies on startle reflex in PubMed in English-speaking journals and a search using “startle” and “anxiety” as the key words revealed over 440 human studies since 1991, cross-fertilized by over 750 animal studies in the same period. These human studies have developed several startle reflex methodologies which have been applied to the examination of emotional and motivational states and various factors in humans that are of interest in the studies of emotional disorders. The startle reflex is a non-invasive translational tool of research that bridges the gap between animal and human investigations. Startle is used to study fear and anxiety, fear learning, affective disturbances, sensitization, motivational states, homeostasis and to contrast affective states and emotional processing across diagnostic groups (for reviews see Grillon 2002; Grillon and Baas 2003).

The fear inhibited light reflex

It has been shown shown that the amplitude of the pupillary light reflex is reduced when the light stimulus is presented in the presence of a cue (e.g. a tone) that has been

previously associated with an electric shock (Bitsios et al 1996). Importantly, these changes in pupillary activity are accompanied by increases in subjective alertness and anxiety. This phenomenon was termed “fear-inhibited light reflex” and the threat-induced decrease in light reflex response amplitude was proposed as a potential laboratory model for human anxiety (Bitsios et al., 1996). The paradigm of the fear-inhibited light reflex is methodologically and conceptually similar to the paradigm of the fear-potentiated startle reflex. In both tests the conditioned response is considered to be a state of fear. The conditioned fear in humans can thus be operationally defined as the augmentation of the startle reflex, or the inhibition of the light reflex, in the presence of a cue associated with a shock. Indeed, during simultaneous recording of the startle and the light reflexes, the cue signalling the possibility of the delivery of a shock modifies both reflexes in the predicted direction (Bitsios et al., 1999). Moreover, the fear-inhibited light reflex, in common with the fear-potentiated startle reflex, is dose-dependently sensitive to the anxiolytic drug diazepam (Bitsios et al., 1998, 1999), suggesting that a common mechanism may mediate the effect of fear in the case of both reflex paradigms (Bitsios *e*t al., 1999).

The inhibition of LRA by threat is best explained by the fear with which the threat of a shock is presumably associated, rather than with the anticipation of the shock per se. Indeed, anticipation of an alerting but emotionally neutral event (acoustic tone) did not reduce the light reflex amplitude, although it caused a small increase in pupil diameter, consistent with a psychophysiological dissociation between the two measures (Bitsios et al 2004).

In keeping with the above, a population study showed that the greatest within-subject decreases in light reflex amplitude (but not the within-subject increases in

pupil diameter) occurred in subjects with the greatest score in Spielberger's State Anxiety Inventory (Bitsios et al 2002).

Following the administration of the threat-signalling cue, apart from a reduction in light reflex amplitude, there is also an increase in initial pupil diameter (IPD), a sympathetically mediated response (Bitsios et al., 1996). Despite the close temporal proximity of the two pupillary changes, there is mounting evidence for dissociation between the two responses to threat:

- Anxious patients have similar IPDs but smaller light reflex amplitudes across a range of light intensities, compared to sex- and age-matched healthy controls (Bakes et al 1990)

- The threat-induced increase in IPD and the threat-induced reduction in light reflex response amplitude do not systematically covary (Bitsios et al 1996)

- Only the light reflex response amplitude correlates with subjective and state anxiety (Bitsios et al 1996, 2002).

- Both pupillary responses also appear when subjects are asked to perform an attention task requiring effortful processing (Steinhauer et al 2000). However, that a very easy, non-anxiety provoking, attention task increased only the IPD but did not affect the light reflex response amplitude (Steinhauer et al 2000, Bitsios et al 2004).

- The most compelling evidence perhaps up to date comes from drug studies. The anxiolytic drug diazepam reduced the effect of threat on the light reflex response amplitude but did not affect the threat-induced increase in IPD (Bitsios et al 1998, 1999).

- As opposed to diazepam, the sedative/sympatholytic drug clonidine, consistent with its clinically ambiguous anxiolytic profile, did not have such a threat-specific and

selective effect. Along with reduction in subjective alertness (but not anxiety), clonidine antagonised both the threat-induced increase in pupil diameter and the threat-induced decrease in light reflex amplitude, but these effects were not threat-specific, since they also occurred in the "safe" condition (Bitsios et al 1998a).

IPD therefore increases also in response to the attentional properties of a stimulus (Bitsios et al 2004), or when a mental task becomes difficult (and thus possibly stressful) (Steinhauer 2000), or overtly stressful (Simpson and Moloy 1971). The above taken together suggest that the increase in IPD is non-specifically sensitive to the cumulative alerting/attentional/anxiogenic properties of a stimulus or a task, while the reduction in light reflex amplitude is a more specific correlate of the anxiogenic properties of a stimulus. We believe that the two pupillary responses to threat reflect the operation of separate neural mechanisms.

The fear-inhibited light reflex appears to be a valid laboratory model of human anxiety, with face, predictive and even construct validity which derives from the model's plausible neurobiological foundations. The FILR has yielded comparable results to the human and animal fear-potentiated startle reflex, lending support to the contention that they share a common mechanism (Bitsios et al 1999).

All animal models of anxiety have in common the use of induced fear as an analogy of human anxiety. However, direct comparisons with human anxiety with similar methods in humans are not possible, and these models suffer from anthropomorphic assumptions inherent to their interpretation, which can limit the utility of this approach for studying the neural substrates of human psychopathology. An exception to this rule are the fear-potentiated startle reflex and the fear-inhibited light reflex, paradigms with high face, predictive and even construct validity.

METHODOLOGICAL ADVANTAGES OF THE FILR

- Anxiety is not measured by a change in baseline activity; it is measured by a change in a simple reflex which is reliably elicited, highly controlled, easily quantified and recorded in the laboratory.
- The separate activity of the sympathetic and parasympathetic systems can be seen and studied on the pupillogram.
- Averaging three to five signals are enough to elicit a reliable response. There is no need for averaging hundreds of trials as in the case of event-related potentials.
- The pupil is the only autonomically innervated tissue, which, by means of pupillometry, can be measured directly and with great accuracy. As opposed to skin conductance or heart rate variability, signal transformations and misleading, arbitrary scoring criteria, are entirely redundant. This makes interpretation and scoring reliable and straightforward.
- No voluntary response is required from the subjects, unlike in other tests used in clinical pharmacology (e.g. reaction time). Thus, drug-induced effects (i.e. sedation, motivational changes etc.) that might alter subjective reports, voluntary responses or performance are circumvented.
- No shocks are used during testing, since all the observed effects are recorded prior to shock delivery. Thus, drug effects observed in testing cannot be attributed to drug-induced changes in sensitivity to shock.
- One can measure within-subject differences in the presence versus the absence of the fear-provoking situation, circumventing problems caused by between-subject variability.

IMPLICATIONS OF THE FILR

This paradigm may be very useful in

- Testing the anxiolytic properties of novel, putative anxiolytic drugs.
- General arousal and anxious aversion are reflected separately, in the threat-induced increase in pupil diameter and the threat-induced decrease in response amplitude respectively. Thus it is predicted that this paradigm may distinguish between sedative and anxiolytic drugs.
- Exploring neurotransmission mediating normal and pathological anxiety, human conditioning and contextual learning, using pharmacological prompts with known receptor properties.

2. AIM OF THE PRESENT THESIS

This thesis capitalizes on previous research with the pupil and the inhibition of light reflex by threat, as delineated in the previous chapter. We examined if the serotonergic drugs ketanserin and buspirone and the beta~~alpha~~ adrenergic receptor blocker propranolol reduce arousal levels using the resting pupil diameter in darkness as our main test system. Then we examined if ketanserin and buspirone have an anxiolytic profile in the fear-inhibited light reflex paradigm, a model that taps on the level of acute fear from an imminent threat which is relevant to phobic/panic anxiety disorders. Progress in the understanding of normal and pathological anxiety depends on our ability to develop models for both phasic i.e. panic and phobias as well as sustained fear and anxiety i.e. generalized anxiety disorder, agoraphobia. Because the fear-inhibited light reflex protocol taps on acute, phasic anxiety, we developed a new psychophysiological protocol that explores the time course of anticipatory fear as reflected in light reflex inhibition by threat and we validated this against the startle potentiation by threat. The aim was to tap more on fear onset and offset, two processes that are relevant to sustained anxiety which is more relevant to non-phobic, generalized anxiety disorders.

3. EXPERIMENTAL PART

3.1 EXPERIMENTS 1, 2 AND 3: Manipulation of 5HT2, 5HT1a and beta receptors by Ketanserin, Buspirone and Propranolol respectively: effects on the pupil and the pupillary light reflex

Introduction

It has long been known that any decrease in arousal is accompanied by a decrease in pupil diameter (Loewenfeld 1993), and assessment of pupil diameter is routinely used by anaesthetists when gauging the depth of anaesthesia (Aitkenhead et al. 2001). Pupil size in darkness has been successfully used as a single physiological measure of arousal in patients suffering from excessive daytime sleepiness (EDS) due to obstructive sleep apnea (Bitsios et al. 2006); compared to age- and sex-matched controls, the sleepy patients showed smaller pupil size, which correlated with objective indexes of apnea severity and subjective measures of sleepiness, the differences becoming more apparent during the afternoon circadian nadir (Bitsios et al. 2006). Moreover, pupil size was sensitive to the alerting effects of modafinil in patients with EDS as a result of obstructive sleep apnea (Nikolaou et al. 2008). Recent evidence points to the importance of metabolic factors, hypertension and depression in the aetiology of EDS (Bixler et al. 2005), and treatment for these conditions is not uncommon among these patients. If monitoring of resting pupil size is to be more regularly incorporated in future studies as a clinical tool for the objective assessment of EDS, it would be important to understand the pupillary effects of the various drugs prescribed for these patients. In this study, we sought to determine the effects of single doses of ketanserin, buspirone and propranolol on pupillary behaviour of healthy subjects in three separate experiments. If these drugs alter pupil size, they

might interfere with the pupillometric determination of alertness in patients suffering from EDS (Bitsios et al. 2006; Nikolaou et al. 2008) if the above drugs are prescribed for treatment of comorbid hypertension or mood disorders. Ketanserin is an anti-hypertensive agent with sympatholytic effects, via central 5HT2-mediated modulation of the sympathetic system (Cameron et al. 1987). It is also an α1 adrenergic and histamine receptor antagonist (Dollery 1999). Ketanserin 20 mg has been reported to reduce critical flicker fusion frequency (CFFF; Graham et al. 2002) and sustained attention (Wingen et al. 2007) and it is considered a sedative drug (Dollery 1999) although clinically, its effects on arousal may not be as profound (Herrmann and Baumgartner 1986). Propranolol is another widely used antihypertensive agent with sympatholytic properties via peripheral beta adrenoceptor blockade on the vascular bed. It can also behave as a 5HT1a antagonist and a 5HT1b agonist in the rat cortex (Pierson et al. 1989). Propranolol is not considered a sedative drug (Currie et al. 1988), and there are mixed reports regarding its ability to reduce arousal; impaired psychomotor performance has been reported after single doses (Landauer et al. 1979; Salem and McDevitt 1984), but other studies have failed to show such effects (Currie et al. 1988; Harmer et al. 2001; Ogle et al. 1976; Tyrer and Lader 1974). Buspirone is a non-sedative anxiolytic and a partial agonist at the 5-HT1a receptor (Andrade and Nicoll 1987), with some affinity for the dopamine D2 receptor (Jann 1988; Peroutka 1985; Riblet et al. 1982). Buspirone has a dose-dependent miotic effect in healthy human subjects (Fanciullacci et al. 1995; Phillips et al. 1999), but the mechanism remains unclear (Phillips et al. 1999). A reduction in pupil size by a drug may be due to the reduction of the sympathetic input to the iris, increase of the parasympathetic input to the iris or both. In order to examine the relative contributions of the sympathetic and the parasympathetic systems in a putative effect of these drugs on

pupil size, we examined their effect on the pupillary light reflex. The pupillary light reflex may help to elucidate the effects of a drug on the sympathetic and parasympathetic inputs to the iris, since the time course of the light reflex response is determined by the successive activation of the parasympathetic and sympathetic inputs; the amplitude reflects activation of the midbrain parasympathetic Edinger–Westphal nucleus (Barbur 2004; Gamlin et al. 1997), while the recovery time reflects mainly sympathetic activation, which resumes at the end of the light stimulus and recovers the pupil to its original levels (Bitsios et al. 1998a; Loewenfeld 1999; Smith and Smith 1999). However, because recovery time also depends, by definition, on initial (baseline) pupil diameter (Loewenfeld 1993) and is, in the experience of our lab, susceptible to blinks, we measured only latency and amplitude of the light reflex. Finally, we also examined the effects of these drugs on other measures of arousal such as CFFF (Smith and Misiak 1976) and visual analogue scales (VAS) (Bond and Lader 1974) and cardiovascular indices of autonomic function such as heart rate and blood pressure.

Materials and methods

Subjects

In all three experiments, subjects were between 18 and 30 years old, with a body mass index (BMI) in the normal range. Inclusion criteria included written informed consent, absence of personal history of head trauma, medical and neurological conditions or use of prescribed and recreational drugs and absence of personal or family (up to second-degree relatives) history of Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition Axis I disorders. All participants underwent physical and psychiatric assessment using the Mini-International Neuropsychiatric

Interview (Sheehan et al. 1998), an ophthalmological examination and a urine drug screen test. All subjects were regular caffeine (two to three cups of coffee per day on average) and occasional alcohol consumers. Subjects' demographic characteristics in experiments I (ketanserin), II (buspirone) and III (propranolol) are shown in Table 1. The study was approved by the University of Crete Ethics Committee.

Design and drugs

Ketanserin, buspirone and propranolol were administered in a placebo-controlled, within-subject design in three separate experiments, using separate groups of subjects but identical experimental procedures. In each experiment, subjects participated in two weekly sessions (returning to the laboratory at the same time each week for each session). Subjects were allocated to treatments and sessions according to a double-blind, balanced, crossover design. Ketanserin 20 mg, buspirone 10 mg, propranolol 40 mg and placebo were prepared in identical capsules and administered orally. The choice of dose of each drug was based on centrally bioactive doses reported in published studies [ketanserin (Graham et al. 2002), buspirone (Phillips et al. 1999) and propranolol (Grillon et al. 2004)].

Tests and apparatus

Resting pupil diameter

A binocular infrared video pupillometer (Procyon P2000D, Procyon, London, UK; sampling rate, 25 Hz; spatial resolution, >0.05 mm; accuracy, >±3%) was used to monitor RPD in darkness. Our methodology of recording pupil diameter [5-min Pupillary Alertness Test (5-min PAT)] has been described in detail previously (Bitsios et al. 2006, Nikolaou et al. 2008). Pupil diameter was sampled for 15 consecutive 20-s

periods, and thus, the total monitoring time was 300 s. The outcome measures were the average RPDs for each one of the 15 20-s periods and the collapsed RPD for the entire 300-s recording. Data were stored for off-line cleaning from spontaneous blinks, scoring and statistical analysis.

The light reflex

The light reflex was elicited and recorded in darkness, following testing with the 5-min PAT. The stimuli were light flashes of 200-ms duration delivered via a light emitting diode, presented to the subject's right and left eye in an alternating fashion, as a white disc of 8° diameter, providing 'full retinal field' light stimulation (at four levels of stimulus luminance, 0.35, 5, 50 and 140 cd m−2), while the non-stimulated eye was fixating a target dot projected at a distance of approximately 10 m. Each one of the 4 levels of stimulus luminance was presented in a block of three stimuli, the average of which was the response for that luminance level. The inter-stimulus interval within blocks was fixed at 7 s. Therefore, the total time for the elicitation and recording of the light reflex was 80 s. Stimulus presentation was computer controlled, and pupillary measures were digitised and stored for off-line analysis. Using the automated manufacturers' software, the parameters studied were light reflex latency (i.e. the time elapsed from onset of the light flash until the onset of a pupillary response) and light reflex response amplitude [i.e. the difference between the baseline (defined as the mean pupil diameter recorded over 500 ms before the onset of the light stimulus) and the diameter reached at the trough of the pupillary response to the light stimulus].

Critical flicker fusion frequency

The Leeds Psychomotor Tester (Psychopharma, Surrey, UK) was used to collect CFFF measurements, defined as the frequency at which a flickering light appears to be continuous (Smith and Misiak 1976). The CFFF is sensitive to sedative drugs. Subjects viewed the stimulus through a 2-mm 'artificial pupil'. The CFFF test was conducted conventionally, with eight threshold measurements collected per session: four with increasing frequencies and four with decreasing frequencies. The mean of the eight measurements was taken as the value of the CFFF (see Samuels et al. 2006).

Visual analogue scales

A computerised version of the VAS was used to collect self-ratings of alertness, contentedness and anxiety. Nine contrasting statements were rated along a continuous 10-cm line to represent the participant's subjective alertness (Norris 1971). The ratings on the nine items were multiplied by their respective factor loadings based on a factor analysis carried out by Bond and Lader (1974) and the mean of the weighted values entered the analysis.

Cardiovascular measures

Blood pressure and heart rate recordings were taken in the sitting position using an electroaneroid sphygmomanometer.

Procedure

After arrival in the laboratory, each subject had a 15-min acclimatisation period, after which the pretreatment tests (recordings of heart rate, blood pressure and VAS) were carried out. The testing was completed in 5 min. On completion of pretreatment tests,

the subjects ingested the capsule containing either the active drug or placebo. The pretreatment tests were repeated post-ingestion, together with recordings of the 5-min PAT and the pupillary light reflex (post-treatment tests). The time course of the sessions was based on the pharmacokinetics of the active drugs: tmax is 1 h following oral administration of single doses of propranolol (Hardman et al. 2001), ketanserin (Brogden and Sorkin 1990) and buspirone (Sakr and Andheria 2001).

Data analysis

In each experiment, the average RPDs for each one of the 15 20-s periods were analysed with a mixed model analysis of variance (ANOVA) with period (15 levels) as the within and order (placebo then drug and drug then placebo), treatment (placebo and active drug), gender (male and female) and smoking status (smokers and non-smokers) as the between-subject factors. Light reflex variables (latency and amplitude) were analysed with separate 2×4 (treatment×luminance level) repeated measures ANOVAs. The pre-post treatment differences in CFFF, VAS and cardiovascular measures were subjected to paired samples t tests.

Results

Resting pupil diameter (5-min PAT) Figure 1 shows the RPD values for each one of the 15 20-s periods for the drug treatment conditions in experiments I, II and III. In all three experiments, RPDs were becoming progressively smaller from the first to the 15th period in all treatment conditions. In experiment I (ketanserin), a 2×2×2×2×15 (order × treatment × gender × smoking status × period) ANOVA showed significant main effects of treatment [$F(1,5)=13.3$, $p<0.015$] and period [$F(14,70)=3.02$, $p<0.001$]. All other main effects and interactions were non-significant (all p values

>0.09). Identical analysis in experiment II (buspirone) showed significant treatment [$F(1,5)=7.0$, $p<0.05$] and period [$F(14,70)=2.9$, $p<0.002$] main effects. All other main effects and interactions were not significant (all p values >0.2). Finally, identical analysis in experiment III (propranolol) did not reveal any significant main effects or interactions (all p values >0.3). A 3×2×2×2×15 (treatment group × order × gender × smoking status × period). ANOVA of the placebo data only showed that RPDs in the three treatment groups did not differ in the placebo condition [group main effect and all interactions involving group ($F<1$)].

Light reflex. In all three experiments, latency was reduced, and amplitude was increased with increasing light intensity, as evidenced by expected significant main effects of light intensity for these measures [latency; $F_{propranolol}(3,33)=26.7$, $p<0.001$; $F_{ketanserin}(3,33)=19.8$, $p<0.001$; $F_{buspirone}(3,33) = 18.1$, $p<0.001$; amplitude: $F_{propranolol}(3,33)=287.0$, $p<0.001$; $F_{ketanserin}(3,33)=102.1$, $p<0.001$; $F_{buspirone}(3,33)=71.3$, $p< 0.001$] (Figure 2). There was no significant treatment main effect for latency in any experiment (all $p>0.1$). Ketanserin increased light reflex amplitude as evidenced by a significant treatment main effect [$F(1,11)=8.1$, $p=0.016$] in experiment I, while propranolol and buspirone had no effect on this measure [$F(1,11)=1.2$, $p>0.3$, and $F<1$, respectively]. There was no significant treatment by light intensity interaction for any measure in any experiment.

Critical flicker fusion frequency The post-pre treatment differences are shown in Table 2. There were significant treatment effects in experiment I (ketanserin) ($t=3.48$, df 11, $p<0.005$) but not in experiments II (buspirone) and III (propranolol; $t=0.15$, df 11, $p>0.88$ and $t=-1.27$, df 11, $p> 0.23$, respectively).

VAS alertness The post-pre treatment differences are shown in Table 2. There were significant treatment effects in experiment I (ketanserin; $t=3.1$, df 11, $p<0.01$) but not in experiments II (buspirone) and III (propranolol; $t=-0.34$, df 11, $p>0.7$ and $t=-0.04$, df 11, $p>0.9$, respectively).

Cardiovascular measures The post–pre treatment differences in heart rate, systolic and diastolic blood pressure are shown in Table 2. Heart rate was significantly reduced in experiment III (propranolol; $t=2.6$, df 11, $p<0.025$), while diastolic but not systolic blood pressure was significantly reduced in experiments I (ketanserin) and II (buspirone; $t=3.55$,df 11, $p<0.005$; and $t=2.28$ df:11, $p<0.05$ respectively).

Discussion

Table 2 shows that in the doses used, all treatments showed some evidence of bioactivity. Ketanserin reduced diastolic blood pressure, consistent with its antihypertensive properties, which are thought to be centrally mediated via the suppression of sympathetic nerve activity (McCall and Schuette 1984; Ramage 1985). Ketanserin reduced RPD and increased the light reflex amplitude, suggesting a role of 5HT2 receptors in mediating pupil size and the light reflex. Two previous reports failed to show an effect of ketanserin on the pupil (Costagliola et al. 1991; Tekat et al. 2001), but the observed ketanserin-induced miosis in our study is in agreement with previously reported miotic effects of the selective 5HT2 antagonists ICI 169,369 and ICI 170,809 (Millson et al. 1991; Millson et al. 1992). Although preclinical studies in rabbits would support a direct involvement of 5-HT2 receptors in controlling pupillary responses (Tobin et al. 1988), local application of ketanserin has been

reported to have no effect in human subjects (Costagliola et al. 1993). Therefore, the observed pupillary effects of ketanserin cannot be easily attributed to peripheral effects of the drug on the iris. The reduction in pupil size by ketanserin is consistent with a postulated sympatholytic effect of this drug, but this cannot account for the increase in light reflex amplitude, since the latter is a parasympathetically mediated response (Loewenfeld 1993). This pattern of effects on pupillary size and kinetics is similar to that observed by clonidine (Bitsios et al. 1998a). Clonidine is an a2-adrenoceptor agonist whose effects in man are generally attributed to a sympatholytic action resulting from stimulation of pre-synaptic inhibitory a2adrenoceptors located on the cell bodies and dendrites of central noradrenergic neurones; these neurones have an excitatory effect on sympathetic function (Szabadi and Bradshaw 1996). Central noradrenergic neurones located in the locus coeruleus send an inhibitory projection to the Edinger–Westphal nucleus (Szabadi and Bradshaw 1996). Thus, the 'switching off' of the central noradrenergic neurones due to the activation of inhibitory a2adrenoceptors by clonidine results not only in a decrease in sympathetic outflow but also in the removal of the noradrenergic inhibition of the pupillary light reflex, leading to an increased miotic response of the pupil after a light stimulus. The observed clonidine-like effects of ketanserin on pupil size and kinetics raise the possibility that this drug may attenuate central inhibition of Edinger– Westphal neurones, leading to an enhancement of the effect of light on the pupil. Ketanserin showed a sedative profile in the CFFF and VAS-rated alertness, and it is interesting in this respect that this drug, similarly to clonidine, reduced salivation and alertness and had a clonidine-like profile in the waking electroencephalogram (Reimann et al. 1986). All the above, taken together, strengthen the notion that pupil size is a physiological correlate of central arousal levels and suggest that ketanserin reduces

alertness and pupil size, via an action on the 'arousal/pupil control interface', which is likely to be the locus coeruleus and its connections (Szabadi and Bradshaw 1996; Hou et al. 2005, Hou et al. 2007b). The precise mechanism and neuronal circuitry involved remain to be elucidated, but it is likely that these ketanserin effects were mediated through antagonism at the 5-HT2a receptor for which the drug shows high affinity (pKi of 2.5; Branchek et al. 1990) which is 50-fold higher than its affinity on 5HT2c receptors. Ketanserin is an antagonist at the α1-adrenoceptor but its affinity (Ki ~40 nM) (Coyne 2008) is 20 times lower than that at the 5HT2a receptor. However, a contribution from antagonistic activity at the α1-adrenoceptor cannot be entirely excluded because such antagonism could have caused miosis by an action on the α1-rich iris dilator muscle or in the pre-ganglionic sympathetic neurons (Szabadi and Bradshaw 1996). In this context, it is important that local application of ketanserin in the iris did not cause miosis (Costagliola et al. 1993) because it speaks against participation of the α1-adrenoceptor in the ketanserin-induced miosis observed in the present study. Finally, ketanserin also possesses appreciable anti-histaminergic activity (Dollery 1999), which could have also contributed to its sedative and miotic effects. Indeed, antagonism of H_1 histamine receptors by diphenhydramine resulted in sedation and miosis but light reflex amplitude was not affected (Hou et al. 2006, 2007a), thus the characteristic clonidine-like effect of ketanserin observed here was not present with H_1 antagonism. Buspirone reduced RPD consistent with previous observations (Fanciullacci et al. 1995; Phillips et al. 1999). In contrast to ketanserin, the buspirone-induced miosis is unlikely to be related to any sedative properties of this drug, since buspirone is not a sedative agent (Newton et al. 1982), and it did not affect the VAS and CFFF measures of alertness used in this study. Buspirone also reduced diastolic blood pressure consistent with previous results (Fanciullacci et al.

1995) and in agreement with the effect of flenoxisan, another 5HT1a agonist. This drug decreased blood pressure in hypertensive patients and normotensive subjects without reflex tachycardia (De Voogd and Prager 1990). Buspirone exhibits affinity for dopamine D_2 family of receptors and while, this is 16-fold and 3-fold lower for the D_2 and D_3 R respectively than that for 5HT1a receptors, its affinity for the D_4 receptor equals that for 5HT1a (Roth and Driscol 2011); therefore, a contribution from the D_4 receptors cannot be entirely excluded. 5-HT1a receptor agonists reduce sympathetic outflow (Connor et al. 1991; Ramage and Fozard 1987), an effect that is reversed by 5-HT antagonists (McCall et al. 1987). Therefore, the observed buspirone-induced miosis and hypotension are fully consistent with a central sympatholytic effect of the drug. It is surprising that we could not find any effect of buspirone on the amplitude of the pupillary light reflex, since previous work (Phillips et al. 1999) has shown that the buspirone induced miosis was light dependent consistent with the involvement of the light reflex mechanism. It is possible that this was a result of the low buspirone dose (10 mg) used in the present study, since the light dependent miosis in the study of Phillips et al. (1999) was observed with 20 mg but not 5 or 10 mg of buspirone. Buspirone effects on the light reflex are informative in this respect, as buspirone did not affect light reflex amplitude, a parasympathetically mediated response. Thus, the miosis caused by buspirone, at least for the dose used in the present study, is likely to have been mediated by a reduction in sympathetic activity rather than an increase in parasympathetic activity. The way in which the activation of central 5-HT1a receptors may lead to a sympatholytic effect is not clear, but it could be mediated via central noradrenergic neurons, since the latter receive modulatory input from the serotonergic system (Maeda et al. 1991; Vertes and Kocsis 1994). On the other hand, there is evidence that the periaqueductal grey (PAG)

may have an integrative function in the sympathetic and parasympathetic control of the pupil (Klooster and Vrensen 1998) and may also participate in the modulation of the cardiac sympathetic function (Farkas et al. 1998); indeed, stimulation of the 5-HT1a-rich (Brandao et al. 1991; Pazos and Palacios 1985; Pompeiano et al. 1992) and highly responsive to 5HT1a agonists (Behbehani et al. 1993) dorsomedial PAG produces hypotension without tachycardia (Pajolla and de Aguiar Correa 2004; Pajolla et al. 2005) in line with our observations. Therefore, it is also possible that the pupillary and cardiovascular effects of buspirone are mediated via a direct drug effect in the PAG, but in any case, the neuronal circuitry involved and the location of the 5-HT1a receptors within this circuitry remain to be elucidated. Consistent with its beta-blocking properties, propranolol reduced heart rate, suggesting bioactivity at 40 mg, but it did not affect pupil size and kinetics or any other measure of alertness, suggesting that beta adrenergic receptors are not involved in central regulation of arousal and pupillary functions. These data favour the view that propranolol is not a sedative drug (Currie et al. 1988; Harmer et al. 2001; Ogle et al. 1976; Tyrer and Lader 1974). It is worth noting that no active treatment affected the light reflex latency, which is an index of speed of signal processing by the retina and the integrity of the afferent branch of the reflex (Loewenfeld 1993). This suggests that treatments did not interfere with afferent light signal processes. These results contribute to our understanding of the central pupillary control, but they also show that pupil size may not be used unequivocally as an index of the level of alertness in the case of drug-induced changes, when the drugs directly influence, by a separate pharmacological action, the pupil control mechanism, e.g. buspirone (this study), pramipexole (Samuels et al. 2006) and diazepam (Bitsios et al. 1998b; Hou et al. 2006; Hou et al. 2007b for a discussion on the relationship between drug-induced sedation and miosis).

These considerations aside and given that pharmacological sedation in normal individuals may not be assumed to equate to the EDS seen in patient populations, elucidation of the central pupillary control via drug-induced sedation may be of relevance to sleep disorders characterised by reduced alertness and small pupils (Bitsios et al. 2006; Nikolaou et al. 2008). The practical implication of our study is that the pupillometric assessment of alertness in patients with EDS may be confounded by buspirone treatment for a comorbid anxiety/mood disorder or by ketanserin but not propranolol treatment for comorbid hypertension. Our study also suggests that due to its sedative properties, ketanserin may not be the drug of choice for the treatment of comorbid hypertension in patients suffering from EDS. In conclusion, 5HT2 but not 5HT1a receptors or beta adrenoceptors appear to be involved in central regulation of arousal together with pupillary functions, although 5HT1a receptors may participate in central pupillary control alone. More detailed dose–response studies are required, but these results help to elucidate the central regulation of pupil size and alertness and they are informative to (a) drug studies using the pupil as a test system of alertness and (b) clinical studies using the pupil as a test system of alertness in patients with sleep disorders, who are concurrently medicated with ketanserin, propranolol or buspirone.

Tables

Table 1 Subjects' demographic characteristics in each experiment

	Experiment I, Ketanserin	Experiment II, Buspirone	Experiment III, Propranolol	*p* value
Sample size	12	12	12	NA
Male/female[a]	6:6	6:6	6:6	1
Age (years)	24.8(4.0)	25.3(3.0)	24.8(3.6)	>0.9
BMI	24.3(3.9)	23.5(3.1)	22.2(2.9)	>0.3
Education (years)	16.3(2.4)	17.3(1.6)	17.3(1.8)	>0.4
Smokers/non-smokers[a]	4:8	4:8	8:4	>0.1

Figures in brackets are SD

[a] For these measures, chi-square comparisons were applied. All other variables were analysed with one-way ANOVAs

Table 2 Critical flicker fusion frequency (CFFF), visual analogue scales (VAS), heart rate and blood pressure post–pretreatment differences [group means(SD)] in each experiment

Test	Experiment I		Experiment II		Experiment III	
	Placebo	Ketanserin	Placebo	Buspirone	Placebo	Propranolol
Δ CFFF	0.13 (0.4)	−1.4 (1.7)*	−0.09 (0.8)	−0.16 (1.7)	−0.2 (0.7)	0.1 (1.5)
Δ VAS alertness	−0.0 (0.06)	−0.13 (0.1)*	−0.0 (0.04)	0.0 (0.06)	−0.0 (0.04)	−0.0 (0.08)
Δ Heart rate	−3.3 (6.6)	−4.7 (5.6)	−7.7 (4.7)	−3.7 (6.5)	−5.3 (6.6)	−12.7 (9.0)*
Δ Systolic BP	−6.3 (7.1)	−10.0 (6.7)	−2.1 (5.0)	−4.6 (5.8)	−7.9 (8.4)	−7.5 (6.9)
Δ Diastolic BP	5.0 (7.4)	−5.0 (8.3)*	1.3 (5.7)	−2.9 (3.3)*	−2.1 (5.4)	0.0 (8.5)

*$p<0.05$

Figures

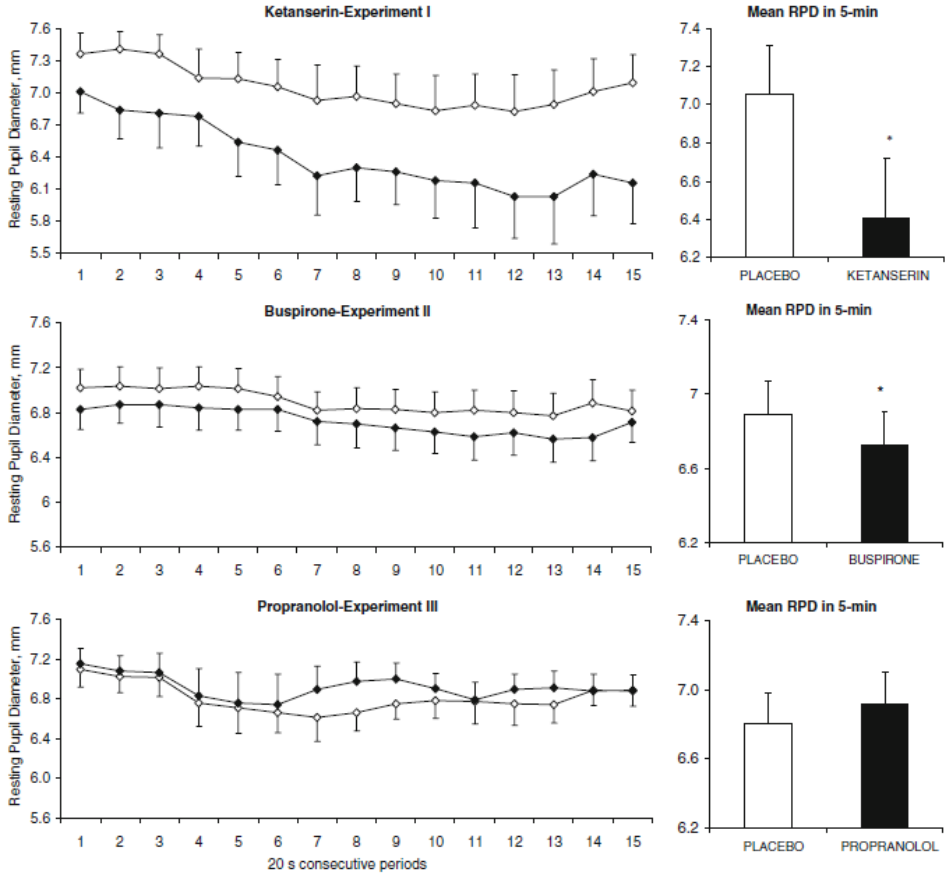


Fig. 1 Resting Pupil Diameter (RPD) values for the 15 20-s periods (*left*) and mean RPD in 5 min (*right*) for the placebo (*white diamonds*) and the drug treatment conditions (*black diamonds*) in the three experiments

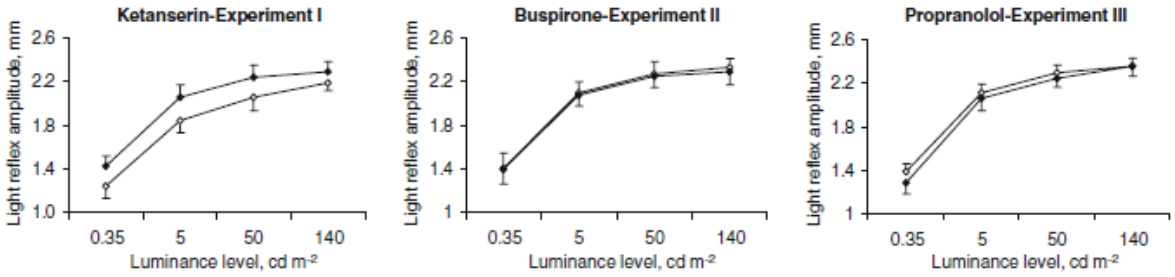


Fig. 2 Light reflex amplitude for the placebo (*white diamonds*) and the drug treatment conditions (*black diamonds*) in the three experiments

3.2 EXPERIMENTS 4 AND 5: The Effects of Ketanserin and Buspirone on the Fear-Inhibited Light Reflex

Introduction

The pupillary light reflex is a homeostatic parasympathetic reflex and consists of a brisk and transient contraction of the smooth iris sphincter muscle in response to rapid increments in light flux to the retina, thus reflecting the amount of light captured by the eye. Its evolutionary purpose is the optimization of vision under all environmental light conditions. Previous research showed that the pupillary light reflex has characteristics that make it a suitable and useful tool for the study of normal and pathological anxiety. Using a verbal threat paradigm, it was shown that the reflex is inhibited when the light probe is presented after a warning cue that signifies threat and elicits subjective fear – thence the initial term "Fear-inhibted light reflex" (Bitsios et al 1996). This reflex inhibition was found proportional to the level of state anxiety (Bitsios et al 2002), and correlated to other valid experimental paradigms in animals and humans such as the Fear-potentiated startle (Bitsios et al 1999); a common property of the fear-inhibited light reflex and fear-potentiated startle reflex was their dose-dependency on the anxiolytic diazepam (Bitsios et al 1998, 1999), but not to clonidine which simply reduces the central noradrenergic sympathetic responses and central arousal levels (Bitsios et al 1998a). These results taken together suggest that the fear-inhibited light reflex is a valid laboratory model of human anxiety. Based on the well-established role of the amygdala in the mediation of fear acquisition and expression, it was argued that verbal threat as used in the verbal protocol, activates the amygdala which in turn inhibits directly or indirectly (via established connections with the locus ceruleus and the hypothalamus) the Edinger-Westphal nucleus, the

motor centre of the pupillary light reflex. Other studies exploratory and validating studies have shown that the light reflex is inhibited by different kinds of threat and the level of inhibition reflects the "amount" of induced fear by various fear stimuli, independent of the sensory modality of these stimuli (Hourdaki et al 2005). While non-threatening cues will not inhibit the light reflex, they can increase the baseline pupil diameter especially when they have high novelty value (Bitsios et al 2004, Hourdaki et al 2005); this increase in pupil diameter can also be observed under threat but in contracts to threat-induced reduction in light reflex amplitude, it is not sensitive to diazepam (Bitsios et al 1998, 1999), while it is sensitive to clonidine (Bitsios et al 1998) and noradrenergic drugs (Bitsios et al 1999a). It has been argued that the increase in pupil diameter by threat or novel cues reflects the organism's orienting arousal levels and as such it always precedes the inhibition of the reflex by threat/fear. The arousal-induced increase in baseline pupil diameter during threat is a pure sympathetically mediated response, since it can be abolished pharmacologically with blockade of the tone of sympathetic iris sphingter muscle via dapiprazole (an a1 antagonist) eye drops. In contrast, the sympathetic system does not participate in the the fear-inhibited light reflex since reflex inhibition is not affected by dapiprazole eye-drops. Therefore, the inhibition of the light reflex by fear/threat is mediated by central parasympathetic inhibition, most probably at the level of the Edinger-Westphal nucleus, the motor centre of the reflex which receives inhibition from the subcortical defense system (amygdala, hypothalamus, central grey etc) (Giakoumaki et al 2005).

All the studies above have validated the fear-inhibited light reflex as a model of imminent danger that is akin to phobic or panic anxiety. The model appears to be able to separate between threat-induced fear and threat-induced arousal or alertness. Using

pharmacological probes with known properties it is possible to explore the neurotransmission of the type of anxiety modeled by this paradigm. The central aim of the following experiments was to examine the effects of ketanserin and buspirone on the fear-inhibited light reflex and the arousal-induced increase in pupil diameter during threat. Ketanserin is an agent with sympatholytic properties, via central 5HT2-mediated modulation of the sympathetic system (Cameron et al. 1987) and while it is used as an anti-hypertensive drug, it is interesting to examine the role of 5HT2a/c blockade, if any, in our model, because 5HT2 receptors are thought to be involved in the control of anxiety behaviors in a complex way; their activation in the dorsolateral septum increases anxiety behaviors (de Paula et al 2012), while their activation in the dorsal PAG facilitates anxiolysis (Nunes-de-Suza et al 2011) and both these anxiolytic and anxiogenic effects were blocked by ketanserin; ketanserin also has affinity for the 5HT1c receptor which is also involved in the control of anxiety behaviors as their activation in the ventromedial hypothalamus reduces inhibitory avoidance which is blocked by ketanserin (da Silva et al 2011). Ketanserin was found to have an anxiogenic profile in a zebrafish novelty exposure model (Nowicki et al 2014) and has no anxiolytic effects in human anxiety disorders. We showed in our previous experiment that ketanserin 20mg reduced arousal and pupil diameter and increased light reflex amplitude (a clonidine-like action) in a non-threatening setting. We hypothesized that we would replicate these findings in light reflex amplitude and pupil diameter in the safe condition of the present experiment but given the mixed findings of 5HT2a/c blockade on anxiety behaviors, it was hard to predict the net effect of ketanserin on fear-inhibition of the light reflex;

Buspirone is a partial agonist at the 5-HT1A receptor (Andrade and Nicoll 1987), with some affinity for the dopamine D2 receptor (Jann 1988; Peroutka 1985; Riblet et al.

1982). Buspirone is used as a non-sedative anxiolytic and therefore is of a priori interest for anxiety studies. We showed that buspirone 10mg reduced pupil size without reducing alertness or affecting light reflex amplitude in a non-threatening setting. We hypothesized that we would replicate these findings in light reflex amplitude and pupil diameter in the safe condition of the present experiment and consistent with a role of 5HT1a receptors in human anxiety, we also hypothesized that their agonism by buspirone, would attenuate the effects of threat on light reflex amplitude.

Materials and Methods

Subjects

In both experiments, subjects were between 18 and 30 years old, with a body mass index (BMI) in the normal range. We recruited two separate groups of 12 subjects (6 female) for each experiment. Inclusion criteria included written informed consent, absence of personal history of head trauma, medical and neurological conditions or use of prescribed and recreational drugs and absence of personal or family (up to second-degree relatives) history of Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition Axis I disorders. All participants underwent physical and psychiatric assessment using the Mini-International Neuropsychiatric Interview (Sheehan et al. 1998), an ophthalmological examination and a urine drug screen test. All subjects were regular caffeine (two to three cups of coffee per day on average) and occasional alcohol consumers. The study was approved by the University of Crete Ethics Committee.

Design and drugs

Ketanserin and buspirone were administered in a placebo-controlled, within-subject design in two separate experiments, using separate groups of subjects but identical experimental procedures. In each experiment, subjects participated in two weekly sessions (returning to the laboratory at the same time each week for each session). Subjects were allocated to treatments and sessions according to a double-blind, balanced, crossover design. Ketanserin 20 mg, buspirone 10 mg and placebo were prepared in identical capsules and administered orally. The choice of dose of each drug was based on centrally bioactive doses reported in published studies [ketanserin (Graham et al. 2002) and buspirone (Phillips et al. 1999)].

Pupillometry

The recordings took place in a dark, sound-attenuated room. A binocular infrared television pupillometer (Procyon, P2000D, Procyon Biopharma Inc., Dorval, Canada) was used to elicit and record the light reflex response in darkness in previously dark-adapted eyes. The sampling rate of the pupillometer was 25 Hz, the spatial resolution was better than 0.05 mm and the accuracy was better than ±3%. The stimuli were weak light flashes of 200 ms in duration (stimulus luminance: 50 cd m^{-2}), delivered via a light emitting diode, presented to the subject's placebo-treated eye as a white disk of 8° diameter, providing 'full field' light stimulation while the unstimulated eye was fixating a target dot projected at a distance of approximately 10 m. Stimulus presentation was computer controlled, and pupillary measures were digitized and stored for offline analysis. The parameters studied were: IPD (i.e. the mean pupil diameter recorded over 500 ms before the onset of the light stimulus) and light reflex

response amplitude (i.e. the difference between the IPD and the diameter reached at the trough of the pupillary response to the light stimulus).

Procedures

Training session

Upon their arrival in the laboratory, the subjects received a detailed description of all procedures and a demonstration of all apparatus. Then the subjects underwent a brief training session (application of a few light flashes in the dark to evoke the pupillary light reflex), in order to familiarize them with pupillometry.

Experimental sessions

At the beginning of each experimental session, after a 10-min rest period, the subjects ingested the capsule. Forty minutes after drug ingestion, the subjects entered the adaptation phase. During the adaptation phase, the subjects first wore red goggles for 15 min in order to adapt to dim red illumination. Following this, the light reflex was elicited in darkness with 12 light flashes, in order to familiarize them with pupillometry (5 min). At the end of the adaptation phase (1 h after the ingestion of the capsule), the skin on the subjects' left wrist was prepared, the electrodes were applied, and the main phase was started. This time course was based on the pharmacokinetics of the active drugs: tmax is 1 h following oral administration of single doses of ketanserin (Brogden and Sorkin 1990) and buspirone (Sakr and Andheria 2001). The main phase comprised twelve identical, consecutive blocks of four light flashes of the same intensity and duration (60 light flashes in total, per session). In the main phase, responses in each block were recorded either during anticipation of an electric shock

(Threat condition) or without anticipation (Safe condition). Half of the subjects started with a Safe and the rest with a Threat block and blocks alternated regularly thereafter. Half of the subjects started with a Safe block, and the remaining half with a Threat block. The Safe and Threat conditions alternated regularly in the remaining 11 blocks. The main phase lasted approximately 20 min.

Data analysis

The pupillary measures [initial pupil diameter (IPD) and light reflex amplitude (LRA)] for each block were obtained by averaging the four light reflex responses and taking the measures from the averaged response. Data for each pupillary measure were collapsed across blocks for the two conditions (Safe, Threat) and the two treatments (active drug vs. placebo). Two-way analysis of variance with treatment and condition as the within-subject factors was used to analyze the pupillary measures.

Results

Ketanserin Experiment

The IPD was larger under the Threat than under the Safe condition, under both treatments but reduced with ketanserin treatment in both psychological conditions (Figure 1). Analysis of variance revealed a significant main effect of treatment [$F(1,11) = 12.37$; $P < 0.005$), condition [$F(1,11) = 26.89$; $P < 0.001$) and block [$F(5,55) = 3.25$; $P < 0.001$]. There was a weak treatment by condition interaction [$F(1,11) = 3.4$; $P = 0.09$) which became significant ($F=4.3$, $P=0.05$) after taking Delta STAI-S (Ket) as the covariate; separate follow up analyses for each treatment revealed significant effects of condition with both treatments but the ANCOVA revealed a significant condition effect only with the ketanserin treatment, suggesting

that those with the greatest ketanserin-induced reduction in STAI-S had the greatest IPD reductions in the safe relative to the threat condition.

Analysis of variance of the amplitude data revealed only a significant treatment by condition [$F(1,11) = 10.99$; $P < 0.007$) and treatment by block interaction [$F(5,55) = 3.03$; $P < 0.0052$), consistent with the visible amplitude reductions by ketanserin in the safe condition (Figure 2). When the ketanserin-induced reduction in IPD was taken as the covariate this effect was lost ($P>0.2$) and a significant effect of condition was revealed. This suggests that the reduction in light reflex amplitude by ketanserin in the safe condition was attributable to IPD reduction caused by this drug.

State anxiety (Figure 3) was reduced post- compared to pre- treatment by both treatments and ketanserin appeared to have a greater effect compared to placebo but this was not significant as revealed by a 2x2 (treatment by occasion) ANOVA of the STAI-State data; occasion main effect ($p<0.05$), treatment main effect ($F<1$), interaction ($p>0.1$).

Buspirone Experiment

The IPD was larger under the Threat than under the Safe condition, under both treatments but reduced with buspirone treatment in both psychological conditions (Figure 4). Analysis of variance revealed a significant main effect of treatment [$F(1,11) = 4.6$; $P < 0.05$), condition [$F(1,11) = 10.4$; $P < 0.01$) and block [$F(5,55) = 3.8$; $P < 0.02$]. There were no significant interactions.

Analysis of variance of the amplitude data revealed a significant block main effect [$F(5,55)=24.1$; $p<0.001$] and a significant treatment by condition [$F(1,11) = 5.8$; $P < 0.05$) and treatment by condition by block interaction [$F(5,55) = 3.3$; $P < 0.05$),

consistent with the visible amplitude increases by buspirone in the threat condition particularly after the first two blocks (Figure 5).

State anxiety (Figure 6) was reduced post- compared to pre- treatment by both treatments and placebo appeared to have a greater effect compared to buspirone as revealed by a significant interaction {$F(1,11) = 6.2$; $p<0.05$] but this was due to greater anxiety levels prior to the placebo session. The effects of occasion and treatment were not significant [$F(1,11) = 2.44$; $p>0.1$ and $F<1$ respectively).

Discussion

This is the first study that examined the effects of ketanserin and buspirone on the fear-inhibited light reflex. We found that ketanserin reduced pupil diameter in both safe and threat conditions, consistent with its sedative and sympatholytic properties and in agreement with our previous using the 5-min PAT. Regardless of the pharmacological mechanisms involved, the final path for reduction in pupil size is either reduction in the sympathetic input to the iris dilator muscle (sympatholysis) or increase in the parasympathetic input to the iris sphincter muscle following disinhibition of the EW nucleus (expressed with increase in LRA) or both as we argued for ketanserin based on the previous experiment. In contrast to our previous findings, here we found that ketanserin reduced rather than increase light reflex amplitude in the safe condition and that this effect was attributable to the miosis caused by this drug as evidenced by the analysis of covariance. This finding strongly suggests the operation of a “floor effect” in the “Ketanserin Safe” condition whereby a miotic pupil cannot respond to a strong light stimulus with a large amplitude due to mechanical constraints inherent in the pupillary system. This problem could have been partially overcome by a lower ketanserin dose or a study design that would have

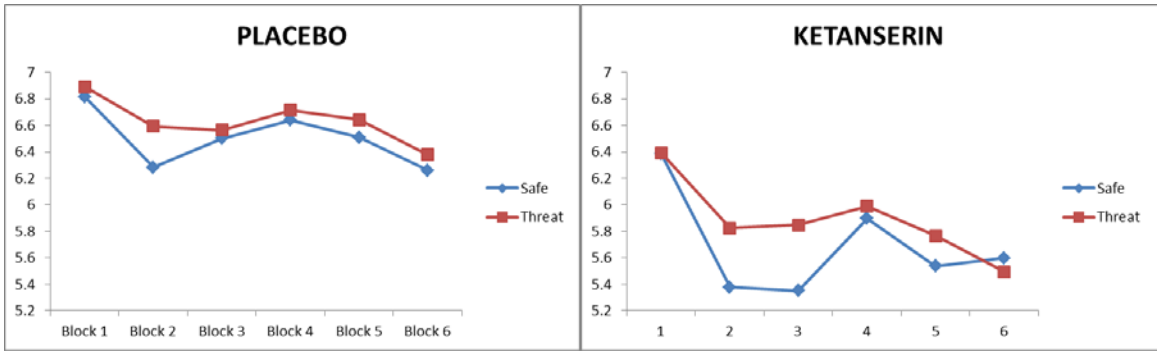
involved the use of much weaker light stimuli, which would generate weak light reflex; such light reflexes of low amplitude could have circumvented the problem of mechanical limitations and we might have witnessed the anticipated increase in amplitude in the safe condition under ketanserin. This effect of ketanserin on the light reflex in the safe condition confounds interpretation of results.

Ketanserin did not reduce the effects of threat on pupil diameter or light reflex amplitude and as confounded as the latter result may be, it seems certain that despite its sedative properties, ketanserin did not affect subjects' arousal and anxiety in the threat compared to the safe condition which remained the same as those observed in the placebo treatment. Based on these results, it can be concluded that acute ketanserin treatment has no anxiolytic effects at least for the type of anxiety modeled by the fear-inhibited light reflex paradigm, and this is consistent with animal studies that show no effect of 5HT2 antagonists on the conceptually similar fear-potentiated startle paradigm (Davis et al 1988) and its lack of clinical effectiveness in anxiety disorders. Our results confirm ketanserin's sedative effect which was reflected in a decrease in pupil size, consistent with a role of 5HT2a receptor antagonism in sleep (van Laar et al 2001, Morairty et al 2008) but also with the anti H1 and a1 properties of the drug (Dollery 1999). Most importantly, our results show that caution is required when interpreting the putative antagonism of the fear-inhibited light reflex and a drug as evidence for anxiolysis if the drug itself has some intrinsic agonistic activity in the test system, in our case the amplitude of the pupillary light reflex.

Buspirone presented with a clear anxiolytic profile on the other hand. We observed a miotic effect of the drug consistent with our previous study with the 5-min PAT and studies from other groups. In contrast to ketanserine-, buspirone-induced miosis was not as profound and there was no floor effect in operation for LRA which allowed

reliable comparisons between the Safe and Threat conditions under placebo and active treatments. There were no effects of buspirone on the Threat-induced increase in PD suggesting that the arousal component of threat was unaffected by the drug. Critically however, there was a clear effect on Threat-induced reduction in LRA; indeed, LRA in the treat condition reached Safe levels in all blocks; this was unlike the placebo treatment where LRA was inhibited in the treat compared to safe condition, especially in the second part of the session, consistent with a reported effect of this drug in accelerating extinction on fear-conditioned autonomic responses (Hellewell et al 1999). These results reveal an anxiolytic but no sedative properties of buspirone on the fear-inhibited light reflex model, consistent with an anxiolytic profile of buspirone on phasic fear with the conceptually similar fear-potentiated startle paradigm (Miles et al 2011) and the clinical utility of buspirone in anxiety disorders. Buspirone is an agonist of 5HT1a receptors and it reduces activity in serotonergic neurons. 5HT1a receptor activation in the dorsal periaqueductal grey (PAG) suppresses neural activity and reduces escape behavior (a defensive behavior associated with panic anxiety) (de Paula Soares et al 2004, de Oliveira Sergio et al 2011). 5HT1a receptor activation in the dorsomedial hypothalamus (DMH) also reduced the increase in escape behavior caused by electrical stimulation of the DMH (de Bortoli et al 2013). However, rat studies have shown that buspirone's anxiolytic effects on the fear-potentiated startleare not mediated by actions at 5HT1a receptors alone (Davis et al 1988) and instead they suggest a synergistic effect of 5HT1a agonism and Dopamine receptor antagonism in modulating the expression of fear-potentiated startle (Davis 1993)

Figure 1 (Top) Initial Pupil Diameter (IPD) in the six safe and six threat blocks for placebo and ketanserin treatments. (Bottom) IPD collapsed across blocks.



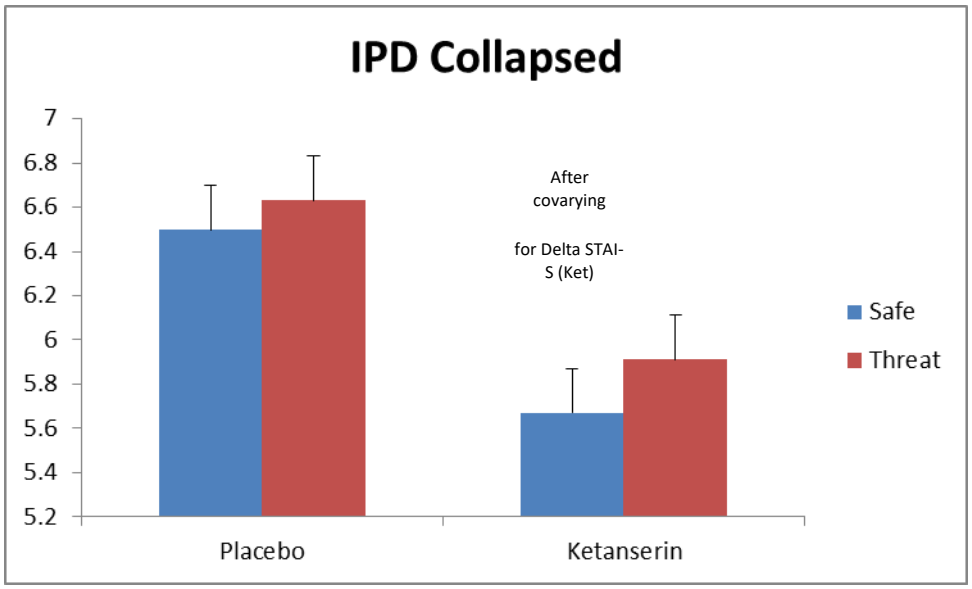


Figure 2 (Top) Light reflex amplitude in the six safe and six threat blocks for placebo and ketanserin treatments. (Bottom) Light reflex amplitude collapsed across blocks.

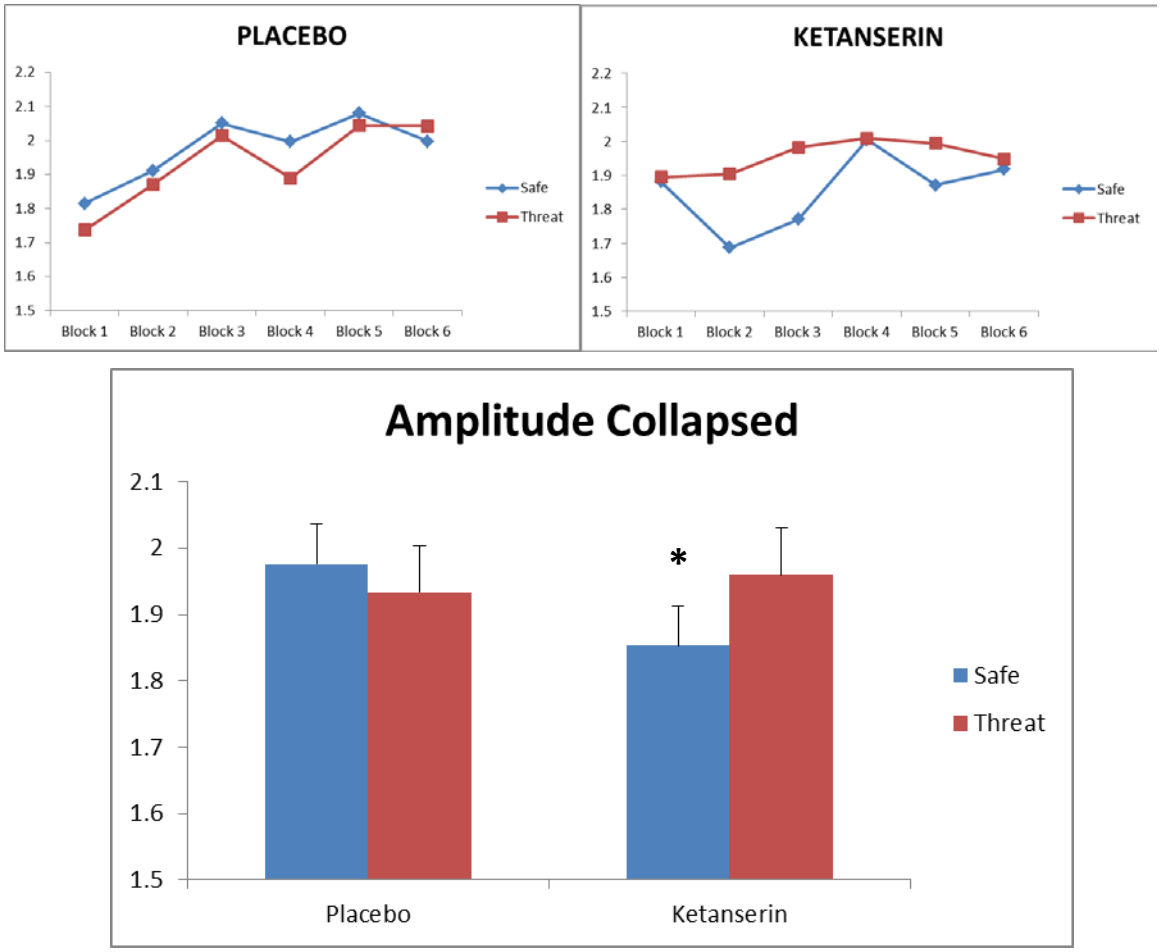


Figure 3 State anxiety pre- and post-treatment for the two treatments

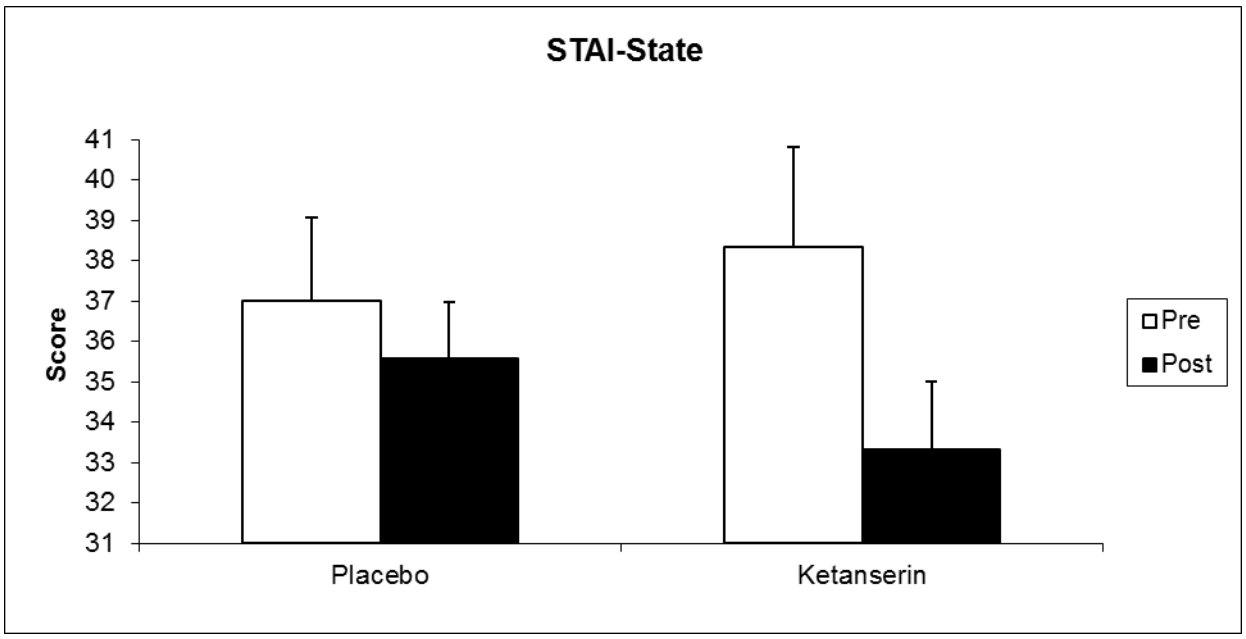
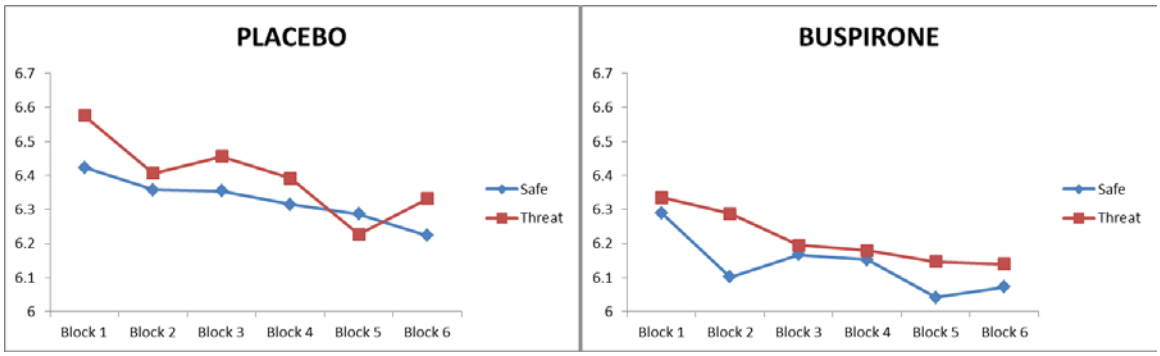


Figure 4 (Top) Initial Pupil Diameter (IPD) in the six safe and six threat blocks for placebo and buspirone treatments. (Bottom) IPD collapsed across blocks.



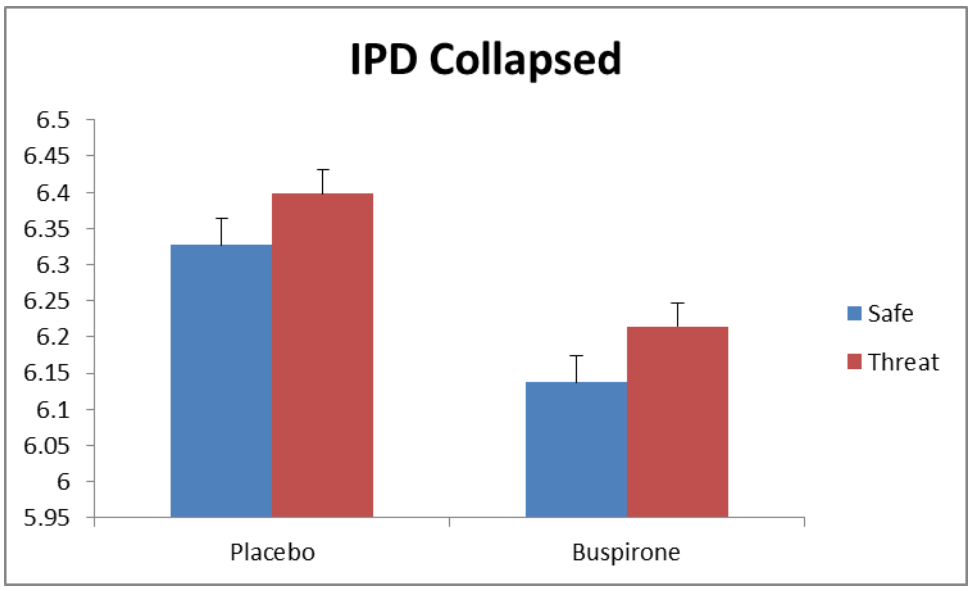
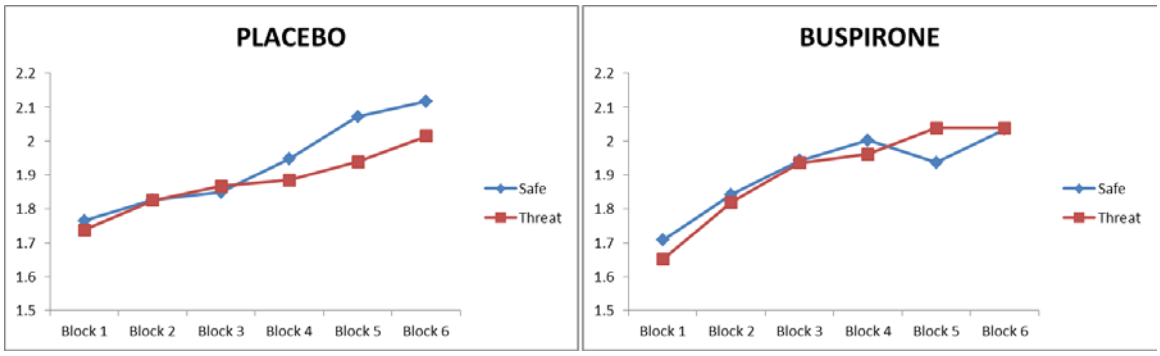


Figure 5 (Top) Light reflex amplitude in the six safe and six threat blocks for placebo and buspirone treatments. (Bottom) Light reflex amplitude collapsed across blocks.



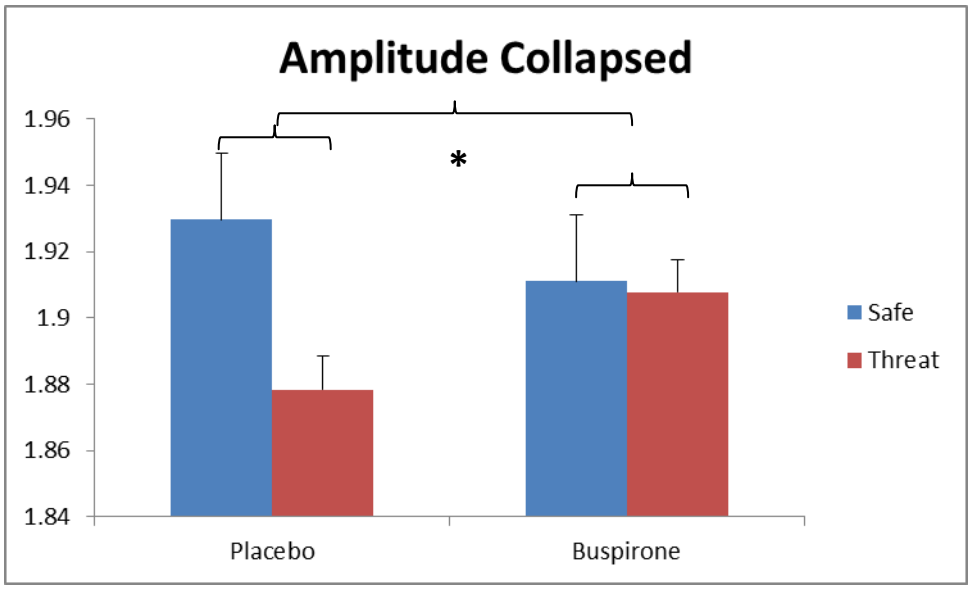
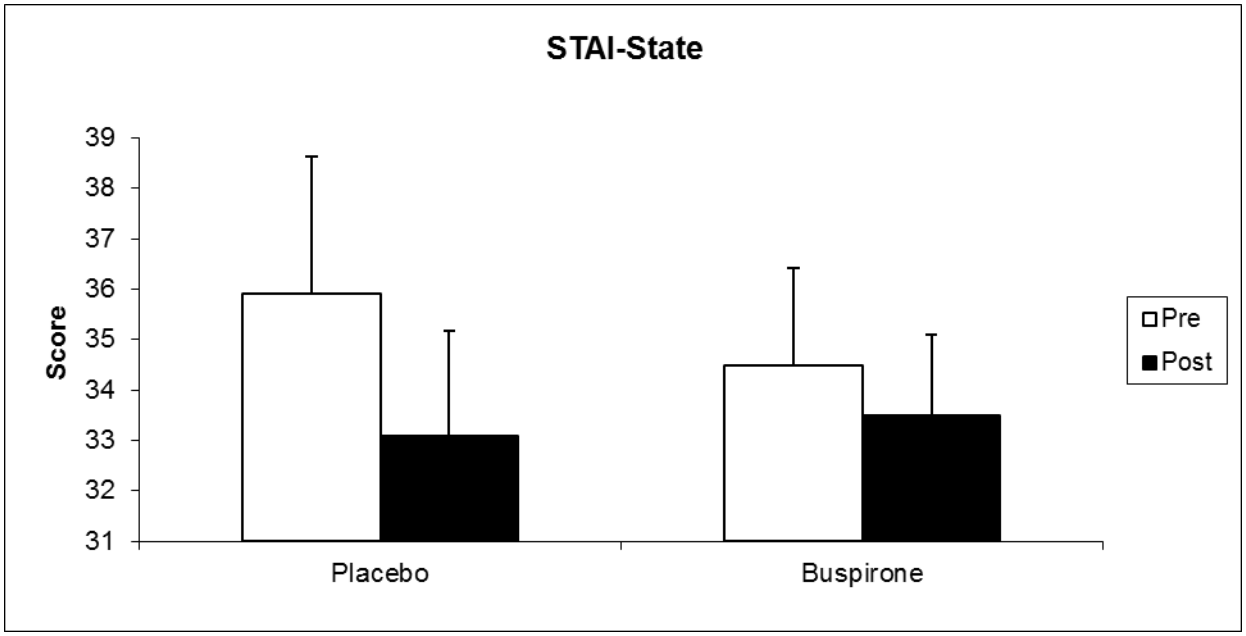


Figure 6 State anxiety pre- and post-treatment for the two treatments



3.3 EXPERIMENT 6. Development and validation of a modified fear-inhibited light reflex protocol measuring fear onset, time-course and offset.

Introduction

Preclinical data indicates that threat stimuli elicit two classes of defensive behaviors, those that are associated with imminent danger and are characterized by phasic fear or panic (more relevant to phobias and panic disorder), and those that are associated with temporally uncertain danger and are characterized by sustained apprehension and hypervigilanc (more relevant to anticipatory and generalised anxiety disorder) (review in Grillon 2008).

The fear-inhibited light reflex is thought to result from a central state of fear, the amygdala being an established critical structure of the defence system that projects directly to the reflex pathway and inhibits the reflex in the threat condition (Bitsios et al 1996, 1998, 1999). The rapid onset of the fear-inhibited light reflex and the ability to present the light stimuli at various time intervals after the onset and the offset of an aversive stimulus suggest that this phenomenon could be used as a method to delineate the time course of the onset and offset of a state of fear. This has been confirmed in the conceptually similar paradigm of fear-potentiated startle (Grilon et al 1993). The goal of the present study was to investigate the temporal specificity of the fear-inhibited light reflex in human subjects and compare it to the established temporal specificity of the fear-potentiated startle reflex (Grillon et al 1993) in a combined protocol using a non-conditioning technique. Subjects were told that shocks would be administered only during the 3-s at the end of a long waiting (25-s) period in the threat conditions. Startle and light stimuli were delivered at various times preceding and following this period.

Methods

Subjects

The study was approved by the Ethics Committee of the University of Crete and all participants gave their written informed consent prior to their participation. Twelve healthy male volunteers, all medical students 19-29 years (Mean ± SD, 22.5 ± 2.78) participated in the study.

Stimuli and Apparatuses

Pupillometry

The recordings took place in a dark, sound-attenuated room. A binocular infrared television pupillometer (PROCYON, P2000D) was used to elicit and record the light reflex response in darkness, in previously dark-adapted eyes. The sampling rate of the pupillometer was 25 Hz, the spatial resolution was better than 0.05 mm and the accuracy was better than ±3%. The stimuli were 200 ms duration bright light flashes (stimulus luminance: 50 cd m^{-2}), as it has been shown previously that the manifest effect of threat on light reflex amplitude is greater with increasing light intensity (Hourdaki et al 2005). The light stimuli were delivered via a light emitting diode, presented to the subject's left or right eye in an alternating fashion as a white disk of 8^{o} diameter, providing "full field" light stimulation, while the unstimulated eye was fixating a target dot projected at a distance of approximately 10 m. Stimulus presentation was computer controlled, and pupillary measures were digitized and stored for off-line analysis. The parameters studied were: initial pupil diameter (IPD) (i.e. the mean pupil diameter recorded over 500 ms prior to the onset of the light stimulus) and light reflex response amplitude (i.e. the difference between the IPD and the diameter reached at the trough of the pupillary response to the light stimulus).

EMG-recording

A commercially available electromyographic startle system (EMG SR-LAB, San Diego Instruments, San Diego, Calif, USA) was used to examine the eyeblink component of the acoustic startle response. Acoustic stimuli were administered binaurally through headphones (model TDH-39-P, Maico Minneapolis, MN). Electromyographic recordings were taken while subjects were seated comfortably in an armchair and instructed to relax but stay awake. The eyeblink component of the startle reflex was indexed by recording EMG activity of the orbicularis oculi muscle directly beneath the right eye, by positioning 2 miniature silver/silver chloride electrodes filled with Signa Gel electrolyte paste (Parker Laboratories, inc, New Jersey USA). The ground electrode was attached behind the right ear on the mastoid. A background of 70 dB broadband white noise was present throughout the recording period. Pulses consisted of 40 ms, 106 dB bursts of white noise with a near instantaneous rise time. The recording took place in the dark environment of the pupillometric chamber.

Procedures

The experiment consisted of a training session and an experimental session.

Training session: Subjects received a training demonstration of apparatuses and procedures in order to familiarise them with pupillometry and recording of the startle reflex. They were then exposed to a mild electric stimulus (constant current square pulse 1.5 mA, 50 ms) delivered to the skin overlying the median nerve of their left wrists, through disposable silver surface electrodes by a Grass stimulator (SD 9). This stimulus is known to cause negligible or only minimal discomfort (Bitsios *et al.,* 1996). They were informed at this point that the shock in the experimental session would be 50

times stronger, and, therefore, the discomfort would be greater than the one they had just experienced. There was no further demonstration of electrical stimuli in the training session.

Experimental session: This took place one or two days after the training session. Following 15 min of dark adaptation, the experiment started with a habituation procedure which comprised a block of 6 light stimuli and a block of 6 startle stimuli in a counterbalanced fashion in order to familiarise the subjects again with the procedures of recording and reduce novelty related arousal. The data from this phase were discarded. This phase was followed by the attachment of electrodes on subjects' left wrists and three minutes later, the light and startle reflexes were recorded under threat and safe conditions in three experimental blocks separated by 4-min rest periods. Each block started with 5 + 5 (light and startle) within-block habituation stimuli, following which ten threat and ten safe conditions were alternated (Figure 1). For half the subjects Blocks 1 and 3 started with a threat condition and Block 2 started with a safe condition and for the other half this order was reversed. For half the subjects the first threat-safe (or safe-threat) pair was associated with recording of the light reflex and the second threat-safe (or safe-threat) pair with recording of the startle reflex. Recording of the light and startle reflex were then regularly alternated in the remaining 8 threat-safe (or safe-threat) pairs of a block. For the rest of the subjects the order of light and startle reflex testing was reversed. Thus, 5 threat-safe pairs were used for testing the light and 5 threat-safe pairs for testing the startle reflex. In each block, the light (or startle) stimuli were presented every 4 s at the following fixed time intervals in the threat and the safe conditions: 1, 5, 9, 13 and 17 s (see Figure 1). Thus, 55 light and 55 startle stimuli were delivered during each block, for a total of 171 (6 +

55 + 55 + 55) light stimuli and a total of 171 (6 + 55 + 55 + 55) startle stimuli during the entire experiment.

A red light signalled the threat condition when the startle reflex was tested but in the case of the light reflex a buzzing low intensity sound was used instead, since light is the unconditioned stimulus for this reflex. The subjects were told that electric shocks could be delivered only in the last 4 s of the threat condition (time interval 17 s) but not in the safe condition. The subjects were also informed about the duration of each condition and the passage of time. A tape recorder counted the time from 0 to 20 s in the threat and safe conditions when the light reflex was tested and a digital timer counted the time from 0 to 20 s in the threat and safe conditions when the startle reflex was tested. The subjects were told that they would receive between one and three shocks and that the second and third shocks, if administered, would be more intense. In fact, the subjects received only one non-painful electric shock as in the training session (see above), which was delivered at time interval 17 s of the last threat condition in Block 2. Thus, Blocks 1 and 2 were recorded prior to shock administration and Block 3 was recorded after shock administration.

Data Reduction and Analysis

Light reflex amplitude data obtained at each one time interval in the alternating five safe and five threat conditions within a block, were collapsed across these five threat and five safe conditions. The resulting collapsed light reflex amplitudes at each time interval in the safe and in the threat conditions were taken as the response of the block and were entered the statistical analysis. This procedure was repeated for the second and third block. Finally, identical procedures were followed for the IPD data (obtained in the same five threat-safe pairs as the light reflex amplitude data) and for the eyeblink amplitude data (obtained in separate five threat-safe pairs). The pupillary

(light reflex amplitude and IPD) and EMG data (eyeblink amplitude) were analysed with separate ANOVAs with repeated measures with block (3 levels), condition (threat, safe) and time interval (1, 5, 9, 13 and 17 s) as the within-subject factors. To compare symmetrical time intervals relative to the time of shock administration, the time intervals 1, 5, 9, 13 and 17 s in the threat condition were compared to the time intervals 17, 13, 9, 5 and 1 s in the safe condition, respectively. The Greenhouse-Geisser correction was used when appropriate, in order to minimize Type I errors. Nominal degrees of freedom along with the epsilon and the corrected p values are reported.

Results

Light Reflex

Amplitude: Figure 2 (top) presents the LRA in the threat and safe conditions at each time-interval over the three blocks and in Figure 2 (bottom) the LRA data are presented averaged across blocks. It can be seen that the light reflex amplitude was slightly reduced during the threat compared to the safe condition, and it became abruptly smaller as the test interval approached the time when shock was anticipated. Following the threat of shock, light reflex amplitude rapidly increased. A three-way ANOVA with block (3 levels), condition (threat, safe) and time interval (1, 5, 9, 13, 17) as the within-subject factors revealed significant main effects of condition and time-interval ($F_{1,11} = 11.97$; $p<0.01$ and $F_{4,44} = 8.69$; $\varepsilon = 0.45$, $p<0.001$ respectively) as well as a significant interaction ($F_{4,44} = 35.31$; $\varepsilon = 0.49$, $p<0.001$) and with linear time-interval trends ($F_{1,11} = 56.57$; $p<0.001$). There was no significant main effect of block ($F_{2,22} = 2.29$; $\varepsilon = 0.72$, $p > 0.1$) or block by condition and block by time interval interactions ($F_s<1.2$). The 3-way interaction was

significant ($F_{8,88} = 3.71$; $\varepsilon = 0.56$, $p < 0.01$). Follow up of this interaction with separate 3 x 5 (block by time interval) ANOVAs for the threat and the safe conditions, revealed a significant block by time interval interaction in the safe condition only ($F_{8,88} = 2.48$; $p < 0.05$) and Bonferoni-corrected t-tests revealed that this was due to increased amplitude at time interval 17 s in block 3.

Initial pupil diameter: Figure 3 (top) presents the IPD in the threat and safe conditions at each time-interval over the three blocks and in Figure 2 (bottom) the IPD data are presented averaged across blocks. It can be seen that, IPD was increased during the threat condition and it became slightly greater as the test interval approached the time when shock was anticipated. In sharp contrast to the amplitude data, IPD was greatly increased at the onset of both the threat and safe conditions rapidly habituating thereafter. Three-way ANOVA with the same factorial design as above, revealed main effects of block ($F_{2,22} = 15.66$; $\varepsilon = 0.96$, $p < 0.001$), condition ($F_{1,11} = 15.01$; $p < 0.003$) and time interval ($F_{4,44} = 49.79$; $\varepsilon = 0.45$, $p < 0.001$) with significant linear and quadratic time-interval trends ($F_{1,11} = 13.91$; $p<0.01$ and $F_{1,11} = 67.97$; $p<0.001$ respectively). There was a significant condition by interval interaction ($F_{4,44} = 52.31$; $\varepsilon = 0.34$, $p < 0.001$) with significant linear ($F_{1,11} = 58.13$; $p<0.01$) but not quadratic trends ($F = 1.2$). The block by condition ($F < 1$), block by interval ($F_{8,88} = 2.13$; $\varepsilon = 0.61$, $p = 0.08$) or the 3-way ($F < 1$) interactions were not significant but there were significant quadratic trends in the 3-way interaction ($F_{1,11} = 9.08$; $p < 0.05$).

Startle reflex

Figure 4 (top) presents the startle reflex in the threat and safe conditions at each time-interval over the three blocks and in Figure 4 (bottom) the startle data are presented averaged across blocks. It can be seen that the eyeblink was larger during the threat compared to the safe condition, and this effect increased as the test interval approached the time when shock was anticipated. Following the threat of shock, eyeblink amplitude rapidly decreased. In addition, eyeblink amplitude habituated over blocks. Three-way ANOVA with the same factorial design as above, revealed significant main effects of condition and time-interval ($F_{1,11} = 12.88$; $p<0.01$ and $F_{4,44} = 6.34$; $\varepsilon = 0.48$, $p<0.007$ respectively) as well as a significant trend for the condition by time interval interaction ($F_{4,44} = 3.10$; $\varepsilon = 0.50$, $p < 0.065$) with significant linear as well as quadratic time-interval trends ($F_{1,11} = 7.69$, $p<0.05$ and $F_{1,11} = 6.93$, $p<0.05$ respectively). There was also a significant main effect of block ($F_{2,22} = 13.6$; $\varepsilon = 0.67$, $p < 0.001$) with a trend for a block by condition interaction ($F_{2,22} = 2.94$; $\varepsilon = 0.95$, $p < 0.08$), while the block by interval and the 3-way interactions were not significant ($Fs < 1.6$).

To estimate the time when light reflex amplitude first began to be reduced and the time when IPD and eyeblink amplitude first began to be increased in the threat condition, light and eyeblink amplitudes and IPD in the safe condition at time interval 17 s, immediately preceding the 1 s time interval in the threat condition, were compared with light reflex and eyeblink amplitudes and IPD respectively at each time interval in the threat condition with Bonferroni corrected paired t-tests. Light reflex amplitude was significantly reduced only at time interval 17 s in Blocks 1 and 2, and at time intervals 9 and 17 s in Block 3, while IPD was significantly increased at all time intervals in Blocks 1 and 2 and at time intervals 1, 5 and 17 s in Block 3.

Eyeblink amplitude was significantly increased at time intervals 1, 9 and 17 s in Block 1, at 17 s in Blocks 2 and 3.

Discussion

This study replicates and extends pervious results, indicating that shock anticipation inhibits the light reflex and potentiates startle. Here, light reflex inhibition and startle potentiation showed good temporal specificity reaching a maximum at the time when shock was imminent and declined rapidly thereafter. Overall, the light and startle reflexes behaved similarly when they were tested in the same session and in the same group of subjects. The significant main effect of condition indicated that overall light reflex amplitude was reduced and overall startle amplitude was increased in the threat compared to the safe condition. The significant time-interval main effect indicated that light reflex and startle amplitudes (averaged across conditions) were reduced and increased respectively, as the test interval approached the time when shock was anticipated, while the condition by time-interval interaction indicated that these effects were greater in the threat condition. This interaction was significant for the light reflex and reached trend levels for the startle reflex (but improved after exclusion of Block 1), while the time interval linear trend which confirms the specificity of timing was significant for both the light and the startle reflex.

Temporal specificity for startle potentiation improved across the experiment and was sharper for Blocks 2 and 3 than for Block 1. In Block 1 the startle reflex was overall elevated as confirmed by the significant block main effect and moreover, it was initially increased, then decreased and then increased again. The elevated startle levels in the entire block 1 and its initial increase were probably due to contextual fear (from instructions and the onset of threat) which habituated thereafter and then, a gradual

increase in startle amplitude at the end of Block 1 can be attributed to the shock approaching. This contextual fear and its subsequent habituation in Block 1 might have opposed the specific effects of the threat of shock and this reflects to the improvement of the condition by time interval interaction which became significant when Block 1 was excluded from startle analysis. Indeed, temporal specificity for startle potentiation was much sharper for Blocks 2 and 3. In contrast, temporal specificity for the light reflex inhibition was similar for all blocks with no evidence for confounding habituation from Block 1 to 3. A progressive decrease in light reflex amplitude continued until the time when administration of shock was expected in contrast to our previous protocols when light reflex amplitude was inhibited at a constant level throughout the threat condition (Bitsios et al 1996), indicating that the magnitude of this fear-inhibition is sensitive to the exact instructions that are given. Also, the rapid recovery of light reflex amplitude after threat offset is in striking contrast to other autonomic measures that take minutes until return to baseline after threat offset (Farha & Sher 1989, Folkins 1970).

One interpretation of the present study is that the threat of shock created a state of fear that was responsible to the modulation of light and startle reflexes. However, the reflexes may have been modulated by a diffuse attentional effect (Deane 1969, Putnam 1990), rather than by fear, or subjects' attention may have increased as the aversive stimulus approached (Deane 1969, Lacey & Lacey 1974, Wood & Obrist 1968). It is important in this context that previous startle (Grillon et al 1993) and light reflex studies (Bitsios et al 2004, Hourdaki et al 2005) have demonstrated that instructions to attend to a barely perceptible, non-threatening stimulus did not affect startle or light reflex amplitudes compared to a "non-attend" condition suggesting that the observed modulation of the reflexes here was not due to stimulus anticipation per

se but instead to the aversive quality of the stimulus and the fear inherent to shock anticipation. Therefore, while anticipatory attention cannot entirely be ruled out, anticipatory anxiety seems to be the most plausible explanation for the present findings.

It is important in this context that the initial pupil diameter presented with a very different pattern; while it was overall increased in the threat compared to the safe condition replicating previous results (Bitsios et al 1996), it was not sensitive to the offset of threat. Instead, it increased remarkably whenever the condition was alternated independent of the nature of the condition and it quickly habituated within the threat and the safe conditions. These results suggest that pupil diameter captured an attention-to-context effect. In contrast to light reflex amplitude, pupil diameter also habituated from Block 1 to 3 suggesting that this measure was sensitive to the overall level of arousal related to novelty and aversion of experimental procedures. While non-threatening cues will not inhibit the light reflex (Bitsios et al 2004, Hourdaki et al 2005), they can increase the baseline pupil diameter especially when they have high novelty value or they pose attentional demands (Bitsios et al 2004); the increase in pupil diameter that is observed under threat is not sensitive to diazepam in contrast to threat-induced reduction in light reflex amplitude (Bitsios et al 1998, 1999), but it is sensitive to clonidine (Bitsios et al 1998) and noradrenergic drugs (Bitsios et al 1999a). It has been argued that the increase in pupil diameter by threat or novel cues reflects the organism's orienting arousal levels. This arousal-induced increase in baseline pupil diameter during threat is a pure sympathetically mediated response, since it can be abolished pharmacologically with blockade of the tone of sympathetic iris sphingter muscle via dapiprazole (an a1 antagonist) eye drops. In contrast, the sympathetic system does not participate in the the fear-inhibited light reflex since

reflex inhibition is not affected by dapiprazole eye-drops. Therefore, the inhibition of the light reflex by fear/threat is mediated by central parasympathetic inhibition, most probably at the level of the Edinger-Westphal nucleus, the motor centre of the reflex which receives inhibition from the subcortical defence system (amygdala, hypothalamus, central grey etc) (Giakoumaki et al 2005). Here we showed that a) light reflex amplitude may be equally or even more sensitive than startle amplitude in detecting changes in anticipatory anxiety and b) the light reflex allows the dissociation of specific anxiety effects from non-specific anxious arousal.

There is now a large body of evidence which shows that the potentiation of the startle reflex is mediated by the amygdala, a structure implicated in fear and anxiety (Gloor 1960; Mishkin and Aggleton 1981; Kapp et al. 1984; Sarter and Markovwsitsch 1985; Le Doux et al. 1988; Davis 1992). The central nucleus of the amygdala which has direct neural connections with the nucleus reticularis pontis caudalis (an obligatory point of the startle reßex pathway), has been especially implicated in the potentiation of the startle reflex (see Davis 1993 for a review). Thus, lesions of the central nucleus of the amygdala block the fear-potentiated startle reflex, without affecting the baseline startle reflex (Hitchcock and Davis 1986, 1991). We demonstrated here that a common property between startle potentiation and light reflex inhibition by threat of shock is their temporal specificity. Another common property of the fear-potentiated startle reflex and fear-inhibited light reflex is their dose-dependency on the anxiolytic diazepam (Bitsios et al 1998, 1999). It is therefore an intriguing possibility that similar mechanisms operate both in the fear-inhibited light reflex and the “fear-potentiated startle reflex” (Grillon et al. 1991; Davis 1992; Davis et al. 1993). It is known that the pupillary light reflex is under tonic inhibitory control from the hypothalamus (Loewenfeld 1958, 1993, pp 407.480; Koss 1984, 1986) via

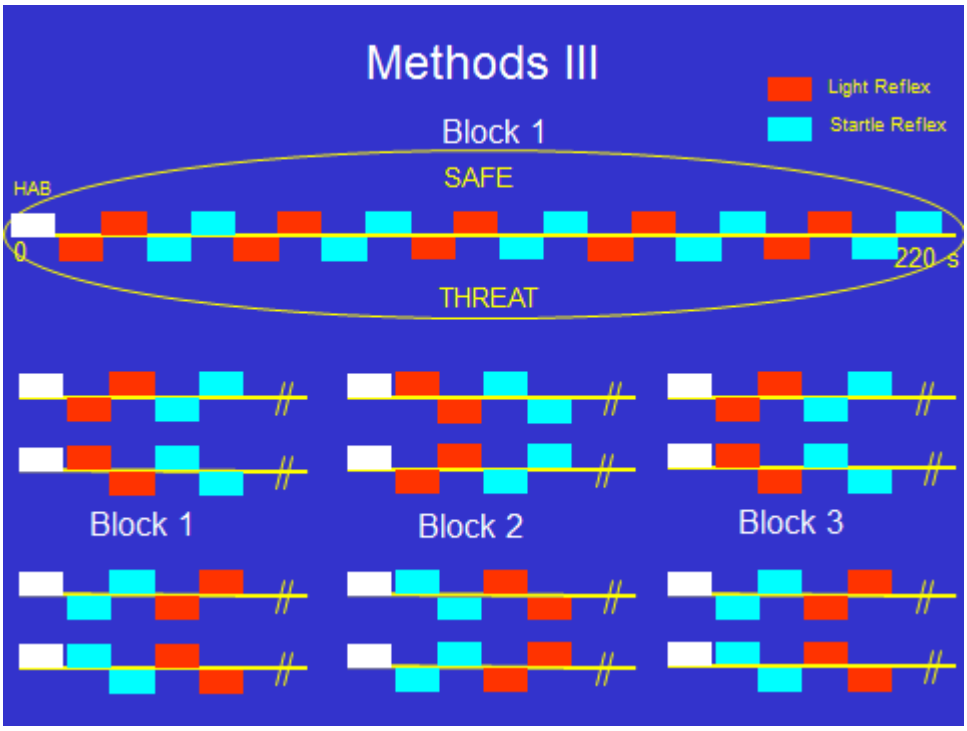
connections between the hypothalamus and the Edinger-Westphal nucleus (Saper et al. 1976). There is also evidence that stimulation of the amygdala causes pupillary dilatation in the cat (Koikegami and Yoshida 1953; de Molina and Hunsberger 1962), probably via well-known excitatory amygdalo-hypothalamic connections (Le Doux 1988; Davis 1992). It is thus possible that stimulation of the amygdala by conditioned aversive stimuli enhances the inhibitory influence of the hypothalamus on the Edinger-Westphal nucleus, resulting in enhancement of the inhibition of the pupillary light reflex. Furthermore, diazepam may antagonize the threat-evoked reduction in light reflex amplitude and the threat-evoked increase in subjective anxiety by preventing the activation of the amygdala-hypothalamus complex by threat (Bitsios et al 1998, 1999), as has been shown to be the case with the fear-potentiated startle reflex.

We have shown in this study that, similar to animal research, shock anticipation in human subjects potentiates startle and inhibits light reflex and that both these phenomena show good temporal specificity. The present protocol could provide a sensitive tool for exploring both normal and pathological anxiety. Some individuals may have a high level of startle potentiation or light reflex inhibition, yet have a normal time course of both the onset and the offset of these changes in startle and light reflex. Other individuals may have normal magnitude of startle potentiation or light reflex inhibition but an abnormal time course of fear onset or offset. Fear onset and offset are active processes mediated by specific brain structures and sensitive perhaps to different classes of drugs, and this paradigm could lead to better treatments for certain types of anxiety disorders. While the older protocol modeled phasic fear to imminent threat, the current protocol appears to better model sustained anxiety to temporally distant or uncertain danger. Psychopharmacological and behavioral

evidence from ethological studies distinguishes between these two forms of anxiety which can be traced back to distinct neuroanatomical systems, the amygdala and the bed nucleus of the stria terminalis (Grillon 2008). Progress in our understanding of normal and abnormal anxiety is critically dependent on our ability to model sustained aversive states to temporally uncertain threat. The current protocol may be an experimental model of anxiety, not fear/panic, and more relevant to non-phobic anxiety disorders.

Figure 1

Top panel: first line: Safe/Threat blocks of light and startle reflex. Second and third lines: The beginnings of each of the three blocks are shown in order to illustrate the counterbalancing of safe and threat blocks. Bottom: Experimental procedure during a block that started with a threat condition. The expanded portion of the figure (bottom) shows the position of the light (or startle) stimuli.



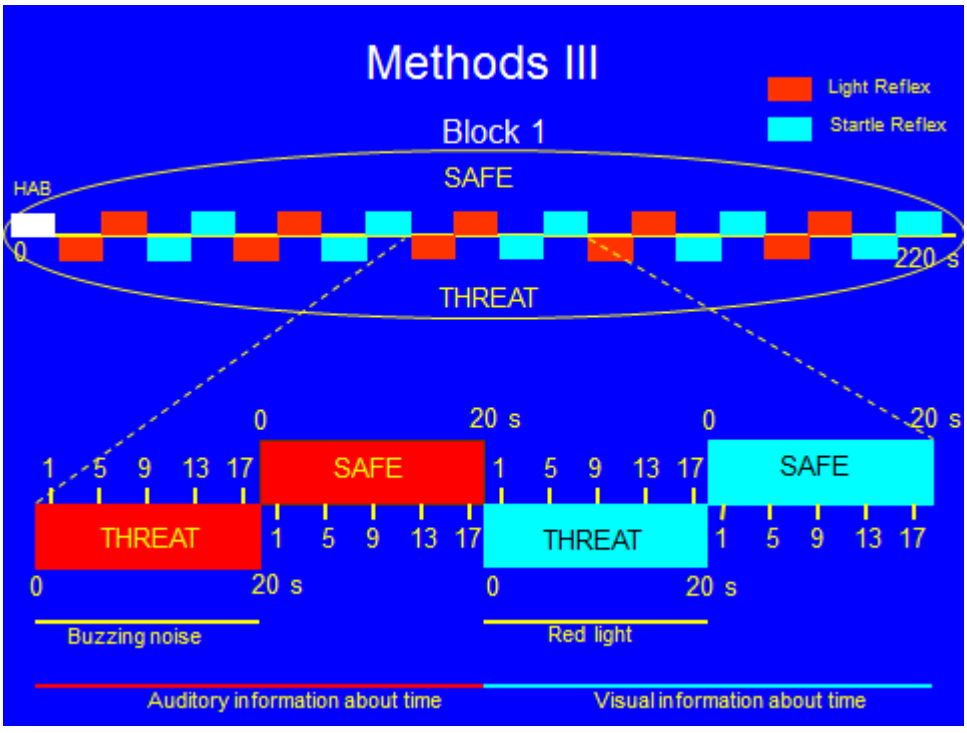


Figure 2

Light reflex amplitude over successive time intervals in the threat (top left) and safe (top right) conditions over the three blocks and collapsed across blocks (bottom). The vertical line indicates offset of the threat and onset of the safe condition. Note the increased light reflex inhibition as the time of the expected shock administration came closer in the threat condition and the rapid cessation of this effect in the safe condition. Top: Blue for Block 1, Yellow for Block 2 and Red for Block 3.

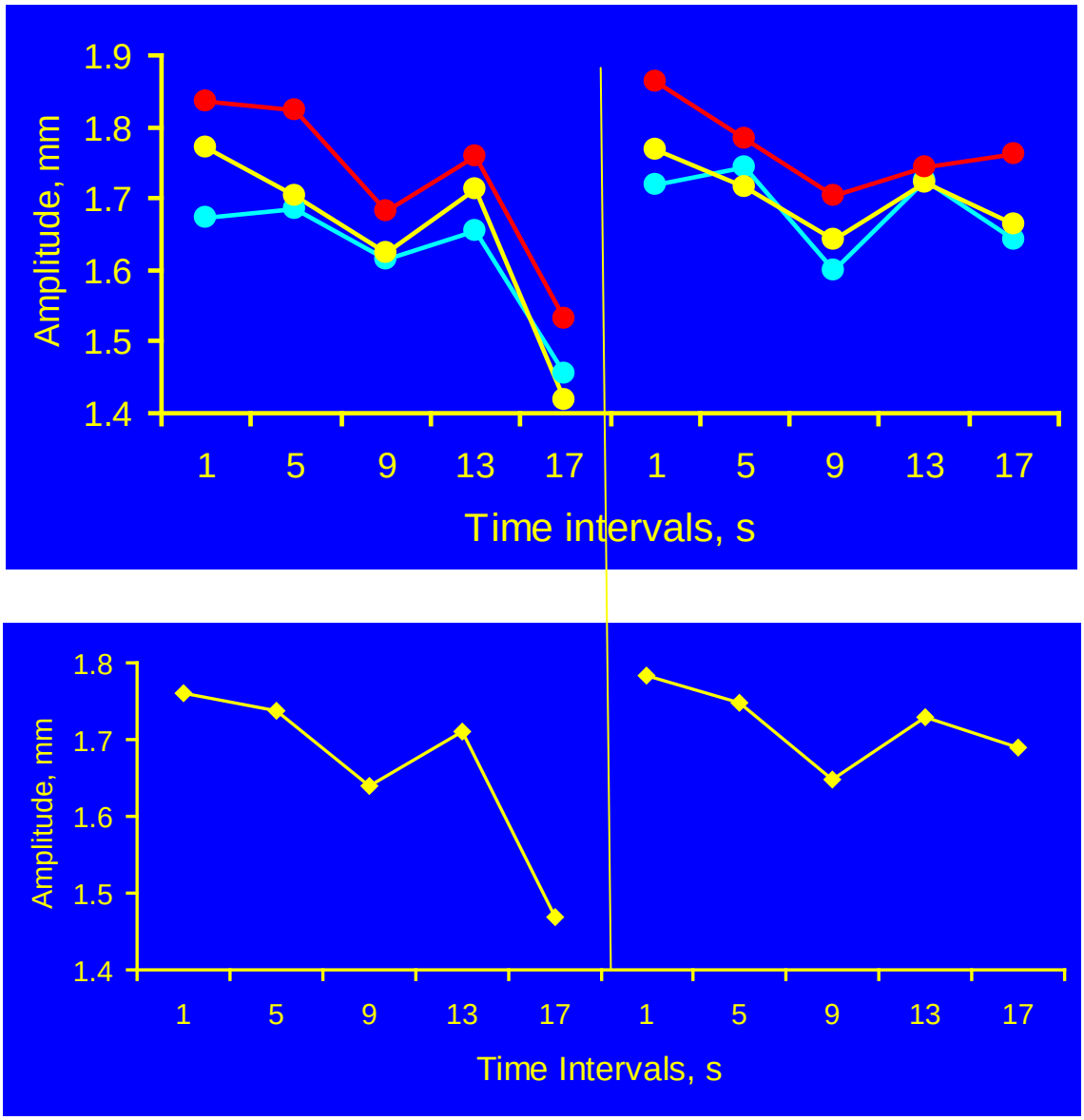


Figure 3

IPD over successive time intervals in the threat (top left) and safe (top right) conditions over the three blocks and collapsed across blocks (bottom). The vertical line indicates offset of the threat and onset of the safe condition. Note the increased IPD at the onset of both the threat and the safe conditions and its rapid habituation thereafter. Compare to this effect, the increase in IPD as the time of the expected shock administration came closer in the threat condition was relatively small. Top: Blue for Block 1, Yellow for Block 2 and Red for Block 3.

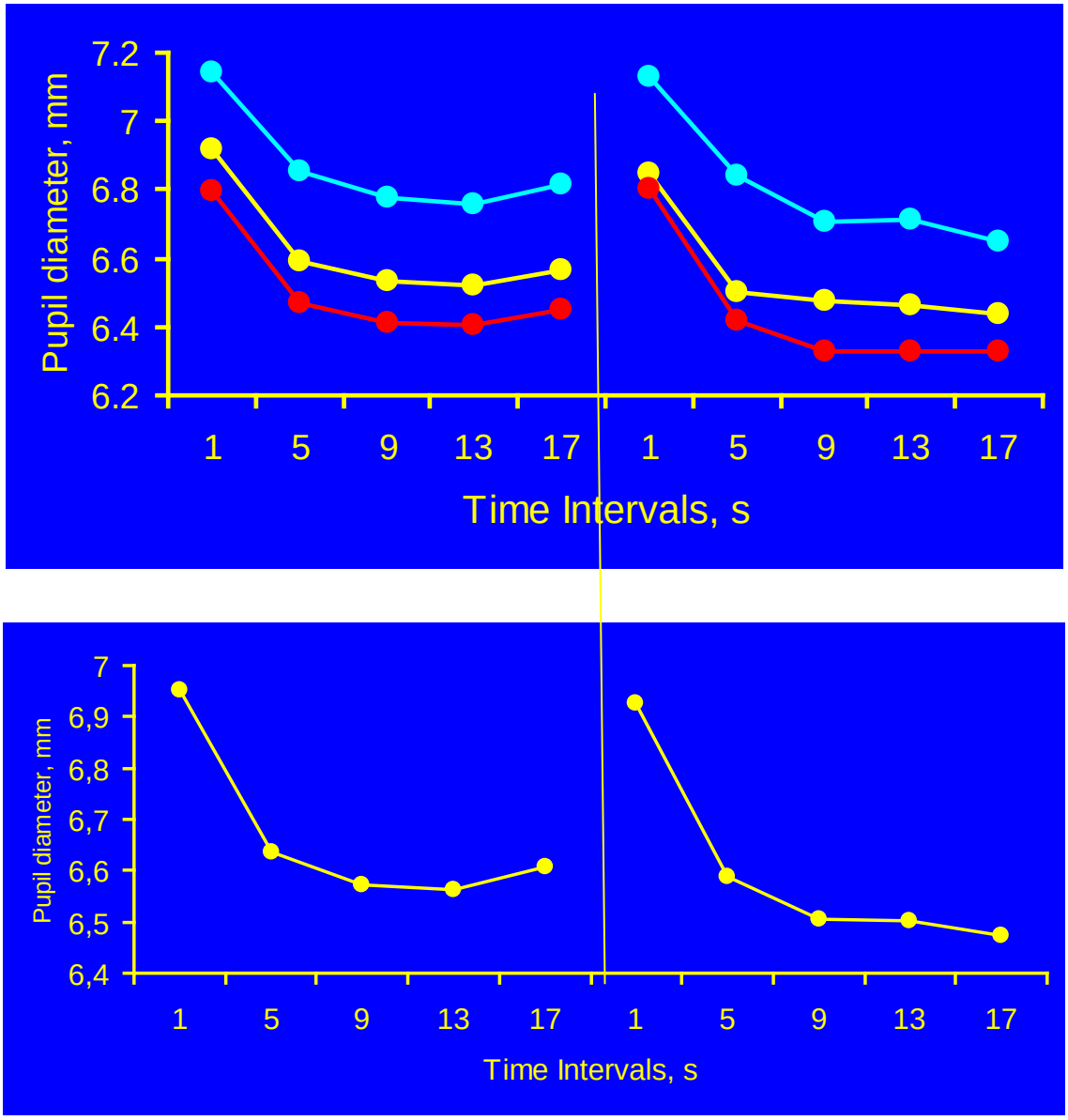
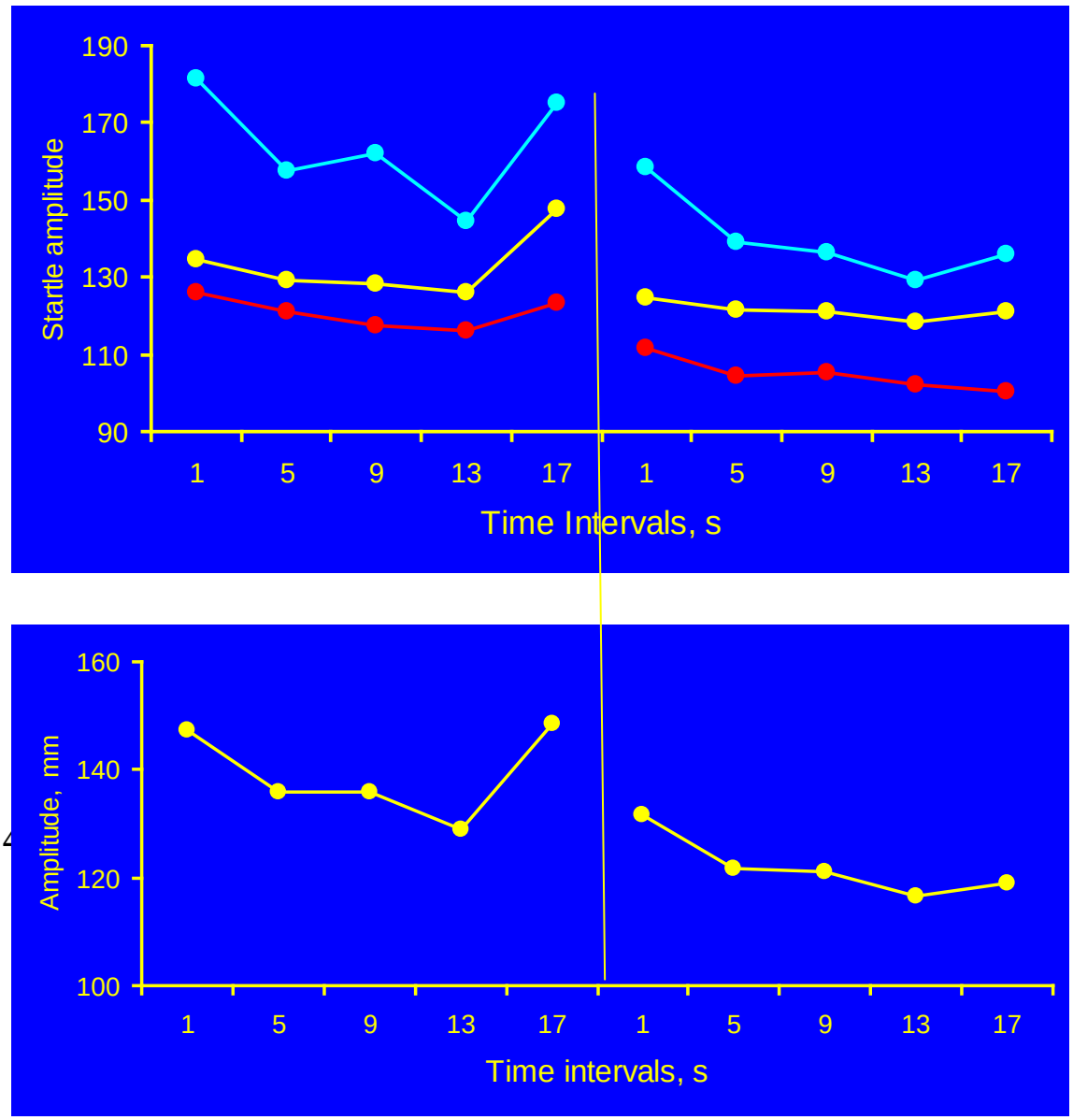


Figure 4

Startle reflex amplitude over successive time intervals in the threat (top left) and safe (top right) conditions over the three blocks and collapsed across blocks (bottom). The vertical line indicates offset of the threat and onset of the safe condition. Note the increased startle inhibition as the time of the expected shock administration came closer in the threat condition and the rapid cessation of this effect in the safe condition. Top: Blue for Block 1, Yellow for Block 2 and Red for Block 3.



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Poster 174
ONE WEEK TOLCAPONE TREATMENT IN PSYCHOTIC PATIENTS: EFFECTS ON GATING, WORKING MEMORY AND CLINICAL PICTURE

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Background: A single dose of the COMT inhibitor tolcapone improves gating and working memory in partially remitted psychotic patients. We explored the therapeutic potential of tolcapone, by examining the effects of one week administration on gating, working memory and clinical picture in psychotic patients.
Methods: Fourteen medicated partially remitted psychotic patients received placebo and tolcapone-200mg/day for one week each, according to a double-blind, crossover design. At the end of each treatment, we assessed PPI with 75- and 85-dB prepulses at 30-, 60- and 120-ms intervals, working memory with the letter-number sequencing (LNS) task and symptom improvement with the Clinical Global Impression-Improvement (CGI-I) scale.
Results: Tolcapone did not affect startle amplitude or habituation. A 2×2×3×2 (treatmentXprepulseXintervalXgender) ANOVA of the PPI data showed a significant treatmentXintervalXgender interaction ($p = 0.014$), indicating tolcapone-induced PPI increases at 60 ms and 120 ms in males and at 30 ms in females. These results survived when SANS total score (which correlated negatively with delta tolcapone effect on mean PPI) as well as antipsychotic treatment expressed as chlorpromazine equivalents were taken as covariates. In addition, a significant treatment main effect ($p < 0.04$) was revealed. Separate 2×2 (treatmentXgender) ANOVAs showed that tolcapone improved LNS performance (treatment $p = 0.012$) and CGI-I score ($p < 0.001$).
Discussion: Weekly tolcapone administration improved gating and working memory and, importantly, the clinical profile of non-genotyped psychotic patients. These preliminary findings have intriguing therapeutic implications in the targeted treatment of cognitive deficits and symptoms of psychosis. More impressive effects are anticipated when patient samples are stratified for COMT status.

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Poster 175
LURASIDONE IN THE TREATMENT OF ACUTE SCHIZOPHRENIA: RESULTS OF THE DOUBLE-BLIND, PLACEBO-CONTROLLED PEARL 2 TRIAL

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Background: Lurasidone is a new psychotropic agent with high affinity for D_2 and 5-HT_{2A} receptors, and for receptors implicated in enhancement of cognition, mood and negative symptoms (5-HT_7, 5-HT_{1A} and α_{2C}). The aim of this study was to evaluate the efficacy and safety of lurasidone in patients with an acute exacerbation of schizophrenia.
Methods: Hospitalized patients 18-75 years old who met DSM-IV criteria for schizophrenia and were acutely ill with a PANSS total score ≥80 were eligible for enrollment. Subjects were tapered off psychotropic medication and after a 7-day placebo washout period were randomized to 6-weeks of once-daily double-blind treatment with lurasidone 40 mg or 120 mg, olanzapine 15 mg or placebo. After 3 weeks, patients were eligible for discharge if sufficiently stable and improved. A mixed model repeated measures (MMRM) analysis was performed for the efficacy measures: the Positive and Negative Symptoms of Schizophrenia Scale (PANSS) total and subscale scores and the Clinical Global Impression, Severity scale (CGI-S).
Results: Demographic and clinical characteristics were similar at baseline among patients randomized to the four treatment groups: lurasidone 40 mg (n = 119; mean PANSS total, 96.6); lurasidone 120 mg (n = 118; mean PANSS total, 97.9); olanzapine 15 mg (n = 122; mean PANSS total, 96.3); and placebo (n = 114; mean PANSS total, 95.8). Treatment with lurasidone was associated with significantly greater improvement on the PANSS total score versus placebo (-16.0) among patients in the 40 mg (-25.7; $P < 0.001$) and 120 mg (-23.6; $P = 0.011$) dosage groups at Week 6. Treatment with lurasidone was also associated with significantly greater improvement on the PANSS positive subscale score versus placebo (-5.4) in the 40 mg (-7.7; $P = 0.018$) and 120 mg (-7.5; $P = 0.035$) dosage groups; and on the PANSS negative subscale score versus placebo (-3.6) in the 40 mg (-6.0; $P = 0.002$) and 120 mg (-5.2; $P = 0.045$) dosage groups. On the CGI-S, significant improvement was observed versus placebo (-1.1), during treatment with both the 40 mg (-1.5; $P = 0.006$) and 120 mg (-1.4; $P = 0.040$) doses of lurasidone. Olanzapine 15 mg/day produced significantly greater improvements than placebo on both the PANSS total score (-28.7 vs. -16.0; $P < 0.001$), PANSS positive subscale (-9.3 vs. -5.4; $P < 0.001$), PANSS negative subscale (-6.2 vs. -3.6; $P < 0.001$), and CGI-S (-1.5 vs. -1.1; $P < 0.001$). A weight increase > 7% was observed in 7.2% of patients treated with lurasidone (combined doses), 40.2% treated with olanzapine, and 8.7% treated with placebo. Change in median cholesterol was similar during treatment with lurasidone (-7.0 mg/dL, combined doses) and placebo (-5.0 mg/dL), but was increased during treatment with olanzapine (+9.0 mg/dL). Change in median triglycerides was also similar during treatment with lurasidone (-1.0 mg/dL, combined doses) and placebo (+1.0 mg/dL), but was increased during treatment with olanzapine (+24.0 mg/dL).
Discussion: The results of this multicenter, double-blind, placebo-controlled, Phase 3 trial indicate that lurasidone, at fixed doses of 40 and 120 mg/day, is a safe and effective treatment for patients with an acute exacerbation of schizophrenia. The efficacy of both doses of lurasidone was established based on results for both the PANSS total and CGI-S scores, and for the PANSS positive and negative subscale scores. No dose-response relationship was observed on the PANSS total or subscale scores, or the CGI-S scores. Treatment with lurasidone was not associated with adverse effects on weight, metabolic, or ECG parameters. Based on these findings and results of previous trials, lurasidone may be a useful addition to the treatment armamentarium for schizophrenia. ***Funded by Dainippon Sumitomo Pharma.***

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Poster 176
COMMUNITY TREATMENT ORDERS, ETHNICITY, CONDITIONS AND PSYCHOTROPIC MEDICATION: THE FIRST SIX MONTHS (N = 126)

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Background: Community treatment order (CTO) legislation was initiated in November 2008 in England and Wales. Due to high rates of uptake, a shortage of second opinion appointed doctors (SOADs) to

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ORIGINAL INVESTIGATION

Comparison of ketanserin, buspirone and propranolol on arousal, pupil size and autonomic function in healthy volunteers

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Abstract
Rationale The human pupil may be a suitable physiological test system for the assessment of excessive daytime sleepiness (EDS), but pupillometric assessment could be confounded by medication for comorbid hypertension and mood disorders.
Objectives We examined the profile of the 5HT-2/α1/H1 antagonist ketanserin, the 5HT1a agonist buspirone and the beta adrenoceptor antagonist propranolol on pupillary and other measures of arousal.
Materials and methods Ketanserin (20 mg), buspirone (10 mg) and propranolol (40 mg) were administered in three independent experiments according to a crossover, placebo-controlled, double-blind design. Resting pupil diameter (RPD) was sampled over 5-min in darkness with infrared pupillometry. Tests also included critical flicker fusion frequency (CFFF), visual analogue scales (VAS), the pupillary light reflex and heart rate/blood pressure.
Results Ketanserin reduced RPD, CFFF, VAS-rated arousal and blood pressure and increased the light reflex amplitude. Buspirone reduced RPD and blood pressure. Propranolol reduced heart rate but had no effects on pupillary functions or any arousal measure.
Conclusions Ketanserin but not propranolol had a fully sedative profile and may confound pupillometric assessment of EDS. Beta adrenergic receptors do not appear to participate in arousal and pupillary functions, while 5HT1a receptors reduce pupil size without affecting arousal. Pupil size may not be used unequivocally as an index of the level of alertness in the case of drug-induced changes, when drugs interfere with the central pupil control mechanism in ways that are unrelated to their effects on arousal.

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Keywords Pupil size · Light reflex · Arousal · Pupillary alertness test · Critical flicker fusion frequency · Drug-induced sedation

Introduction

It has long been known that any decrease in arousal is accompanied by a decrease in pupil diameter (Loewenfeld 1993), and assessment of pupil diameter is routinely used by anaesthetists when gauging the depth of anaesthesia (Aitkenhead et al. 2001). Pupil size in darkness has been successfully used as a single physiological measure of arousal in patients suffering from excessive daytime sleepiness (EDS) due to obstructive sleep apnea (Bitsios et al. 2006); compared to age- and sex-matched controls, the sleepy patients showed smaller pupil size, which correlated with objective indexes of apnea severity and subjective measures of sleepiness, the differences becoming more apparent during the afternoon circadian nadir (Bitsios et al. 2006). Moreover, pupil size was sensitive to the alerting effects of modafinil in patients with EDS as a result of obstructive sleep apnea (Nikolaou et al. 2008).

Recent evidence points to the importance of metabolic factors, hypertension and depression in the aetiology of EDS (Bixler et al. 2005), and treatment for these conditions is not uncommon among these patients. If monitoring of resting pupil size is to be more regularly incorporated in future studies as a clinical tool for the objective assessment

of EDS, it would be important to understand the pupillary effects of the various drugs prescribed for these patients. In this study, we sought to determine the effects of single doses of ketanserin, buspirone and propranolol on pupillary behaviour of healthy subjects in three separate experiments. If these drugs alter pupil size, they might interfere with the pupillometric determination of alertness in patients suffering from EDS (Bitsios et al. 2006; Nikolaou et al. 2008) if the above drugs are prescribed for treatment of comorbid hypertension or mood disorders.

Ketanserin is an anti-hypertensive agent with sympatholytic effects, via central 5HT2-mediated modulation of the sympathetic system (Cameron et al. 1987). It is also an α_1 adrenergic and histamine receptor antagonist (Dollery 1999). Ketanserin 20 mg has been reported to reduce critical flicker fusion frequency (CFFF; Graham et al. 2002) and sustained attention (Wingen et al. 2007) and it is considered a sedative drug (Dollery 1999) although clinically, its effects on arousal may not be as profound (Herrmann and Baumgartner 1986). Propranolol is another widely used antihypertensive agent with sympatholytic properties via peripheral beta adrenoceptor blockade on the vascular bed. It can also behave as a 5HT1a antagonist and a 5HT1b agonist in the rat cortex (Pierson et al. 1989). Propranolol is not considered a sedative drug (Currie et al. 1988), and there are mixed reports regarding its ability to reduce arousal; impaired psychomotor performance has been reported after single doses (Landauer et al. 1979; Salem and McDevitt 1984), but other studies have failed to show such effects (Currie et al. 1988; Harmer et al. 2001; Ogle et al. 1976; Tyrer and Lader 1974). Buspirone is a non-sedative anxiolytic and a partial agonist at the 5-HT1A receptor (Andrade and Nicoll 1987), with some affinity for the dopamine D2 receptor (Jann 1988; Peroutka 1985; Riblet et al. 1982). Buspirone has a dose-dependent miotic effect in healthy human subjects (Fanciullacci et al. 1995; Phillips et al. 1999), but the mechanism remains unclear (Phillips et al. 1999).

A reduction in pupil size by a drug may be due to the reduction of the sympathetic input to the iris, increase of the parasympathetic input to the iris or both. In order to examine the relative contributions of the sympathetic and the parasympathetic systems in a putative effect of these drugs on pupil size, we examined their effect on the pupillary light reflex. The pupillary light reflex may help to elucidate the effects of a drug on the sympathetic and parasympathetic inputs to the iris, since the time course of the light reflex response is determined by the successive activation of the parasympathetic and sympathetic inputs; the amplitude reflects activation of the midbrain parasympathetic Edinger–Westphal nucleus (Barbur 2004; Gamlin et al. 1997), while the recovery time reflects mainly sympathetic activation, which resumes at the end of the light stimulus and recovers the pupil to its original levels (Bitsios et al. 1998a; Loewenfeld 1999; Smith and Smith 1999). However, because recovery time also depends, by definition, on initial (baseline) pupil diameter (Loewenfeld 1993) and is, in the experience of our lab, susceptible to blinks, we measured only latency and amplitude of the light reflex. Finally, we also examined the effects of these drugs on other measures of arousal such as CFFF (Smith and Misiak 1976) and visual analogue scales (VAS) (Bond and Lader 1974) and cardiovascular indices of autonomic function such as heart rate and blood pressure.

Materials and methods

Subjects

In all three experiments, subjects were between 18 and 30 years old, with a body mass index (BMI) in the normal range. Inclusion criteria included written informed consent, absence of personal history of head trauma, medical and neurological conditions or use of prescribed and recreational drugs and absence of personal or family (up to second-degree relatives) history of Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition Axis I disorders. All participants underwent physical and psychiatric assessment using the Mini-International Neuropsychiatric Interview (Sheehan et al. 1998), an ophthalmological examination and a urine drug screen test. All subjects were regular caffeine (two to three cups of coffee per day on average) and occasional alcohol consumers. Subjects' demographic characteristics in experiments I (ketanserin), II (buspirone) and III (propranolol) are shown in Table 1. The study was approved by the University of Crete Ethics Committee.

Design and drugs

Ketanserin, buspirone and propranolol were administered in a placebo-controlled, within-subject design in three separate experiments, using separate groups of subjects but identical experimental procedures. In each experiment, subjects participated in two weekly sessions (returning to the laboratory at the same time each week for each session). Subjects were allocated to treatments and sessions according to a double-blind, balanced, crossover design. Ketanserin 20 mg, buspirone 10 mg, propranolol 40 mg and placebo were prepared in identical capsules and administered orally. The choice of dose of each drug was based on centrally bioactive doses reported in published studies [ketanserin (Graham et al. 2002), buspirone (Phillips et al. 1999) and propranolol (Grillon et al. 2004)].

Table 1 Subjects' demographic characteristics in each experiment

	Experiment I, Ketanserin	Experiment II, Buspirone	Experiment III, Propranolol	*p* value
Sample size	12	12	12	NA
Male/female[a]	6:6	6:6	6:6	1
Age (years)	24.8(4.0)	25.3(3.0)	24.8(3.6)	>0.9
BMI	24.3(3.9)	23.5(3.1)	22.2(2.9)	>0.3
Education (years)	16.3(2.4)	17.3(1.6)	17.3(1.8)	>0.4
Smokers/non-smokers[a]	4:8	4:8	8:4	>0.1

Figures in brackets are SD

[a] For these measures, chi-square comparisons were applied. All other variables were analysed with one-way ANOVAs

Tests and apparatus

Resting pupil diameter

A binocular infrared video pupillometer (Procyon P2000D, Procyon, London, UK; sampling rate, 25 Hz; spatial resolution, >0.05 mm; accuracy, >±3%) was used to monitor RPD in darkness. Our methodology of recording pupil diameter [5-min Pupillary Alertness Test (5-min PAT)] has been described in detail previously (Bitsios et al. 2006, Nikolaou et al. 2008). Pupil diameter was sampled for 15 consecutive 20-s periods, and thus, the total monitoring time was 300 s. The outcome measures were the average RPDs for each one of the 15 20-s periods and the collapsed RPD for the entire 300-s recording. Data were stored for off-line cleaning from spontaneous blinks, scoring and statistical analysis.

The light reflex

The light reflex was elicited and recorded in darkness, following testing with the 5-min PAT. The stimuli were light flashes of 200-ms duration delivered via a light-emitting diode, presented to the subject's right and left eye in an alternating fashion, as a white disc of 8° diameter, providing 'full retinal field' light stimulation (at four levels of stimulus luminance, 0.35, 5, 50 and 140 cd m^{-2}), while the non-stimulated eye was fixating a target dot projected at a distance of approximately 10 m. Each one of the 4 levels of stimulus luminance was presented in a block of three stimuli, the average of which was the response for that luminance level. The inter-stimulus interval within blocks was fixed at 7 s. Therefore, the total time for the elicitation and recording of the light reflex was 80 s. Stimulus presentation was computer controlled, and pupillary measures were digitised and stored for off-line analysis. Using the automated manufacturers' software, the parameters studied were light reflex latency (i.e. the time elapsed from onset of the light flash until the onset of a pupillary response) and light reflex response amplitude [i.e. the difference between the baseline (defined as the mean pupil diameter recorded over 500 ms before the onset of the light stimulus) and the diameter reached at the trough of the pupillary response to the light stimulus].

Critical flicker fusion frequency The Leeds Psychomotor Tester (Psychopharma, Surrey, UK) was used to collect CFFF measurements, defined as the frequency at which a flickering light appears to be continuous (Smith and Misiak 1976). The CFFF is sensitive to sedative drugs. Subjects viewed the stimulus through a 2-mm 'artificial pupil'. The CFFF test was conducted conventionally, with eight threshold measurements collected per session: four with increasing frequencies and four with decreasing frequencies. The mean of the eight measurements was taken as the value of the CFFF (see Samuels et al. 2006).

Visual analogue scales A computerised version of the VAS was used to collect self-ratings of alertness, contentedness and anxiety. Nine contrasting statements were rated along a continuous 10-cm line to represent the participant's subjective alertness (Norris 1971). The ratings on the nine items were multiplied by their respective factor loadings based on a factor analysis carried out by Bond and Lader (1974) and the mean of the weighted values entered the analysis.

Cardiovascular measures

Blood pressure and heart rate recordings were taken in the sitting position using an electroaneroid sphygmomanometer.

Procedure

After arrival in the laboratory, each subject had a 15-min acclimatisation period, after which the pretreatment tests (recordings of heart rate, blood pressure and VAS) were

carried out. The testing was completed in 5 min. On completion of pretreatment tests, the subjects ingested the capsule containing either the active drug or placebo. The pretreatment tests were repeated post-ingestion, together with recordings of the 5-min PAT and the pupillary light reflex (post-treatment tests). The time course of the sessions was based on the pharmacokinetics of the active drugs: t_{max} is 1 h following oral administration of single doses of propranolol (Hardman et al. 2001), ketanserin (Brogden and Sorkin 1990) and buspirone (Sakr and Andheria 2001).

Data analysis

In each experiment, the average RPDs for each one of the 15 20-s periods were analysed with a mixed model analysis of variance (ANOVA) with period (15 levels) as the within- and order (placebo then drug and drug then placebo), treatment (placebo and active drug), gender (male and female) and smoking status (smokers and non-smokers) as the between-subject factors. Light reflex variables (latency and amplitude) were analysed with separate 2×4 (treatment×luminance level) repeated measures ANOVAs. The pre-post treatment differences in CFFF, VAS and cardiovascular measures were subjected to paired samples t tests.

Results

Resting pupil diameter (5-min PAT) Figure 1 shows the RPD values for each one of the 15 20-s periods for the drug treatment conditions in experiments I, II and III. In all three experiments, RPDs were becoming progressively smaller from the first to the 15th period in all treatment conditions. In experiment I (ketanserin), a 2×2×2×2×15 (order × treatment × gender × smoking status × period) ANOVA showed significant main effects of treatment [$F(1,5)=13.3$, $p<0.015$] and period [$F(14,70)=3.02$, $p<0.001$]. All other main effects and interactions were non-significant (all p values >0.09). Identical analysis in experiment II (buspirone) showed significant treatment [$F(1,5)=7.0$, $p<0.05$] and period [$F(14,70)=2.9$, $p<0.002$] main effects. All other main effects and interactions were not significant (all p values >0.2). Finally, identical analysis in experiment III (propranolol) did not reveal any significant main effects or interactions (all p values >0.3). A 3×2×2×2×15 (treatment group × order × gender × smoking status × period). ANOVA of the placebo data only showed that RPDs in the three treatment groups did not differ in the placebo condition [group main effect and all interactions involving group ($F<1$)].

Light reflex In all three experiments, latency was reduced, and amplitude was increased with increasing light intensity, as evidenced by expected significant main effects of light intensity for these measures [latency; $F_{propranolol}(3,33)=26.7$, $p<0.001$; $F_{ketanserin}(3,33)=19.8$, $p<0.001$; $F_{buspirone}(3,33)=18.1$, $p<0.001$; amplitude: $F_{propranolol}(3,33)=287.0$, $p<0.001$; $F_{ketanserin}(3,33)=102.1$, $p<0.001$; $F_{buspirone}(3,33)=71.3$, $p<0.001$] (Fig. 2).

There was no significant treatment main effect for latency in any experiment (all $p>0.1$). Ketanserin increased light reflex amplitude as evidenced by a significant treatment main effect [$F(1,11)=8.1$, $p=0.016$] in experiment I, while propranolol and buspirone had no effect on this measure [$F(1,11)=1.2$, $p>0.3$, and $F<1$, respectively]. There was no significant treatment by light intensity interaction for any measure in any experiment.

Critical flicker fusion frequency The post-pre treatment differences are shown in Table 2. There were significant treatment effects in experiment I (ketanserin) ($t=3.48$, df 11, $p<0.005$) but not in experiments II (buspirone) and III (propranolol; $t=0.15$, df 11, $p>0.88$ and $t=-1.27$, df 11, $p>0.23$, respectively).

VAS alertness The post-pre treatment differences are shown in Table 2. There were significant treatment effects in experiment I (ketanserin; $t=3.1$, df 11, $p<0.01$) but not in experiments II (buspirone) and III (propranolol; $t=-0.34$, df 11, $p>0.7$ and $t=-0.04$, df 11, $p>0.9$, respectively).

Cardiovascular measures The post–pretreatment differences in heart rate, systolic and diastolic blood pressure are shown in Table 2. Heart rate was significantly reduced in experiment III (propranolol; $t=2.6$, df 11, $p<0.025$), while diastolic but not systolic blood pressure was significantly reduced in experiments I (ketanserin) and II (buspirone; $t=3.55$, df 11, $p<0.005$; and $t=2.28$ df:11, $p<0.05$ respectively).

Discussion

Table 2 shows that in the doses used, all treatments showed some evidence of bioactivity. Ketanserin reduced diastolic blood pressure, consistent with its antihypertensive properties, which are thought to be centrally mediated via the suppression of sympathetic nerve activity (McCall and Schuette 1984; Ramage 1985). Ketanserin reduced RPD and increased the light reflex amplitude, suggesting a role of 5HT2 receptors in mediating pupil size and the light reflex. Two previous reports failed to show an effect of ketanserin on the pupil (Costagliola et al. 1991; Tekat et al. 2001), but the observed ketanserin-induced miosis in our study is in agreement with previously reported miotic effects of the selective 5HT2 antagonists ICI 169,369 and ICI 170,809 (Millson et al. 1991; Millson et al. 1992).

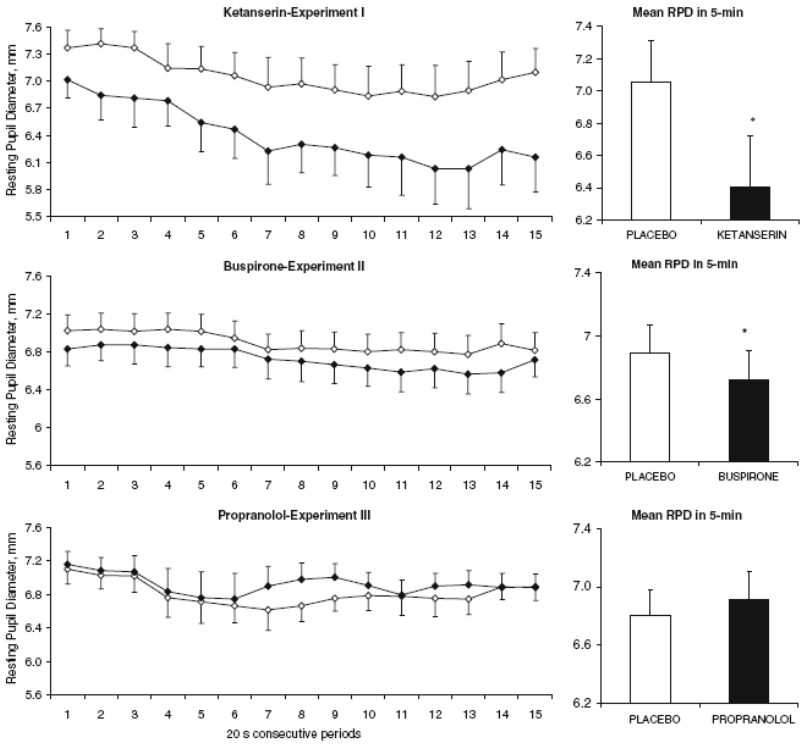

Fig. 1 Resting Pupil Diameter (RPD) values for the 15 20-s periods (*left*) and mean RPD in 5 min (*right*) for the placebo (*white diamonds*) and the drug treatment conditions (*black diamonds*) in the three experiments

Although preclinical studies in rabbits would support a direct involvement of 5-HT2 receptors in controlling pupillary responses (Tobin et al. 1988), local application of ketanserin has been reported to have no effect in human subjects (Costagliola et al. 1993). Therefore, the observed pupillary effects of ketanserin cannot be easily attributed to peripheral effects of the drug on the iris. The reduction in pupil size by ketanserin is consistent with a postulated sympatholytic effect of this drug, but this cannot account for the increase in light reflex amplitude, since the latter is a parasympathetically mediated response (Loewenfeld 1993). This pattern of effects on pupillary size and kinetics is similar to that observed by clonidine (Bitsios et al. 1998a). Clonidine is an a2-adrenoceptor agonist whose effects in

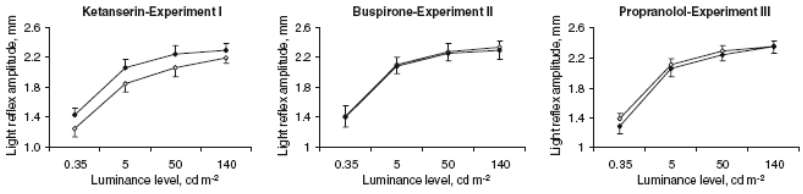

Fig. 2 Light reflex amplitude for the placebo (*white diamonds*) and the drug treatment conditions (*black diamonds*) in the three experiments

Table 2 Critical flicker fusion frequency (CFFF), visual analogue scales (VAS), heart rate and blood pressure post–pretreatment differences [group means(SD)] in each experiment

Test	Experiment I		Experiment II		Experiment III	
	Placebo	Ketanserin	Placebo	Buspirone	Placebo	Propranolol
Δ CFFF	0.13 (0.4)	−1.4 (1.7)*	−0.09 (0.8)	−0.16 (1.7)	−0.2 (0.7)	0.1 (1.5)
Δ VAS alertness	−0.0 (0.06)	−0.13 (0.1)*	−0.0 (0.04)	0.0 (0.06)	−0.0 (0.04)	−0.0 (0.08)
Δ Heart rate	−3.3 (6.6)	−4.7 (5.6)	−7.7 (4.7)	−3.7 (6.5)	−5.3 (6.6)	−12.7 (9.0)*
Δ Systolic BP	−6.3 (7.1)	−10.0 (6.7)	−2.1 (5.0)	−4.6 (5.8)	−7.9 (8.4)	−7.5 (6.9)
Δ Diastolic BP	5.0 (7.4)	−5.0 (8.3)*	1.3 (5.7)	−2.9 (3.3)*	−2.1 (5.4)	0.0 (8.5)

*$p<0.05$

man are generally attributed to a sympatholytic action resulting from stimulation of pre-synaptic inhibitory a2-adrenoceptors located on the cell bodies and dendrites of central noradrenergic neurones; these neurones have an excitatory effect on sympathetic function (Szabadi and Bradshaw 1996). Central noradrenergic neurones located in the locus coeruleus send an inhibitory projection to the Edinger–Westphal nucleus (Szabadi and Bradshaw 1996). Thus, the 'switching off' of the central noradrenergic neurones due to the activation of inhibitory a2-adrenoceptors by clonidine results not only in a decrease in sympathetic outflow but also in the removal of the noradrenergic inhibition of the pupillary light reflex, leading to an increased miotic response of the pupil after a light stimulus. The observed clonidine-like effects of ketanserin on pupil size and kinetics raise the possibility that this drug may attenuate central inhibition of Edinger–Westphal neurones, leading to an enhancement of the effect of light on the pupil. Ketanserin showed a sedative profile in the CFFF and VAS-rated alertness, and it is interesting in this respect that this drug, similarly to clonidine, reduced salivation and alertness and had a clonidine-like profile in the waking electroencephalogram (Reimann et al. 1986). All the above, taken together, strengthen the notion that pupil size is a physiological correlate of central arousal levels and suggest that ketanserin reduces alertness and pupil size, via an action on the 'arousal/pupil control interface', which is likely to be the locus coeruleus and its connections (Szabadi and Bradshaw 1996; Hou et al. 2005, Hou et al. 2007b).

The precise mechanism and neuronal circuitry involved remain to be elucidated, but it is likely that these ketanserin effects were mediated through antagonism at the 5-HT2 receptor for which the drug shows high affinity (p*Ki* of 9.5; Branchek et al. 1990). However, a contribution from antagonistic activity at the α_1-adrenoceptor cannot be excluded, since ketanserin has appreciable affinity (p*Ki*, 8.0; Israilova et al. 2002) for these receptors as well and such antagonism could have caused miosis by an action on the α_1-rich iris dilator muscle or in the pre-ganglionic sympathetic neurons (Szabadi and Bradshaw 1996). In this context, it is important that local application of ketanserin in the iris did not cause miosis (Costagliola et al. 1993) because it speaks against participation of the α_1-adrenoceptor in the ketanserin-induced miosis observed in the present study. Finally, ketanserin also possesses some anti-histaminergic activity (Dollery 1999), which could have also contributed to its sedative and miotic effects. Indeed, antagonism of H1 histamine receptors by diphenhydramine resulted in sedation and miosis (Hou et al. 2006, 2007a).

Buspirone reduced RPD consistent with previous observations (Fanciullacci et al. 1995; Phillips et al. 1999). In contrast to ketanserin, the buspirone-induced miosis is unlikely to be related to any sedative properties of this drug, since buspirone is not a sedative agent (Newton et al. 1982), and it did not affect the VAS and CFFF measures of alertness used in this study. Buspirone also reduced diastolic blood pressure consistent with previous results (Fanciullacci et al. 1995) and in agreement with the effect of flenoxisan, another 5HT1a agonist. This drug decreased blood pressure in hypertensive patients and normotensive subjects without reflex tachycardia (De Voogd and Prager 1990).

Buspirone exhibits affinity for dopamine D2 receptors; however, this is 16-fold lower than that for 5HT1a receptors, and therefore, while a contribution from the D2 receptors cannot be entirely excluded, it is likely that the observed effects of buspirone were primarily the result of activity at the 5HT1a receptor. 5-HT1a receptor agonists reduce sympathetic outflow (Connor et al. 1991; Ramage and Fozard 1987), an effect that is reversed by 5-HT antagonists (McCall et al. 1987). Therefore, the observed buspirone-induced miosis and hypotension are fully consistent with a central sympatholytic effect of the drug. It is surprising that we could not find any effect of buspirone on the amplitude of the pupillary light reflex, since previous work (Phillips et al. 1999) has shown that the buspirone-induced miosis was light dependent consistent with the involvement of the light reflex mechanism. It is possible that this was a result of the low buspirone dose (10 mg) used in the present study, since the light dependent miosis

in the study of Phillips et al. (1999) was observed with 20 mg but not 5 or 10 mg of buspirone. Buspirone effects on the light reflex are informative in this respect, as buspirone did not affect light reflex amplitude, a parasympathetically mediated response. Thus, the miosis caused by buspirone, at least for the dose used in the present study, is likely to have been mediated by a reduction in sympathetic activity rather than an increase in parasympathetic activity.

The way in which the activation of central 5-HT1a receptors may lead to a sympatholytic effect is not clear, but it could be mediated via central noradrenergic neurons, since the latter receive modulatory input from the serotonergic system (Maeda et al. 1991; Vertes and Kocsis 1994). On the other hand, there is evidence that the periaqueductal grey (PAG) may have an integrative function in the sympathetic and parasympathetic control of the pupil (Klooster and Vrensen 1998) and may also participate in the modulation of the cardiac sympathetic function (Farkas et al. 1998); indeed, stimulation of the 5-HT1a-rich (Brandao et al. 1991; Pazos and Palacios 1985; Pompeiano et al. 1992) and highly responsive to 5HT1a agonists (Behbehani et al. 1993) dorsomedial PAG produces hypotension without tachycardia (Pajolla and de Aguiar Correa 2004; Pajolla et al. 2005) in line with our observations. Therefore, it is also possible that the pupillary and cardiovascular effects of buspirone are mediated via a direct drug effect in the PAG, but in any case, the neuronal circuitry involved and the location of the 5-HT1a receptors within this circuitry remain to be elucidated.

Consistent with its beta-blocking properties, propranolol reduced heart rate, suggesting bioactivity at 40 mg, but it did not affect pupil size and kinetics or any other measure of alertness, suggesting that beta adrenergic receptors are not involved in central regulation of arousal and pupillary functions. These data favour the view that propranolol is not a sedative drug (Currie et al. 1988; Harmer et al. 2001; Ogle et al. 1976; Tyrer and Lader 1974). It is worth noting that no active treatment affected the light reflex latency, which is an index of speed of signal processing by the retina and the integrity of the afferent branch of the reflex (Loewenfeld 1993). This suggests that treatments did not interfere with afferent light signal processes.

These results contribute to our understanding of the central pupillary control, but they also show that pupil size may not be used unequivocally as an index of the level of alertness in the case of drug-induced changes, when the drugs directly influence, by a separate pharmacological action, the pupil control mechanism, e.g. buspirone (this study), pramipexole (Samuels et al. 2006) and diazepam (Bitsios et al. 1998b; Hou et al. 2006; Hou et al. 2007b for a discussion on the relationship between drug-induced sedation and miosis). These considerations aside and given that pharmacological sedation in normal individuals may not be assumed to equate to the EDS seen in patient populations, elucidation of the central pupillary control via drug-induced sedation may be of relevance to sleep disorders characterised by reduced alertness and small pupils (Bitsios et al. 2006; Nikolaou et al. 2008). The practical implication of our study is that the pupillometric assessment of alertness in patients with EDS may be confounded by buspirone treatment for a comorbid anxiety/mood disorder or by ketanserin but not propranolol treatment for comorbid hypertension. Our study also suggests that due to its sedative properties, ketanserin may not be the drug of choice for the treatment of comorbid hypertension in patients suffering from EDS.

In conclusion, 5HT2 but not 5HT1a receptors or beta adrenoceptors appear to be involved in central regulation of arousal together with pupillary functions, although 5HT1a receptors may participate in central pupillary control alone. More detailed dose–response studies are required, but these results help to elucidate the central regulation of pupil size and alertness and they are informative to (a) drug studies using the pupil as a test system of alertness and (b) clinical studies using the pupil as a test system of alertness in patients with sleep disorders, who are concurrently medicated with ketanserin, propranolol or buspirone.

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Evidence of dysregulated affect indicated by high alexithymia in obstructive sleep apnea

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SUMMARY Alexithymia refers to dysregulation of affect characterized by difficulty in identifying and expressing emotions. Obstructive sleep apnea (OSA) is characterized by increased medical/psychiatric comorbidity and possibly by affect dysregulation. In the present case-control study, we examined alexithymia levels with the Toronto Alexithymia Scale (TAS-20) in 23 psychiatrically uncomplicated OSA outpatients and 23 same gender controls one-to-one matched for age, education and subjective depressive symptomatology. General health/quality of life was assessed with the Short-Form 36 Health Survey (SF-36) in the patient group. Hierarchical multivariate regression models were used to evaluate the association of alexithymia with the presence of OSA, and clinical and polysomnographic parameters of this condition. TAS-20 total and subscale scores were associated positively with Beck Depression Inventory (BDI)-21 and negatively with SF-36 scores. After adjusting for all confounders, OSA was positively associated with total TAS-20 score, 'expressing feelings' and 'externally oriented thinking' subscales. The latter was associated with increased sleepiness and reduced blood oxygenation in the OSA group. Finally, 'difficulty describing feelings' and 'externally oriented thinking' significantly predicted risk for OSA. Alexithymia is higher in non-psychiatrically ill patients with OSA compared with carefully matched controls even after adjustment for subjective depressive symptoms and demographic confounders. Total alexithymia is associated with greater subjective depression and poor general health/quality of life, while 'externally oriented thinking' is associated with disease severity and together with 'difficulty describing feelings' may be vulnerability factors for OSA, although reverse causality cannot be excluded.

KEYWORDS affect, alexithymia, case-control study, depression, obstructive sleep apnea

INTRODUCTION

Obstructive sleep apnea (OSA) is a condition characterized by repetitive obstruction of the upper airway often resulting in oxygen desaturation and arousals from sleep. Patients typically show choking or gasping during sleep and recurrent awakenings from sleep; the classic daytime manifestation is excessive sleepiness, but other symptoms such as unrefreshing sleep, poor concentration and fatigue are commonly reported (AASM Task Force, 1999). Patients with OSA show multiple physiological deficits and several neuropsychological comorbidities, including cognitive and emotional deficits. Reports

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show that patients with OSA experience negative mood, which may take the form of elevated depressive symptoms (Anderson *et al.*, 2001; Barnard *et al.*, 2006), anxiety (DeZee *et al.*, 2005; Sforza *et al.*, 2002; Sharafkhaneh *et al.*, 2005) or hostility, impulsivity and aggression in the general adult or in forensic populations with OSA (Bardwell *et al.*, 1999; Booth *et al.*, 2006; Klonoff *et al.*, 1987; Koyumer *et al.*, 2000; Shapiro *et al.*, 2003; Yue *et al.*, 2003). These highly comorbid conditions may reflect a core impairment in self-regulation of affective arousal (Beebe and Gozal, 2002) and decision-making (Harrison and Horne, 2000) in OSA, making this rather common condition a good model for the study of mood/affect dysregulation. This core impairment may stem from daytime sleepiness or sleep deprivation accompanying OSA only partially, because many emotional deficits remain after apnea and arousal resolution (Lane *et al.*, 2000; Taylor and Bagby, 2004). The hypothesis of mood/affect dysregulation in OSA would predict that these patients would have higher alexithymia rates, perhaps even in the absence of clinically significant affective symptoms.

Alexithymia (Nemiah *et al.*, 1976) is viewed as a dimensional personality construct that encompasses a cluster of cognitive and affective characteristics relating to a deficiency in identifying and describing emotions, non-psychologically minded, externally oriented thinking style, limited imaginal capacity, resulting in deficient self-regulation of affective responses and dissociation of emotional and physical responses to life events and bodily sensations (Lane *et al.*, 2000; Luminet *et al.*, 2004; Taylor and Bagby, 2004). These alexithymic characteristics are thought to contribute to the onset or maintenance of several psychosomatic, medical and psychiatric disorders (Taylor and Bagby, 2004), as a risk factor for these disorders (Lumley *et al.*, 2007) but equally, secondary alexithymia can be triggered by trauma, stress and health problems during adolescence or adulthood (Lumley *et al.*, 2007). Although alexithymia has also been viewed as a defense mechanism that protects chronically ill patients from stress and its consequences (McDougall, 1982), it is predominantly considered a deficit rather than a defensive process (Lumley *et al.*, 2007), and this deficit view is gaining increasing support from basic laboratory research (Kano *et al.*, 2003; Mantani *et al.*, 2005; Vermeulen *et al.*, 2006).

There are no studies that compared the levels of alexithymia in patients with OSA with that of a control group. Active mental illness and especially depression could be an important confounder because it is intimately related to both alexithymia and OSA (Akashiba *et al.*, 2002; Friedman *et al.*, 2003; Honkalampi *et al.*, 2001b, 2004; Saarijarvi *et al.*, 2006; Saunamaki and Jehkonen, 2007). The aim of the present case-control study was to compare the levels and prevalence of alexithymia of a non-psychiatrically ill/not clinically depressed sample of OSA outpatients with those of a control group matched not only for demographic variables but also for levels of subjective depressive symptomatology. A secondary goal was to examine whether alexithymia was associated with clinical and polysomnographic parameters of OSA.

MATERIALS AND METHODS

Participants

This study was approved by the Ethics Committee of the University of Crete, and all participants gave their written informed consent prior to screening. Patients were randomly recruited from the Sleep Disorders Unit, Department of Thoracic Medicine, Medical School, University of Crete. Inclusion criteria were written informed consent, a recent (< 10 days) diagnosis of OSA without yet initiation of nocturnal Continuous Positive Airway Pressure treatment for OSA. All patients underwent formal clinical, respiratory and polysomnographic assessment that has been described in detail previously (Bitsios *et al.*, 2006). In addition, all patients underwent a Mini-International Neuropsychiatric Interview (Sheehan *et al.*, 1998), and their general health and quality of life was assessed with the SF-36 (see below; Ware, 1994; Ware *et al.*, 1995). Exclusion criteria were: unrelated active clinically significant disease, a history or presence of any DSM-IV Axis I disorder, including drug/alcohol abuse, head injury and a positive urine drug-screening test. Twenty-three patients (one female) met these criteria and were included in the study. Patients with conditions commonly comorbid to OSA, such as hypertension (five patients), type II diabetes (one patient), hyperlipidemia (three patients) and thyroid dysfunction (two patients) were not excluded. All eligible patients were assessed for subjective depressive symptomatology using the Beck Depression Inventory (BDI-21; see below).

Healthy controls were identified from a list of 587 employees living in Heraklion selected from nurses and office workers of the university hospital and from postal clerks at the central office of the town. A convenience sub-sample closely matched with the OSA patients for gender, age (± 5 years) and years of education was identified. Although none of them had a previous history or presence of OSA and their medical records had been previously reviewed, they underwent a confirmatory Mini-International Neuropsychiatric Interview (Sheehan *et al.*, 1998) to exclude a history or presence of axis I disorders, and they were also assessed for subjective depressive symptomatology with the BDI-21. A final sub-sample of 23 controls was selected following exact one-to-one matching for age, gender, education and subjective depressive symptomatology.

Instruments

Toronto Alexithymia Scale (TAS-20)

Alexithymia was assessed with a validated Greek translation of the 20-item TAS-20 (Anagnostopoulou and Kioseoglou, 2002), which has been the most widely used measure in alexithymia research (Bagby *et al.*, 1994; Lumley *et al.*, 2007). TAS-20 items consist of statements presented in a five-point Likert scale (score, 1–5) along a 'strongly disagree' to 'strongly agree' continuum, with higher scores indicating more alexithymia. The scale comprises three factors: difficulties identifying feelings; difficulties expressing feelings; and

externally oriented thinking, each yielding a subscale score (Anagnostopoulou and Kioseoglou, 2002; Bagby *et al.*, 1994). TAS-20 total scores can be used dimensionally (score range, 20–100) or categorically, indicating yes or no alexithymia (score, ≥60 and < 60, respectively; Kooiman *et al.*, 2000).

BDI-21

All participants were asked to fill in the BDI-21, a widely used and well-validated self-report inventory of depressive symptoms (Beck and Beamesderfer, 1974). The BDI measures severity of depressive symptoms over the past week (American Psychiatric Association, 2000). For each item, the respondent chooses one or more response options rated from 0 (absence of symptom) to 3 (most severe level). Total scores range from 0 to 63 and represent the sum of the highest level endorsed on each item. The BDI has demonstrated adequate reliability and validity (Beck *et al.*, 1988; Katz *et al.*, 1994; Richter *et al.*, 1998).

Short-Form 36 Health Survey (SF-36)

This 36-item questionnaire is a reliable and validated tool for the assessment of general (physical and mental) health and quality of life (Ware, 1994; Ware *et al.*, 1995). The SF-36 encompasses eight domains – physical functioning, social functioning, mental health, role limitations due to physical problems, role limitations due to emotional problems, vitality (energy and fatigue), bodily pain, and general health perceptions – each of which is scored separately from 0 (worse) to 100 (best).

Statistical analysis

The statistical software SPSS 15.0 (SPSS, Chicago, IL, USA) was used for the analysis. Univariable analysis of categorical variables was made using the Pearson chi-square test. Continuous variables were presented as means and standard deviations (SD), they were normally distributed and univariable analysis was made using the parametric independent samples *t*-test. TAS-20 total scores were used dimensionally (score range, 20–100), to take advantage of the entire variance. Alexithymia is best conceptualized as a dimensional rather than as a categorical construct, especially when examining its association with symptom severity (Parker *et al.*, 2008).

Hierarchical, multivariable linear regression models were performed to evaluate the association of TAS-20 total and subscale scores (dependent variables) with age, body mass index (BMI), education, smoking status, and the presence of another medical condition(s) in Block 1, self-rated depressive symptomatology (BDI-21 scores) in Block 2, and the presence of OSA as a dichotomous variable in Block 3. We also report the ΔR^2 value for each block added, to reveal each block's specific contribution in the prediction of total and alexithymia factors. In the patient group, multivariate linear regression models were used to evaluate the association of TAS-20 total and subscale scores with age of OSA onset, duration of untreated illness (time from onset to diagnosis), OSA severity [Epworth Sleepiness Scale (ESS) and polysomnography (PSG) variables], and presence of comorbidities. All hypothesis testing was conducted assuming a 0.05 significance level and a two-sided alternative hypothesis.

RESULTS

The diagnostic, clinical and polysomnographic characteristics of the patients with OSA are described in Tables 1 and 2. Table 3 describes the comparison between patients with OSA and controls along relevant socio-demographic and psychometric variables.

Cases and their matched controls were nearly identical in age, years of education and BDI-21 score. Patients had significantly greater BMI, while a significantly greater number of smoking subjects was found in the control group. Patients with OSA scored significantly higher in TAS-20 total score and TAS subscale 'externally oriented thinking', while their scores in the 'difficulty in expressing feelings' subscale were also higher at trend level. However, because these differences in TAS-20 scores reflect univariate group comparisons where the confounding effect of the depressive state has not been removed, we also performed between-group comparisons in TAS-20 scores taking BDI-21 score as the covariate. These analyses revealed identical results, i.e. greater TAS-20 total ($F = 8.6$, $P < 0.005$) and 'externally oriented thinking' and 'difficulty in expressing feelings' subscale scores ($F = 8.5$, $P < 0.006$ and $F = 3.3$, $P = 0.075$, respectively) in the OSA group. The groups did not differ in the 'Difficulty identifying feelings' subscale ($F = 1.3$, $P > 0.26$).

The prevalence of overall alexithymia (TAS-20 total score ≥60) was 26.1% in patients with OSA (six patients) and 4.3% in the controls (one subject). The hierarchical multivariate linear regression model for the TAS-20 total score, including age, years of education, BMI, smoking status (yes/no) and presence of another medical condition(s) (yes/no) in Block 1, BDI-21 score in Block 2 and OSA group membership in Block 3 was significant and explained 59.8% (adjusted $R^2 = 0.522$) of the TAS-20 variability, with significant predictors being BDI-21 score (beta: 0.657; $t = 5.86$, $P < 0.001$; $\Delta R^2 = 0.355$), OSA presence (beta: 0.514; $t = 3.01$, $P < 0.005$; $\Delta R^2 = 0.101$) and years of education (beta: −0.296; $t = -2.64$, $P < 0.02$; Block 1 $\Delta R^2 = 0.142$). Table 4 presents the full findings of the final hierarchical linear regression models for each of the three TAS subscales, with inclusion of the same confounders. OSA membership was associated with 'difficulty expressing feelings' and 'externally oriented thinking' subscales.

Table 5 shows the correlation matrix between all continuous variables. Because BDI-21 correlated significantly with TAS-20 scores, we removed seven patients and their matched controls with BDI-21 scores equal or greater than 15. The cut-off point of 14/15 has been used to categorize subjects with self-reported depression (total BDI-21 score 15 or higher) or

Table 1 Diagnostic characteristics of 23 patients with OSA

Pt	*Age (years)*	*BMI (kg m^{-2})*	*ESS score*	*AHI*	*AI*	*Mean O_2 sat*	*Lowest O_2 sat*	*Desat per h*
#1	34.00	43.40	2.00	32	30	93	84	35
#2	45.00	40.97	21.00	41	37	92	83	51
#3	41.00	39.66	16.00	62	58	91	80	64
#4	49.00	37.90	18.00	82	80	87	70	88
#5	46.00	30.11	15.00	33	26	91	81	35
#6	41.00	37.23	18.00	43	31	92	83	55
#7	30.00	33.30	9.00	26	35	92	84	39
#8	51.00	38.64	4.00	36	49	93	82	31
#9	34.00	31.10	10.00	34	35	93	82	31
#10	15.00	40.19	5.00	50	42	94	84	66
#11	39.00	30.40	7.00	68	54	94	82	57
#12	46.00	38.46	7.00	20	20	95	90	30
#13	32.00	35.90	5.00	60	45	94	81	39
#14F	36.00	38.60	6.00	70	55	93	80	95
#15	57.00	29.47	21.00	28	26	94	86	24
#16	56.00	25.24	17.00	40	31	94	82	33
#17	42.00	31.20	15.00	23	20	94	89	20
#18	46.00	33.20	4.00	31	25	93	82	25
#19	39.00	37.60	6.00	38	35	92	80	38
#20	47.00	37.90	22.00	77	73	87	68	76
#21	58.00	31.20	16.00	26	31	94	87	24
#22	33.00	34.90	17.00	85	62	91	68	85
#23	28.00	28.50	3.00	56	41	92	81	53
Mean	41.1	35.0	11.5	46.1	40.9	92.5	81.3	47.6
SD	10.2	4.7	6.6	19.9	16.3	2.03	5.7	23.3

Pt, patient; F, female.
AHI, apnea–hypopnea index; AI, arousal index; BMI, body mass index; ESS, Epworth Sleepiness Scale.
Desat per h: the number of episodes where saturation of oxygen drops during sleep more than 3% or 4% from baseline, divided by the hours of sleep.
Mean O_2 sat: the mean value of oxygen saturation during sleep.
Lowest O_2 sat: the nadir value of oxygen saturation during sleep.

Table 2 Other clinical and polysomnographic characteristics of the OSA patient group

	Patients with OSA (n = 23)
Mean duration of untreated illness (SD) in months	38.0 (37.2)
Mean age at onset (SD) in years	38.04 (11.1)
Mean BDI-21 total score (SD)/range	9.65 (7.2)/1–24
Mean SF-36 score (SD)	96.4 (10.0)
% (no) on medication	34.8 (8.0)
% (no) with comorbid conditions	47.8 (11.0)
Mean total sleep time (SD)	307.2 (68.6)
Mean REM sleep (SD)	30.0 (14.4)
Mean non-REM sleep (SD)	263.1 (52.9)
Mean slow-wave sleep (SD)	29.3 (16.3)
Mean sleep efficiency (SD)	72.4 (12.9)

BDI, Beck Depression Inventory; OSA, obstructive sleep apnea; REM, rapid eye movement; SF-36, Short-Form 36 Health Survey.

Table 3 Demographic and psychometric comparison of OSA and control groups

	OSA (n = 23)	*Control (n = 23)*	P
Age (years)	41.1 ± 2.1	42.6 ± 1.9	>0.6
Years of education	12.2 ± 0.9	12.3 ± 0.6	>0.9
BMI	35.0 ± 0.9	27.3 ± 0.7	<0.001
Smokers : non-smokers ratio*	2 : 21	12 : 11	<0.003
BDI-21	9.7 ± 1.5	8.4 ± 1.6	>0.6
TAS-20 total score	46.9 ± 2.8	38.1 ± 1.7	<0.01
TAS–difficulty identifying feelings	14.9 ± 1.4	12.7 ± 1.1	>0.2
TAS–difficulty describing feelings	12.5 ± 1.05	9.9 ± 0.8	<0.07
TAS–externally oriented thinking	19.5 ± 0.9	15.5 ± 0.9	<0.005

BDI-21, Beck Depression Inventory-21; BMI, body mass index; OSA, obstructive sleep apnea; TAS, Toronto Alexithymia Scale.
*Chi-square test was used for this variable.

non-depression (total score 14 or lower; Fountoulakis *et al.*, 2003). Removal of these individuals would allow determination of the relationship between alexithymia and OSA without the strong confound of high self-rated depressive symptomatology. After exclusion of the patients with OSA and their matched controls standing above the 14/15 cut-off point, BDI-21 score was no longer a significant predictor of TAS-20 total

Table 4 Hierarchical multivariable linear regression models of the determinants of TAS-20 subscale scores

	Model predictors	*Unstandardized coefficients*		*Standardized coefficients*		
		B	*SE*	*Beta*	*t*	*P*
TAS-20 Difficulty identifying feelings						
Block 1 $\Delta R^2 = 0.060$	Age	0.011	0.073	0.018	0.15	0.88
	Years of education	−0.308	0.189	−0.186	−1.63	0.11
	BMI	−0.008	0.176	−0.007	−0.05	0.96
	Smoking	−0.348	1.65	−0.026	−0.21	0.83
	Pres. of another dis.	−2.58	2.161	−0.187	1.19	0.24
Block 2 $\Delta R^2 = 0.509$***	BDI-21 score	0.659	0.097	0.772	6.76	0.000
Block 3 $\Delta R^2 = 0.013$	Presence of OSA†	2.26	2.118	0.186	1.07	0.29
TAS-20 Difficulty expressing feelings						
Block 1 $\Delta R^2 = 0.143$	Age	−0.043	0.06	−0.091	−0.71	0.48
	Years of education	−0.096	0.155	−0.078	−0.62	0.54
	BMI	−0.133	0.145	−0.165	−0.92	0.36
	Smoking	4.14	1.35	0.416	3.07	0.004
	Pres. of another dis.	−0.769	1.77	−0.075	−0.43	0.67
Block 2 $\Delta R^2 = 0.213$***	BDI-21 score	0.327	0.08	0.516	4.09	0.000
Block 3 $\Delta R^2 = 0.137$**	Presence of OSA†	5.48	1.73	0.606	3.15	0.003
TAS-20 Externally oriented thinking						
Block 1 $\Delta R^2 = 0.267$*	Age	0.093	0.072	0.186	1.30	0.20
	Years of education	−0.552	0.186	−0.418	−2.97	0.005
	BMI	0.004	0.174	0.005	0.03	0.98
	Smoking	0.209	1.62	0.020	0.13	0.90
	Pres. of another dis.	−1.29	2.13	−0.118	−0.61	0.55
Block 2 $\Delta R^2 = 0.018$	BDI-21 score	0.107	0.096	0.158	1.12	0.27
Block 3 $\Delta R^2 = 0.079$*	Presence of OSA†	4.46	2.08	0.460	2.14	0.04

All models were significant ($P < 0.01$), explaining 58.2%, 49.2% and 36.3% of the variance of 'difficulty identifying feelings', 'difficulty describing feelings' and 'externally oriented thinking', respectively.
BMI, body mass index; BDI-21, Beck Depression Inventory-21; OSA, obstructive sleep apnea; TAS, Toronto Alexithymia Scale.
Significance of *F* delta: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.
†Reference category: healthy controls.

Table 5 Correlation matrix between TAS total and subscale scores and BDI-21 score, age, years of education and BMI in the entire population of patients and controls

	BDI-21	*Age*	*Education*	*BMI*
TAS-20 total	0.705***	0.040	−0.198	−0.044
TAS–Difficulty identifying feelings	0.702***	−0.009	−0.093	0.089
TAS–Difficulty describing feelings	0.538***	−0.116	−0.066	0.156
TAS–Externally oriented thinking	0.093	0.161	−0.390**	0.298*

BDI-21, Beck Depression Inventory-21; BMI, body mass index; TAS, Toronto Alexithymia Scale.
Values represent Pearson's *r*. df: 46.
***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

score or any other subscale, while we obtained an identical pattern of significant results for the other predictors. This suggests that OSA was associated with high alexithymia (total score as well as of the 'difficulty describing feelings' and 'externally oriented thinking' subscales), even in the absence of high self-rated depressive symptomatology.

We also tested the alternative hypothesis, namely that alexithymia was a vulnerability factor for OSA using a logistic regression with OSA presence (yes/no) as the dependent variable, and age, BMI, education, smoking status, BDI-21 score and TAS-20 total or subscale scores as the independent variables. The TAS-20 total (OR 1.31; 95% CI: 1.01–1.7) and especially the 'difficulty describing feelings' and the 'externally oriented thinking' subscales were associated with increased risk for OSA (OR: 1.76; 95% CI: 1.01–3.3 and OR: 1.3; 95% CI: 1.01–3.1, respectively), while the 'difficulty identifying feelings' was not (OR: 0.75; 95% CI: 0.48–1.2). None of the results of all the analyses described above was affected after removal of the female patient and her matched control.

Finally, we examined whether TAS-20 total or subscale scores in the patient group predicted OSA severity as indicated by key measures such as ESS, arousal index, apnea–hypopnea index and O_2 saturation. In this series of regressions, each of the above OSA measures was entered as the dependent variable, while age, education, smoking habit, BMI, duration of untreated illness (DUI), BDI-21 and SF-36 scores and TAS-20 total or the three TAS-20 subscales were entered as the independent variables. These analyses revealed that the

Table 6 Correlation matrix between TAS total and subscale scores and demographic, clinical, polysomnographic and psychometric variables in the OSA patient group

	Age	*Educ*	*BMI*	*DUI*	*BDI-21*	*SF-36*	*ESS*	*Sat*	*AI*	*AHI*
TAS-20 total	0.04	−0.20	−0.04	−0.25	0.71***	−0.65***	0.26	−0.20	0.26	0.16
TAS–Difficulty identifying feelings	−0.25	−0.18	−0.01	−0.19	0.67***	−0.66***	0.09	−0.08	0.22	0.08
TAS–Difficulty describing feelings	−0.71	0.09	−0.09	−0.06	0.69***	−0.41*	0.17	−0.03	0.22	0.21
TAS–Externally oriented thinking	0.24	−0.43*	−0.02	−0.39†	0.34	−0.52**	0.46*	−0.46*	0.23	0.11

Values represent Pearson's *r*. df: 23.
***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, †$P < 0.063$.
AHI, apnea–hypopnea index; AI, arousal index; BDI-21, Beck Depression Inventory-21; BMI, body mass index; DUI, duration of untreated illness; ESS, Epworth Sleepiness Scale; Sat, mean O_2 saturation; SF-36, Short-Form 36 Health Survey; TAS, Toronto Alexithymia Scale.

TAS-20 'externally oriented thinking' subscale was the only significant predictor of the mean O_2 saturation, explaining 19.3% of the variance (beta: −0.381; $t = -2.1$, $P < 0.05$). The same subscale also predicted 22.7% of the variance of the ESS score (beta: 0.628, $t = 2.26$, $P < 0.05$). Table 6 shows the correlations matrix in the patient group.

DISCUSSION

This is the first case-control study to examine alexithymia in a sample of patients with OSA who were free of DSM-IV defined depression or other psychiatric diagnoses. Alexithymia is a normally distributed personality trait with a prevalence varying from 7 to 13% in the general population (Honkalampi *et al.*, 2001b; Kokkonen *et al.*, 2001; Mason *et al.*, 2005), which is in accordance with its prevalence in our control group (4.3%). The prevalence of alexithymia was higher in our OSA group (26.1%). Inclusion of alexithymia as a dimensional variable in the analyses showed higher levels in the patients with OSA compared with healthy controls. The hierarchical regressions show that OSA certainly qualifies as an additional predictor of high total alexithymia, and particularly the 'difficulty expressing feelings' and 'externally oriented thinking' subscales (see below for discussion of this pattern). These results could not be attributed to differences in age, gender or education as the two groups were carefully matched in this respect, and the differences in BMI and smoking status were controlled for in the regressions, while BMI did not correlate with alexithymia. Furthermore, the relationship of OSA to alexithymia cannot be easily attributed to a non-specific effect of chronic illness, as this would have resulted in higher alexithymia scores in patients with the longer illness duration. Contrary to this prediction, however, alexithymia, as measured with the 'externally oriented thinking' subscale at least, correlated negatively with illness duration, suggesting that those with the longer-standing OSA problems were the lowest on a pragmatic style of thinking.

We also checked and ruled out depression as a potential confounder; firstly, depression was an exclusion criterion and the groups were carefully matched for subjective depressive symptomatology which, in addition, was included as a confounder in the regressions; secondly, alexithymia levels in patients with OSA were still higher even when all seven patient–control pairs at the high end of the BDI-21 spectrum were excluded. The frequently observed association between alexithymia and depression has suggested a state-dependent phenomenon (Honkalampi *et al.*, 2001b, 2004; Marchesi *et al.*, 2005), whereby high alexithymia reflects current depressive affect and situational variables that impose on one's cognitive/affective processing capacity. However, a number of studies in various clinical (Luminet *et al.*, 2001; Pinard *et al.*, 1996; Porcelli *et al.*, 1996; Saarijarvi *et al.*, 2006; de Timary *et al.*, 2008) and non-clinical (Mikolajczak and Luminet, 2006; Picardi *et al.*, 2005) samples suggest the relative (the extent to which relative differences among individuals remain the same over time) or even sometimes the absolute (the extent to which alexithymia scores change over time) stability of alexithymia. The relationship of depression to alexithymia is not well understood and, with the exception of the study of Chatzi *et al.* (2009) in patients with type I diabetes, there are no studies in clinical populations with somatic problems, examining to what extend the two conditions are independent. In agreement with previous reports (Honkalampi *et al.*, 2001a, 2004; Saarijarvi *et al.*, 2006), we find that alexithymia may correlate with the severity of depression (Tables 5 and 6), but our study also suggests that the two conditions can be independent.

In the patient group, total alexithymia score was associated with poorer general health and quality of life but not with OSA severity in terms of PSG after adjusting for all possible confounders. This is in agreement with previous observations of alexithymia alone being unrelated to somatic variables in type I diabetes (Chatzi *et al.*, 2009; Friedman *et al.*, 2003), severity of chronic pain (Kosturek *et al.*, 1998) or severity of other serious medical conditions (Wise *et al.*, 1988). The presence of alexithymia in patients with OSA is of concern due to its relationship to diminished quality of life and association with depression (Honkalampi *et al.*, 2001a, 2004; Saarijarvi *et al.*, 2006) and abnormal cardiovascular (Koelsch *et al.*, 2007; Peters and Lumley, 2007) and blood pressure reactivity (O'Connor and Ashley, 2008).

It is not possible to commend, based on this case-control study, if the observed higher alexithymia in OSA is etiologically related to this condition. The relationship of 'externally

oriented thinking' subscale with severity measures such as increased sleepiness in the last month and reduced mean O_2 saturation, but not sleep disturbance by nocturnal arousals, is notable. Importantly, a similar pattern was found for cognitive impairment in OSA, which was related to severity of hypoxia but not nocturnal arousals (Bédard *et al.*, 1991a; b; for review, see Veasey, 2009). Alexithymia (Borsci *et al.*, 2009) and OSA (Alchanatis *et al.*, 2004; Harper *et al.*, 2003; Macey *et al.*, 2002; Morrell *et al.*, 2003) overlap in terms of their association with reduced gray matter volume in the anterior cingulate, orbitofrontal cortex, insula and temporal areas; the anterior cingulate and orbitofrontal cortex are key areas for cognitive and emotional processing/decision-making (Damasio, 1996; LeDoux, 2007; Rolls, 1996), and the anterior cingulate, insular and temporal cortices are rich in mirror neurons, which are important for self- and emotion-awareness and social cognition (Rizzolatti *et al.*, 2006). The above suggest that: (a) gray matter loss may be a consequence of apnea; hypoxic damage to certain areas mediating emotion may contribute to alexithymia and other affective disorders frequently accompanying OSA; or (b) pre-existing alexithymia-related gray matter abnormalities may contribute to the genesis and maintenance of the disorder (see Macey *et al.*, 2002 for discussion).

Notwithstanding, we cannot yet commend on causality. Certainly, alexithymia can be secondary to physical or mental health problems and, although the latter have been carefully controlled, it cannot be excluded that higher alexithymia was secondary to OSA, although the negative correlation with duration of untreated illness does not support this possibility. Although the logistic regressions clearly suggest the intriguing possibility that alexithymia may be a vulnerability factor for OSA, reverse causality cannot be excluded. A necessary condition for alexithymia to be a vulnerability factor is that it is a stable personality construct. In this respect, the pattern of associations is interesting; the 'difficulty describing feelings' and 'externally oriented thinking' subscales that predicted OSA have shown to be stable personality traits (Luminet *et al.*, 2007; de Timary *et al.*, 2008) and immune to variations in depression, especially the 'externally oriented thinking' subscale (Luminet *et al.*, 2007); the 'difficulty identifying feelings', which did not relate to OSA, has been shown to be related to mood variations and depression (Honkalampi *et al.*, 2010; Luminet *et al.*, 2007). Future research should attempt to differentiate secondary and trait alexithymia components in OSA by including premorbid assessment in longitudinal studies. Future studies could also examine other aspects of affect dysregulation in OSA, such as emotional decision-making processes and the affective startle paradigm.

Strengths of this study include: concurrent assessment of alexithymia and depressive symptoms in patients with OSA and one-to-one matched healthy controls for age, education and depressive symptoms, the use of validated self-reported questionnaires, and detailed medical history. Limitations that should also be considered are the cross-sectional nature of the study, which does not allow the assessment of causal relationships and the small samples. Finally, although we cannot entirely rule out biases from individual, social and educational status, we have no indication from our data of differential selection bias in the study groups. Participants were unaware of the hypothesis being tested, so misclassification of exposure by questionnaires is likely to be random with respect to OSA.

In conclusion, the present study suggests that, compared with healthy controls, the levels and prevalence of alexithymia are higher in non-psychiatrically ill patients with OSA, after controlling for confounds such as age, smoking status, BMI, education and, critically, subjective depressive symptomatology. The study validates clinical observations of a relationship of OSA with affective disorders, revealing problems with emotional processing in patients with OSA who do not yet present with a clinical affective disorder. Higher total alexithymia in the patient group was associated with greater subjective depressive symptomatology and poorer general health and quality of life, while the 'externally oriented thinking' subscale specifically predicted disease severity in clinical and PSG measures; these patients were sleepier and had reduced O_2 saturation. Alexithymia may be the common denominator and the earliest sign of affective disorders in patients with OSA and even predate disease onset. Importantly, the 'difficulty describing feelings' and 'externally oriented thinking' predicted increased risk for OSA, suggesting that these TAS subscales may be vulnerability factors for the disease, although reverse causality cannot be excluded. More comprehensive and longitudinal studies with larger samples are required to better understand the physiological mechanisms of the observed associations.

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