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Orexin-A inputs onto visuomotor cell groups in the monkey brainstem

Sarah Schreyer[1], **Jean A. Büttner-Ennever**[1], **Xiaofang Tang**[1], **Michael J. Mustari**[2], and **Anja K. E. Horn**[1]

[1]Institute of Anatomy, Ludwig-Maximilian-University of Munich, D-80336 Munich, Germany

[2]Washington National Primate Research Center, University of Washington, Seattle, WA 98195, USA

Abstract

Orexin-A, synthesized by neurons of the lateral hypothalamus, helps to maintain wakefulness through excitatory projections to nuclei involved in arousal. Obvious changes in eye movements, eyelid position and pupil reactions seen in the transition to sleep led to the investigation of orexin-A projections to visuomotor cell groups to determine whether direct pathways exist that may modify visuomotor behaviors during the sleep/wake cycle. Histological markers were used to define these specific visuomotor cell groups in monkey brainstem sections and combined with orexin-A immunostaining. The dense supply by orexin-A boutons around adjacent neurons in the dorsal raphe nucleus served as a control standard for a strong orexin-A input. The quantitative analysis assessing various functional cell groups of the oculomotor system revealed that almost no input from orexin-A terminals reached motoneurons supplying the singly-innervated muscle fibers of the extraocular muscles in the oculomotor nucleus, the omnipause neurons in the nucleus raphe interpositus and the premotor neurons in the rostral interstitial nucleus of the medial longitudinal fasciculus. In contrast, the motoneurons supplying the multiply-innervated muscle fibers of the extraocular muscles, the motoneurons of the levator palpebrae muscle in the central caudal nucleus, and especially the preganglionic neurons supplying the ciliary ganglion received a strong orexin input. We interpret these results as evidence that orexin-A does modulate pupil size, lid position, and possibly convergence and eye alignment via the motoneurons of multiply-innervated muscle fibres. However orexin-A does not directly modulate premotor pathways for saccades or the SIF motoneurons.

Keywords

oculomotor; eyelid; pupil; accommodation; saccade

The hypothalamus is involved in a wide variety of behavioural, autonomic visceral and endocrine functions. It is composed of numerous diffuse cell groups and a few magnocellular nuclei (for review: Saper, 2004; Niewenhuys et al., 2008). Le Gros Clark divided them rostrocaudally into four regions, the preoptic, supraoptic or anterior, tuberal and the mammillary region lying caudally (Le Cros Clark, 1938), while Crosby and Woodburne (1940) distinguished three zones arranged mediolaterally, the periventricular, medial and

Correspondence to: Dr. Anja Horn, Institute of Anatomy, LMU Munich, Pettenkoferstr. 11, D-80336 Munich, Germany, Phone: -49 89 5160 4880, Fax: -49 89 5160 4802, Anja.Bochtler@med.uni-muenchen.de.
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lateral zones. Orexins, also known as hypocretins, are neuropeptides produced by a rather restricted group of neurons in the lateral hypothalamus, that project throughout the central nervous system demonstrated in various species, e.g. rat (Peyron et al., 1998; Sakurai et al., 1998; de Lecea et al., 1998), hamster (Mintz et al., 2201), cat (Torterolo et al., 2006) and human (Thannickal et al., 2000). Aside from a role in the control of feeding, drug addiction, emotion, muscle tone and arousal (Yamada et al., 2000; Harris et al., 2005; Narita et al., 2006; Harris and Aston-Jones, 2006), orexin participates in the maintenance and stabilization of wakefulness in the sleep-wake cycle (Sakurai et al., 1998;Saper et al., 2005; Sakurai, 2007; Tsujino and Sakurai, 2009). The latter role was implied by the loss of orexin containing neurons in narcolepsy (Peyron et al., 2000; Nishino et al., 2000; Thannickal et al., 2000; Taheri et al., 2002). The control of sleep may be accomplished through strong excitatory projections of orexin neurons to the noradrenergic locus coeruleus (Hagan et al., 1999), serotoninergic cells of the dorsal raphe nucleus (DR) (Brown et al., 2001; Liu et al., 2002; Lee et al., 2005a) and cholinergic neurons of the laterodorsal tegmental and pedunculopontine tegmental nucleus (Peyron et al., 1998; Zhang et al., 2004). These regions are all part of the ascending arousal system that promotes wakefulness (for review: Sakurai, 2007). During sleep, orexin neurons are inhibited by the ventrolateral preoptic nucleus (for review: Saper et al., 2005)

Orexins appear in two forms, orexin-A and orexin-B, which correspond to hypocretin 1 and hypocretin 2, and show the same localization (Date et al., 1999). Both are derived from a common precursor by proteolytic cleavage, and show different binding affinities to two G-protein-coupled receptors. Whereas orexin-A has a high affinity to both orexin receptors, OxR1 and OxR2, OrxB has a much higher affinity to OxR2 than to OxR1 (Sakurai et al., 1998). Recording experiments showed that orexin neurons are most active during wakefulness, and they are virtually silent during non-REM and REM-sleep with only occasional bursts (Mileykovskiy et al., 2005; Lee et al., 2005b). With the onset of sleep there are rapid changes in eye movements, eyelid position and pupillary responses, e.g. loss of eye fixation with slow drifts, loss of conjugacy and drooping eyelids (Henn et al., 1984; Zhou and King 1997; Marquez-Ruiz and Escudero, 2008).

Here, we investigated the orexin-A projections to specific motor and premotor cell groups of the oculomotor system and to preganglionic neurons of the pupillary and accommodation systems in monkey. We wished to find out whether direct orexin-A inputs are present that could account for changes seen in the transition to sleep or for rapid eye movements during sleep. Our study included the presumed twitch-motoneurons in the oculomotor nucleus (nIII), which supply the singly-innervated muscle fibers (SIF) of extraocular muscles and the presumed non-twitch motoneurons in the C- and S-groups, which supply the multiply-innervated muscle fibers (MIF) of the extraocular muscles (Büttner-Ennever et al., 2001; Büttner-Ennever, 2006). Furthermore, we examined the omnipause neurons in the nucleus raphe interpositus (RIP), which stabilize eye position and trigger the generation of saccades, the premotor neurons for vertical saccades in the rostral interstitial nucleus of the medial longitudinal fascicle (RIMLF), the motoneurons of the levator palpebrae superior muscle (LP) and the orbicularis oculi muscle (OO), and the preganglionic neurons supplying the ciliary ganglion found in Edinger-Westphal (EWpg) and anteromedian nuclei (AM) (for review: Büttner-Ennever et al., 1988; Porter et al., 1989; Horn, 2006; Horn et al., 2008; May et al., 2008a). In order to assess the strength of the orexin-A input, a quantitative analysis of orexin-A containing nerve endings associated with the visuomotor cell groups was performed. These data were compared with levels found in the nucleus raphe dorsalis (DR), which is known to receive strong projections from orexin-producing neurons (Peyron et al., 1998; Brown et al., 2001).

Previous studies in monkey have identified specific functional cell groups of the visuomotor system, close to the raphé nuclei of the brainstem, by their location and histochemical properties. The motoneurons of MIFs, SIFs, LP, OO, and the preganglionic neurons of the

ciliary ganglion were identified by the cholinergic marker choline acetyltransferase (ChAT) (Eberhorn et al., 2005), and saccadic burst and omnipause neurons by immunostaining for parvalbumin or non-phosphorylated neurofilaments (Horn et al., 1994; Horn and Büttner-Ennever, 1998). The serotoninergic neurons of the dorsal raphe nucleus were identified with an antibody against tryptophan hydroxylase (Brownstein et al., 1975; Baker et al., 1991). Taken together these studies provide a unique opportunity to assess the significance of orexin inputs onto functionally identifiable cell groups in monkey.

Part of this work has been presented previously (Büttner-Ennever et al., 2007).

Experimental procedures

All animal tissue was obtained in accordance with state regulations and with approval of the appropriate state and university committees.

Antisera and Controls

Orexin-A—Orexin containing structures were detected with a polyclonal orexin-A antibody raised in rabbit (AB3098, Chemicon, Temecula, CA). The appearance of orexin-A-positive staining seen with this antibody in the present study resembles the data of previous reports (Sakurai et al., 1998; de Lecea et al., 1998).

Choline acetyltransferase (ChAT)—Cholinergic motoneurons were identified with a polyclonal ChAT antibody raised in goat (AB144P, Chemicon) against human placental ChAT. The appearance and distribution of ChAT-positive neurons seen with this antibody in the present study resembles the data of previous reports (Ichikawa and Shimizu, 1998).

Parvalbumin (PV)—The calcium-binding protein, parvalbumin, was detected in immunofluorescence and immunoperoxidase staining with a monoclonal parvalbumin antibody raised in mouse produced by hybridization of mouse myeloma cells with spleen cells from mice immunized with parvalbumin purified from carp muscle (235; Swant, Bellinzona, Switzerland; (Celio et al., 1988).

Tryptophan hydroxylase (TRH-PH8)—Serotoninergic neurons were detected with a monoclonal antibody raised in mouse (MAB5278; Chemicon, Temecula, CA) that binds an epitope of tryptophan hydroxylase (TRH), tyrosine hydroxylase and phenylalanine hydroxylase. TRH is the enzyme that converts 5-hydroxytryptophan to serotonin and therefore can be used as a marker for serotonin. The appearance of TRH-PH8-positive neurons seen with this antibody in the present study resembles the data of previous reports (Baker et al., 1991).

Non-phosphorylated neurofilaments (NP-NF)—NP-NFs were detected using a mouse monoclonal anti-nonphosphorylated ‘epitope in neurofilament H’ antibody (clone 02-135; SMI32, Sternberger Monoclonals Inc., Lutherville, MD). This antibody visualizes two bands (200kDa and 180 kDa) in conventional immunoblots (Tsang et al., 2000).

Controls—Controls for each reaction were carried out by the omission of primary antibodies, which in each case led to unstained sections.

Combined immunoperoxidase labeling for orexin-A and different markers

Brainstem sections from macaque monkeys used in other anatomical projects were employed in this study. The animals were euthanized with an overdose of sodium-pentobarbital (80mg/kg body weight) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer. The brains were removed from the skull and

immersed in 10% sucrose in 0.1M phosphate buffer and transferred to 30% sucrose for frozen sectioning. The brainstems were cut at 40μm in the transverse stereotaxic plane. Selected sections through the hypothalamus, midbrain and pons combined immunolabelling were utilized either double-immunoperoxidase or double-immunofluorescence staining to simultaneously detect Orexin-A and one of the following: choline acetyltransferase (ChAT), parvalbumin (PV), non-phosphorylated filaments (NP-NF) or tryptophan hydroxylase (TRPH 8) (Table 1). For immunoperoxidase staining, free-floating sections were washed in 0.1M Tris-buffered saline (TBS, pH 7.4) and treated with 1% H_2O_2 in TBS for 30 min. After several washes, sections were preincubated with 2% normal horse serum containing 0.3% Triton-X 100 in TBS for 1 h at room temperature. The sections were then treated with rabbit anti-orexin-A (1:6000; Chemicon AB3098) in TBS with 2% horse serum and 0.3% Triton X-100 over two nights at room temperature. After washing in 0.1M TBS, the sections were incubated in biotinylated horse anti-rabbit IgG (1:200; Vector laboratories) in TBS containing 2% bovine serum albumin for 1h. For the detection of orexin-A, the sections were incubated in extravidin-peroxidase (1:1000; Sigma) for 1h. Two rinses were followed by one wash with 0.05M Tris-buffer (pH 7.6), and the reaction with 0.025% diaminobenzidine, 0.4% ammonium nickel sulfate and 0.015% H_2O_2 in 0.05M Tris-buffer (pH 7.6), for 10 min. This results in a black staining of orexin-A-positive structures. After a thorough washing and blocking of residual peroxidase activity with 1% H_2O_2 in 0.1M TBS, the sections were preincubated with 2% normal horse serum in 0.3% Triton-X 100 in TBS for 1 h at room temperature. Then the sections were treated with goat anti-ChAT (1:1000; Chemicon, AB144P), mouse anti-parvalbumin (1:2500; Swant, 235), mouse anti-non-phosphorylated neurofilaments (1:6000; Sternberger, SMI32) or mouse anti-tryptophan hydroxylase PH8-1 (1:2000; Chemicon, MAB5278) in TBS with 2% horse serum and 0.3% Triton X-100 overnight at room temperature. After washing in 0.1M TBS, the sections were incubated in biotinylated horse anti-goat IgG or horse anti-mouse IgG (1:200; Vector laboratories) in TBS containing 2% bovine serum albumin for 1h. The antigen binding sites were detected by incubating sections in extravidin peroxidase (1:1000; Sigma) for 1h and a subsequent reaction with 0.025% diaminobenzidine and 0.015% H_2O_2 in 0.05M Tris-buffer (pH 7.6) for 10 min to yield a brown stain. After washing, the sections were mounted, air-dried, dehydrated in alcohol and coverslipped with Depex.

Combined immunofluorescence labeling for orexin-A and other markers

Free floating sections of monkey brainstem were first treated with 2% normal donkey serum in 0.1M TBS (pH 7.4) containing 0.3% Triton-X 100 for 1 hour. Sections were then incubated in a cocktail containing rabbit anti-orexin-A (1:2000; Chemicon AB3098) and mouse anti-parvalbumin (1:2000; Swant, 235) in TBS with 2% normal donkey serum and 0.3% Triton X-100 for two nights at 4°C. Rinsed sections were then reacted with a cocktail of donkey carbocyanine 3 (Cy3)-tagged anti-rabbit (1:200; red) and donkey Alexa-488-tagged anti-mouse (1:200; green) in TBS containing 2% bovine serum albumin for 1h. After washing in 0.1M TBS, the sections were briefly rinsed with distilled water, and coverslipped with Gel/Mount.

Analysis of stained sections

The slides were examined with a Leica microscope DMRB (Bensheim, Germany) equipped with appropriate filters for red fluorescent Cy3 (N2.1) and green fluorescent Alexa- 488 (I3). Photographs of brightfield and fluorescence preparations were taken with a digital camera (Pixera Pro 600 ES; Klughammer, Markt Indersdorf, Germany) mounted on the microscope (Leica DMRB, Bensheim, Germany). The images were captured on a computer with Pixera Viewfinder software (Klughammer) and processed with Photoshop 7.0 software (Adobe). The sharpness, contrast and brightness were adjusted to reflect the appearance of the labeling seen through the microscope. The pictures were arranged and labeled with drawing software (Coreldraw 11.0; Corel Corporation). For a quantitative analysis, all orexin-A-positive endings

associated with the soma and proximal dendrites of a given neuron were counted by focussing through the section, and the number documented in a spreadsheet table (Microsoft Excel, 2002). From there the average and mean terminal density was calculated, the latter was used for the statistical tests. The analysis of each chosen visuomotor cell group was performed on sections from two monkeys. At least 100 neurons taken from all levels of a given cell group were studied for orexin-A inputs. Statistical analysis was performed using a Wilcoxon two sample test.

Results

The application of the orexin-A antibody revealed two main clusters of strongly stained neuronal cell bodies in the tuberal and rostral mammillary region of the monkey hypothalamus (Fig. 1). One orexin-A positive cell group lies around the fornix (FO) in the perifornical region (PF) and spreads out in the lateral hypothalamic area (LHA) (Fig. B, C); the other orexin-A positive cell group occupies the area of the dorsomedial hypothalamic nuclei (DMH) with scattered neurons in the dorsal hypothalamic area (Fig. 1A, B). The orexin-A positive cells were medium-sized and typically give rise to two or three primary dendrites (Fig. 1D). Orexin-A immunostained fibres with numerous varicosities and terminal boutons were distributed throughout the whole brainstem with varying densities. As described before in rat and cat, a particularly strong supply of Orexin-A positive terminals was found in the locus coeruleus (not shown) and the raphe nuclei (Fig. 3G, I), whereas the cerebellar cortex was almost devoid of orexin-A fibres and terminals (Nambu et al., 1999;Zhang et al., 2004). The presence of immunostained structures throughout the whole section thickness indicated complete antibody penetration. This was an important prerequisite for the quantitative analysis that was performed.

Orexin-A input on motoneurons of the extraocular muscles

Immunostaining for choline acetyltransferase (ChAT) revealed the complete population of eye muscle motoneurons within and around the oculomotor nucleus (nIII) in monkey midbrain sections. ChAT-immunoreactive neurons within nIII were recognized as the SIF-motoneurons of the inferior rectus (IR), medial rectus (MR), inferior oblique (IO) and superior rectus muscle (SR), which appear as multipolar, medium-sized neurons (Eberhorn et al., 2005) (Fig. 2A). They received the weakest orexin-A input of the examined cell groups with the least average terminal density per neuron (0.60 boutons/cell; SD: 1.08) (Fig. 2E; 4). ChAT-immunoreactive neurons within the well delineated C-group at the dorsomedial border of nIII represent the MIF-motoneurons of IR and MR, and those in the S-group, sandwiched between the nIII, the MIF-motoneurons of IO and SR (Büttner-Ennever, 2006) (Fig. 2A, B). In contrast to the SIF motoneurons within nIII, the MIF-motoneurons of the C-group and the S-group received a significant higher supply of orexin-A-positive boutons (C-MIF: 6.36 boutons/cell; SD: 3,87; S-MIF: 6.97 boutons/cell; SD: 5.43) (Fig. 2C, D; E; Fig. 4).

Orexin-A input on motoneurons of the eyelid

In ChAT-immunostained sections the motoneurons of the levator palpebrae muscle (LP) can be easily identified as the central caudal nucleus (CCN) dorsomedial to the caudal part of nIII (Porter et al., 1989; Schmidtke and Büttner-Ennever, 1992) (Fig. 3A, E). The quantitative analysis revealed that the LP motoneurons received a substantial input (6.17 boutons/cell; SD: 3.56) of orexin-A-positive terminals (Fig. 3F; Fig. 4). Similarily the motoneurons of the eyelid closing orbicularis oculi muscle (OO) were identified by ChAT- immunoreactivity in the dorsolateral group of the facial nucleus (Porter et al., 1989). A slightly weaker orexin-A input was noted to the motoneurons of the lid closing orbicularis oculi muscle (3.52 boutons/cell; SD: 1.78) compared to those of the levator palpebrae muscle (Fig. 3M; Fig. 4). Interestingly the adjacent dorsomedial subgroup of the facial nucleus containing motoneurons of the frontalis muscle (Welt and Abbs, 1990) exhibit a very dense supply with orexin-A positive boutons

comparable to that in the dorsal raphe nucleus (Fig. 3L), although a systematic quantitative assessment hast not been performed for this group.

Orexin-A input on preganglionic neurons of the ciliary ganglion

In monkey, the preganglionic neurons of the ciliary ganglion form a compact group of ChAT-positive, medium-sized, multipolar cells with well developed dendrites in the Edinger-Westphal nucleus (EWpg) dorsal to nIII (May et al., 2008a; Horn et al., 2008) (Fig. 2A, B). The rostrally adjoining anteromedian nucleus (AM) contains medium-sized ChAT-positive preganglionic neurons as well. These are are located around the midline, with their long axis oriented vertically (Fig. 2F). The ChAT-positive populations of presumed preganglionic neurons in these nuclei received the strongest supply of orexin-A-positive terminals of all the cell groups studied (EWpg: 12.42 boutons/cell; SD: 6.93; AM:11,67 boutons/cell; SD: 6.91). This was approximately twice as many as contact motoneurons of MIFs and the LP (Fig. 2G, H; Fig. 4).

Orexin-A input to premotor cell groups of the saccadic system

In primates, the saccadic omnipause neurons (OPN) lie in the pontine reticular formation around the midline and form the nucleus raphe interpositus (RIP; Fig. 3A) (Büttner-Ennever et al., 1988). The OPNs were identified by their expression of parvalbumin (PV) or non-phosphorylated neurofilaments (NP-NF) (Fig. 3H, K). They appear as two vertical cell columns their long, horizontally-oriented dendrites reaching across the midline, and are found at the same level that rootlets of the abducens nerve (VI) traverse the pons (Horn et al., 1994). At first sight, a considerable number of orexin-A fibres and boutons appeared to be distributed within the OPN area (Fig. 3H, arrows) compared to the adjacent reticular formation. But with close inspection, it became obvious that only few orexin-A positive boutons are directly associated with individual OPNs, and these were mostly associated with dendrites (Fig. 3K). Systematic analysis of OPNs revealed a rather weak orexin-A input (1.04 boutons/cell; 1.23) (Fig. 4).

According to previous studies, the premotor burst neurons for vertical saccades may be identified in the mesencephalic reticular formation within the rostral interstitial nucleus of the medial longitudinal fasciculus (RIMLF) by their immunoreactivity for parvalbumin (PV) (Horn and Büttner-Ennever, 1998). Thereby, the RIMLF appears as a wing-shaped nucleus located ventrolateral to the third ventricle (Fig. 3A, B). In single staining for orexin-A, we found that the RIMLF contains only a few orexin-A-positive traversing fibres and boutons. Accordingly, quantitative analysis revealed only a weak supply of orexin-A positive punctae (1.02 boutons/cell; SD: 1.16) around PV-immunoreactive presumed vertical saccadic burst neurons in the RIMLF (Fig. 3C, D; Fig. 4).

Orexin-A input on serotoninergic neurons of the dorsal raphe nucleus

Staining for tryptophan hydroxylase (TRH) was used to identify the serotoninergic neurons of the dorsal raphe nucleus (DR), which lies immediately caudal to the trochlear nucleus (nIV) and extends dorsally into the ventral periaqueductal gray (Brownstein et al., 1975; Charara and Parent, 1998). Combined immunostaining revealed that orexin-A positive fibres and boutons were densely distributed among the serotoninergic neurons of the dorsal raphe nucleus (Fig. 3G; for location see Fig. 3A). The somata and proximal dendrites of most TRH-positive were densely contacted by orexin-A-positive boutons (15.18 boutons/cell; SD: 7.23). Accordingly this terminal pattern was considered as a very strong orexin-A input. (Fig. 3 G, I; Fig. 4).

Discussion

Using double-immunostaining for orexin-A and histological markers defining functional cell groups of the visuomotor system, we found three orexin-A termination patterns. 1. Weak orexin-A input to cell groups involved in the saccadic system, e.g. SIF-motoneurons of the extraocular muscles, the omnipause neurons in RIP and the premotor burst neurons in the RIMLF. 2. Medium density orexin-A inputs to MIF-motoneurons of the extraocular muscles and the LP and OO motoneurons in the central caudal nucleus and facial nucleus, respectively. 3. Dense orexin-A input to the preganglionic neurons of the ciliary ganglion in the EWpg and AM.

The localization of the orexin neurons appears to be conserved across species when comparing the rat, hamster, cat and human (Nambu et al., 1999; Mintz et al., 2001; Zhang et al., 2001, Thannickal et al., 2004; Saper, 2004). As in these species the orexin neurons of monkey lie in the diffuse cell groups of the tuberal region with one cluster in the perifornical region (PF) scattering in the lateral hypothalamic area (LHA), and another cluster in the adjacent dorsomedial hypothalamus (DMH). Efferents from this hypothalamic region have been carefully studied previously using autoradiographic techniques (Holstege, 1987). They form two different descending fiber bundles, a lateral tract arising mainly from the paraventricular hypothalamic nucleus; and a medial tract, probably originating in part from the orexin neurons, that targets the raphé magnus and raphé pallidus nuclei, lamina X of the spinal cord and the sympathetic preganglionic neurons in the upper thoracic intermediolateral nuclei.

Furthermore, the observed dense distribution of orexin-A fibres and boutons around serotoninergic neurons of the dorsal raphe nucleus in monkey confirms previous pharmacological and anatomical work in cat and rat (Zhang et al., 2004; Lee et al., 2005a) that shows this nucleus to be strongly influenced by orexin-A.

Orexin-A input to motoneurons of the extraocular muscles

As described in rat and cat (Peyron et al., 1998; Nambu et al., 1999; Zhang et al., 2004), only very few orexin-A terminals were present within the motonuclei of extraocular muscles in monkey, which contain the SIF-motoneurons (Büttner-Ennever, 2006). In contrast, the MIF-motoneurons of the C- and S-group received a considerable supply of orexin-A-positive boutons. This implies that orexin-A plays a role in their function. Their differential orexin-A input supports a current hypothesis that SIF- and MIF- motoneurons subserve different functions reflected in the differences in histochemical properties of their target muscle fibers (Spencer and Porter, 2006), as well as differences in afferent input (Büttner-Ennever et al., 1996a; Wasicky et al., 2004; Eberhorn et al., 2005; Ugolini et al., 2006). Based on combined anatomical and physiological work, SIF-motoneurons are considered to be twitch motoneurons, which primarily drive the eye movements including saccades. In contrast, MIF-motoneurons represent non-twitch motoneurons, which may subserve a tonic action in gaze holding or eye alignment (Goldberg et al., 1981; Nelson et al., 1986; Shall and Goldberg, 1992; Büttner-Ennever et al., 2002; Büttner-Ennever, 2006). Transneuronal tract-tracing methods have revealed that in contrast to SIF-motoneurons, the MIF-motoneurons do not receive premotor afferents from areas involved in eye movement generation, e.g. the paramedian pontine reticular formation or the magnocellular region of the medial vestibular nuclei, but do receive input from centers involved in gaze stabilization, e.g. the prepositus hypoglossus nucleus and marginal zone (Büttner-Ennever and Gerrits, 2004; Ugolini et al., 2006; McCrea and Horn, 2006). During the transition from the awake state, which is stabilized by orexin, to sleep, in which the orexin-neurons are silent, there is a loss of precise fixation, and slow “drifting”, “rolling” or “pendular eye movements occur (Henn et al., 1984; Mileykovskiy et al., 2002; Kiyashchenko et al., 2002; Ohno and Sakurai, 2008). Moreover, in the awake state, versional eye movements are strictly conjugate, which requires tightly linked

motor activity of co-acting eye muscles, and also the fine adjustment of muscle activity to obtain a stable retinal image without motion induced blur or double vision (Leigh and Zee, 2006). Recording studies in cat have shown that the discharge pattern of eye muscle motoneurons in the abducens nucleus does not differ between saccades in REM sleep and alertness (Escudero and Marquez-Ruiz, 2008). Similarly, behavioural studies in monkey revealed that rapid eye movements during REM sleep exhibit the same kinematics and temporal synchrony for both eyes as spontaneous saccades in the awake state, and may therefore be generated by the same neuronal circuits. In contrast, binocular coordination of the direction of eye movements is completely lost in REM sleep, when orexin neurons are silent and the rapid eye movements are typically disconjunctive (Zhou and King, 1997). These authors concluded that binocular coordination is an active process related to the attential mechanism associated with alertness. Our findings of a significant orexin-A input on MIF-motoneurons support the concept that eye alignment derives from an active input associated with being awake (Zhou and King, 1997; Lee et al., 2005b). The present data indicate that SIF-motoneurons are not controlled by monosynaptic orexin-A inputs, which is in line with the occurrence of precisely timed eye movements in the awake state and in REM (Zhou and King, 1997; Marquez-Ruiz and Escudero, 2008).

Orexin-A input on motoneurons of the eyelid

In contrast to the relative lack of orexin-A inputs to SIF-motoneurons within the nIII, the LP-motoneurons in the CCN receive a considerable supply of orexin-A-positive boutons. In the awake state, the eyes are kept open by the activation of the LP-motoneurons from an as yet unknown tonical excitatory input (Büttner-Ennever and Horn, 2004; Horn and Büttner-Ennever, 2008). With increasing fatigue, the activation of the LP-motoneurons ceases, and the eyelid lowers without involvement of the orbicularis oculi muscle, which only closes the eye during a blink (Sibony and Evinger, 1998). Orexin-A terminals like those seen in our studies could contribute to the tonic activation of the LP-motoneurons during wakefulness, when orexin-A is released (Lee et al., 2005b; Horn and Büttner-Ennever, 2008). During sleep, the orexin neurons are silent and the lack of activation will support the closure of the eye lids (Vanni-Mercier et al., 1994). This is corroborated by the observation that narcolepsy patients, who lack orexin-producing neurons, can not keep their eyes open during attacks of catalepsy (Peyron et al., 2000; Thannickal et al., 2000; Serra et al., 2008). This fits also well with our observation of a rather strong orexin-A input to the motoneurones in the dorsomedial subgroup of the facial nucleus innervating the frontalis muscle and contributes to eyelid elevation (Skarf, 2005). The orbicularis oculi muscle (OO) provides active eye closure and eye closing during blinks (for review: Skarf, 2005). The modest orexin-A input to OO-motoneurons, similar to that seen in LP-motoneurons, may provide a modulatory excitatory signal that may participate in setting a threshold for activation of the motoneurons during wakefulness.

Orexin-A input on preganglionic neurons of the ciliary ganglion

The preganglionic neurons of the macaque ciliary ganglion are located in the cytoarchitecturally defined EWpg and rostrally adjoining AM (Akert et al., 1980; Ishikawa et al., 1990; May et al., 2008b). Neurons in these areas received the strongest input of orexin-A of all the cell groups of the visuomotor system examined here, implying considerable direct influence over the pupillary and/or accommodation system. The size of the pupil, its responsiveness to light, and the natural occurring fluctuations of its diameter, all vary with the degree of alertness (Wilhelm, 2008), but also with emotional arousal (Bradley et al., 2008). For example the pupillary fluctuations become stronger with increased drowsiness and therefore have been used as an indicator to measure alertness (Lowenstein et al., 1963; Wilhelm et al., 1998; Wilhelm, 2008). In addition, lens accommodation is abolished in the first stage of light sleep, drowsiness. This is when orexin-A neurons become inactive (Henn et al., 1984; Lee et al., 2005b). Interestingly, the naturally appearing fluctuations of the pupil diameter are

less pronounced in patients with narcolepsy compared to controls with an intact orexin-system (O'Neill et al., 1998), which supports a direct orexin influence on both muscles controlling pupillary size. In rat there is a similar strong orexin-A innervation of the sympathetic preganglionic neurons in the first thoracic segment of the spinal cord, most of them projecting to the superior cervical ganglion, which in turn mediates the innervation of the dilatator muscle (Llewellyn-Smith et al., 2003). The strong orexin-A input to preganglionic neurons of both, sphincter and dilatator muscle supports the concept that orexin functions as a modulatory system and not as an information signal (Saper et al. 2005); in this case modulating the signal intensities to the pupillary system during wakefulness, and perhaps more indirectly during emotional arousal (Bradley et al., 2008; Tsujino and Sakurai, 2009). Consequently during sleep, the reduction of orexin-A inputs to preganglionic neurons of both pupillary muscles may be the cause of increasing fluctuations of pupil size observed by Wilhelm et al., (1998).

Here it should be pointed out that although there are similar observations of a rather selective strong expression of the orexin receptor 1 in the Edinger-Westphal nucleus in the rat (Hervieu et al., 2001), these results are related to a very different system from the one in monkey. In the rat, the orexin-A input to the Edinger-Westphal nucleus is thought to be related to feeding behaviour (Willie et al., 2001). In contrast to monkey, in rat the cytoarchitecturally defined Edinger-Westphal nucleus does not contain preganglionic neurons of the ciliary ganglion but represents a cell group expressing the neuropeptide urocortin 1, an endogeneous ligand for the CRF-receptors, which is involved in food intake (Vaughan et al., 1995; Spina et al., 1996; Yamamoto et al., 1998; Weitemier and Ryabinin, 2005; Horn et al., 2009). In monkey, the area containing urocortin-positive forms a cytoarchitecturally inconspicuous cell group next to the EWpg (May et al., 2008a; Horn et al., 2008). We found that it is covered with numerous orexin-A positive fibres and boutons (Horn, personal observation) that were presumably involved in the regulation of the feeding behaviour (Pan and Kastin, 2008).

Orexin-A input to neuronal cell groups of the saccadic system

We did not observe any significant orexin-A input on premotor burst-neurons for vertical saccades in the RIMLF or on the OPNs in the nucleus raphe interpositus (RIP). This finding implies that orexin-A does not exert a direct influence on the immediate premotor cell groups of the saccadic system and or on the execution of saccades per se (Büttner-Ennever et al., 1988; Scudder and Kaneko, 2002). This is in line with the fact that saccadic eye movements occur in alertness, when orexin-A is released, and also during REM sleep, when orexin-A neurons are mostly silent (Lee et al., 2005b). If orexin-A exerts any action on the saccadic system at all, then it must be indirect or through more upstream neurons, e.g. the nucleus raphe magnus, nucleus reticularis pontis oralis or deep layers of the superior colliculus, which all receive orexin-A positive fibres (Peyron et al., 1998; Nunez et al., 2006) and project to omnipause neurons (Langer and Kaneko, 1990; Büttner-Ennever et al., 1999).

Orexin modulates visuomotor function

There are already several lines of evidence from previous tract-tracing experiments that hypothalamic neurons are interconnected with brainstem nuclei involved in visuomotor function: retrograde tract-tracing experiments in monkey revealed neurons in the lateral hypothalamus, which project to the oculomotor complex (Steiger and Büttner-Ennever, 1979). Similarily, experiments with live rabies virus, injected into the lateral rectus muscle of monkey (Ugolini et al., 2006) revealed labelled neurons scattered in the perifornical hypothalamus after 2-3 days survival time (personal observation). In hamster another link between orexin neurons and visuomotor function is implicated by the strong supply with orexin-A positive boutons from the lateral hypothalamus to the intergeniculate leaflet and the medial vestibular nuclei (Horowitz et al., 2005; Vidal et al., 2005). The medial vestibular nuclei, involved in the generation of eye movements during head rotation, have been shown to project

to many areas receiving strong orexin-A input including the intergeniculate leaflet (Horowitz et al., 2004). The intergeniculate leaflet of the hamster is the homologue to the pregeniculate nucleus in monkey (Livingston and Mustari, 2000), which corresponds to the ventral lateral geniculate nucleus (Nakamura and Itoh, 2004). The ventral geniculate nucleus has several subdivisions, some of which have been shown to be involved in visuomotor functions (Büttner and Fuchs 1973; Büttner-Ennever et al., 1996b). In our results the medial division of the pregeniculate nucleus received a strong supply of orexin-A boutons as well (own observations), and confirm the hypothesis that orexin modulates visuomotor systems.

Conclusion

Orexin is thought to promote and stabilize wakefulness by its excitatory inputs to monoaminergic nuclei, e.g. locus coeruleus and raphe nuclei, where neurons fire at the highest rates during wakefulness, slow down during NREM sleep, and stop firing during REM sleep (for review: Saper et al., 2005) – the same firing pattern is seen in orexin-A neurons (Lee et al., 2005b). The present study confirms the orexin-A inputs to the locus coeruleus and raphé system in monkey, through which the orexin pathways can certainly modulate the eye movement system indirectly during alertness. In addition our results revealed a considerable input from orexin-A neurons onto motoneurons of LP, OO, MIF-motoneurons and on the preganglionic neurons of the ciliary ganglion. These targets have a high and stable level of activity during wakefulness to keep the eyes open and blinking, provide eye alignment or fixation, and modulate the pupil and lens. Only little orexin-A input was found to target the neurons directly involved in the generation of saccades, and whose activity is stable during wakefulness. These results provide evidence for the functional role of orexin inputs in stabilizing the activity of neuronal systems during wakefulness.

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Abbreviations

AM
anteromedian nucleus

CCN
central caudal nucleus

ChAT
choline acetyltransferase

DHA
dorsal hypothalamic area

DMH
dorsomedial hypothalamus

DR
dorsal raphe nucleus

EWpg
Edinger-Westphal nucleus (preganglionic neurons)

FO
fornix

H
H field of Forel

IO
inferior oblique muscle

IR
inferior rectus muscle

LHA lateral hypothalamic area

LP levator palpebrae muscle

MIF multiply-innervated muscle fibre

MLF medial longitudinal fasciculus

MR medial rectus muscle

MT mamillothalamic tract

nIII oculomotor nucleus

nVII facial nucleus

NP-NF non-phosphorylated neurofilaments

OO orbicularis oculi muscle

OPN omnipause neurons

PF perifornical region

PV parvalbumin

RIMLF rostral interstitial nucleus of the medial longitudinal fasciculus

RIP nucleus raphe interpositus

SIF singly-innervated muscle fibre

SO superior oblique muscle

SR superior rectus muscle

VMH ventromedial hypothalamus

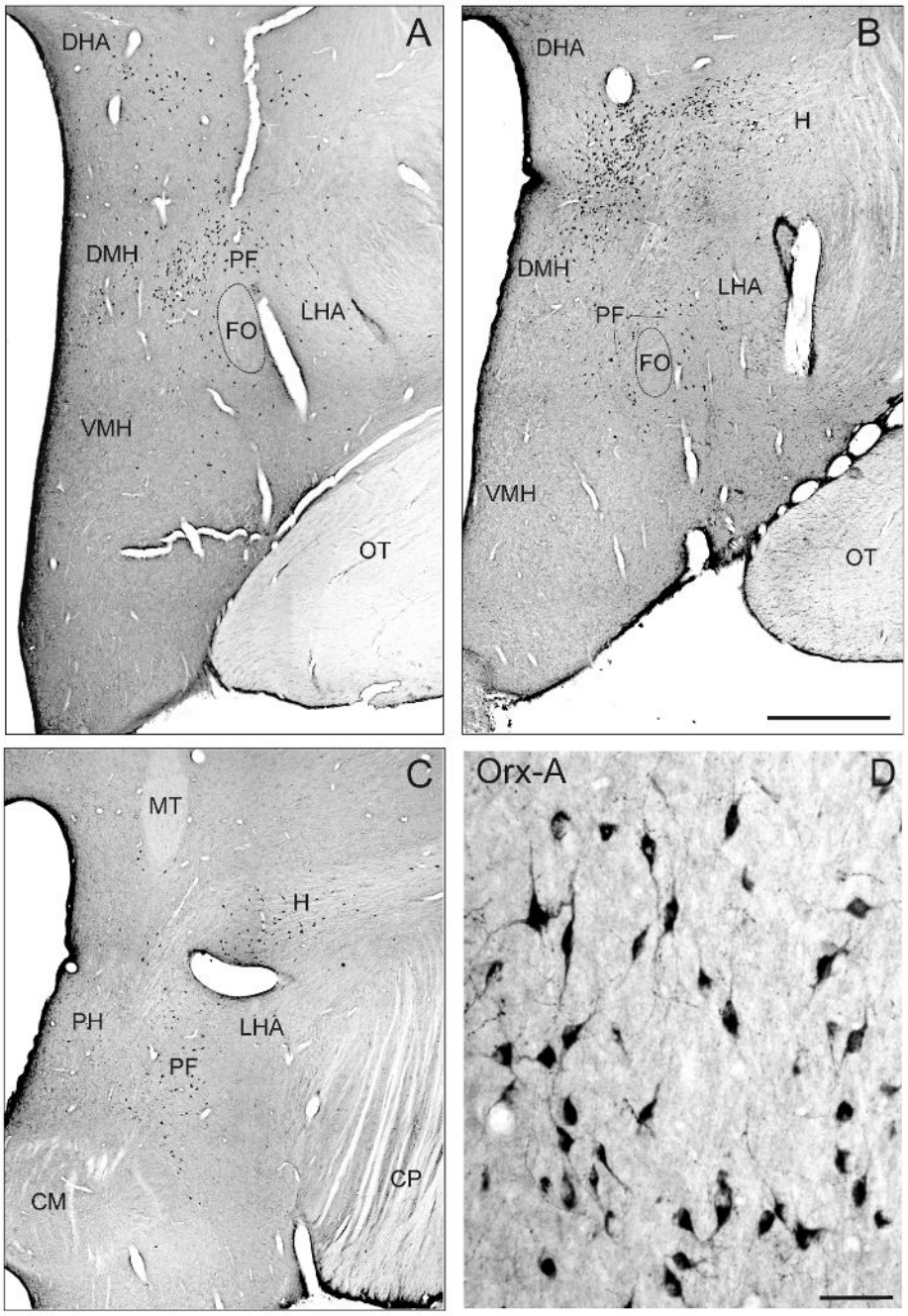

Figure 1.

Frontal sections through 3 planes of the monkey hypothalamus (rostral to caudal) demonstrating the distribution of orexin-A positive neurons within the dorsomedial hypothalamicus (DMH) and in the perifornical region (PF) around the fornix (FO) spreading out in the lateral hypothalamic area (LHA) (A). The high-power photograph shows the morphology of orexin-A positive neurons in the DMH (B). Scale bar: A = 1mm; B = 50μm. CP = cerebral peduncle, DHA = dorsal hypothalamic area; H = H field of Forel; OT = optic tract.

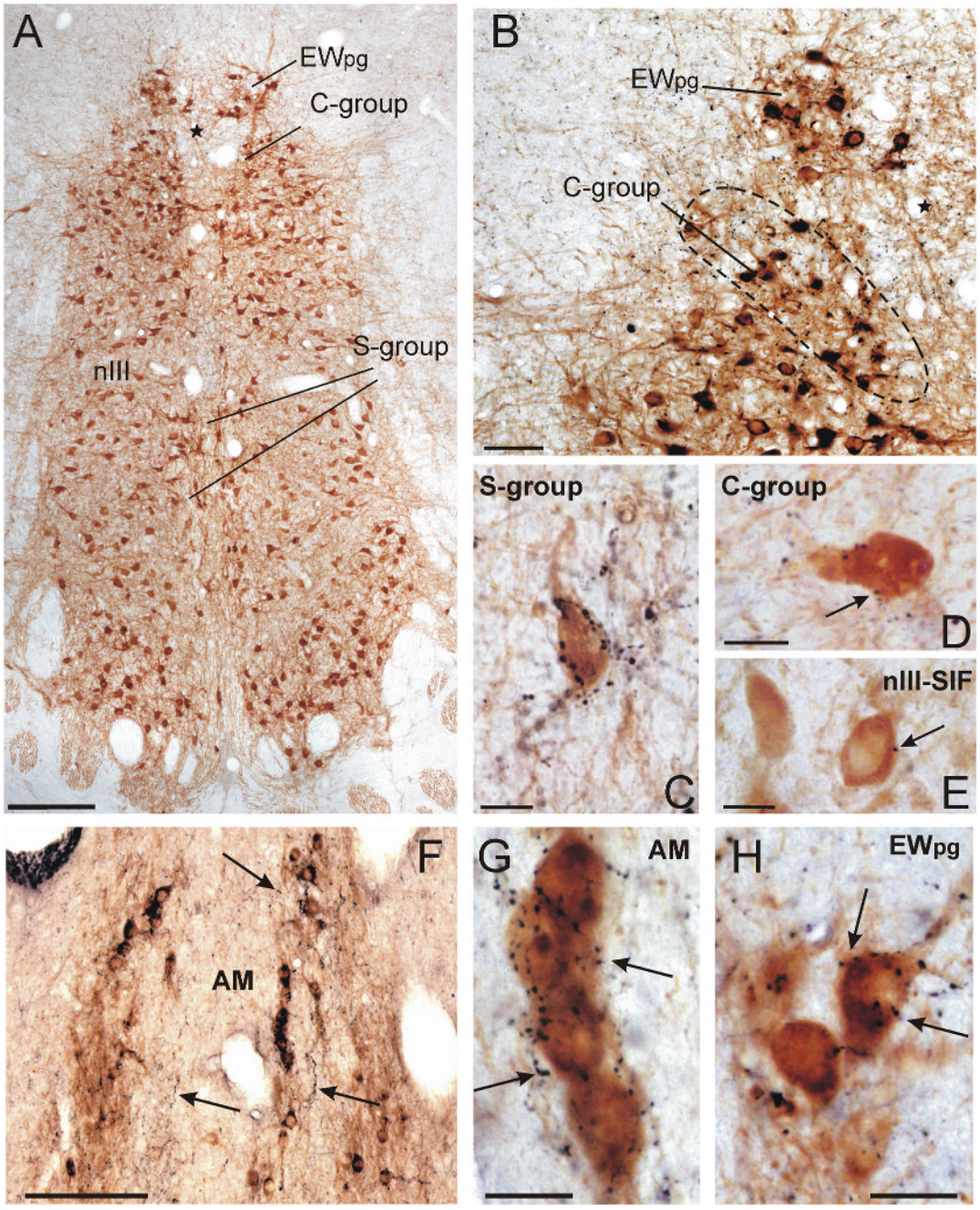

Figure 2.

Frontal sections through the monkey oculomotor nucleus (nIII) stained for choline acetyltransferase (ChAT; brown) and orexin A (Orx-A; black) in the overview (A) and at high magnification (B). The star indicates the same blood vessel in A and B. The high-power photographs (C-H) show that the MIF-motoneurons in the C- and S-group are associated with far more orexin-A-positive terminals (C, D, arrows) than the SIF-motoneurons within nIII (E, arrow). Numerous orexin-A-positive fibres are distributed throughout the ChAT-positive preganglionic neurons of the ciliary ganglion in the Edinger-Westphal nucleus (EWpg) (B) and anteromedian nucleus (AM) (F; arrow). The high-power photographs demonstrate the

dense supply of ChAT-positive preganglionic neurons by orexin-A-positive punctae in the AM and EWpg (G, H, arrows). Scale bar: A, F = 200μm; B = 50μm; C- E; G, H = 25μm

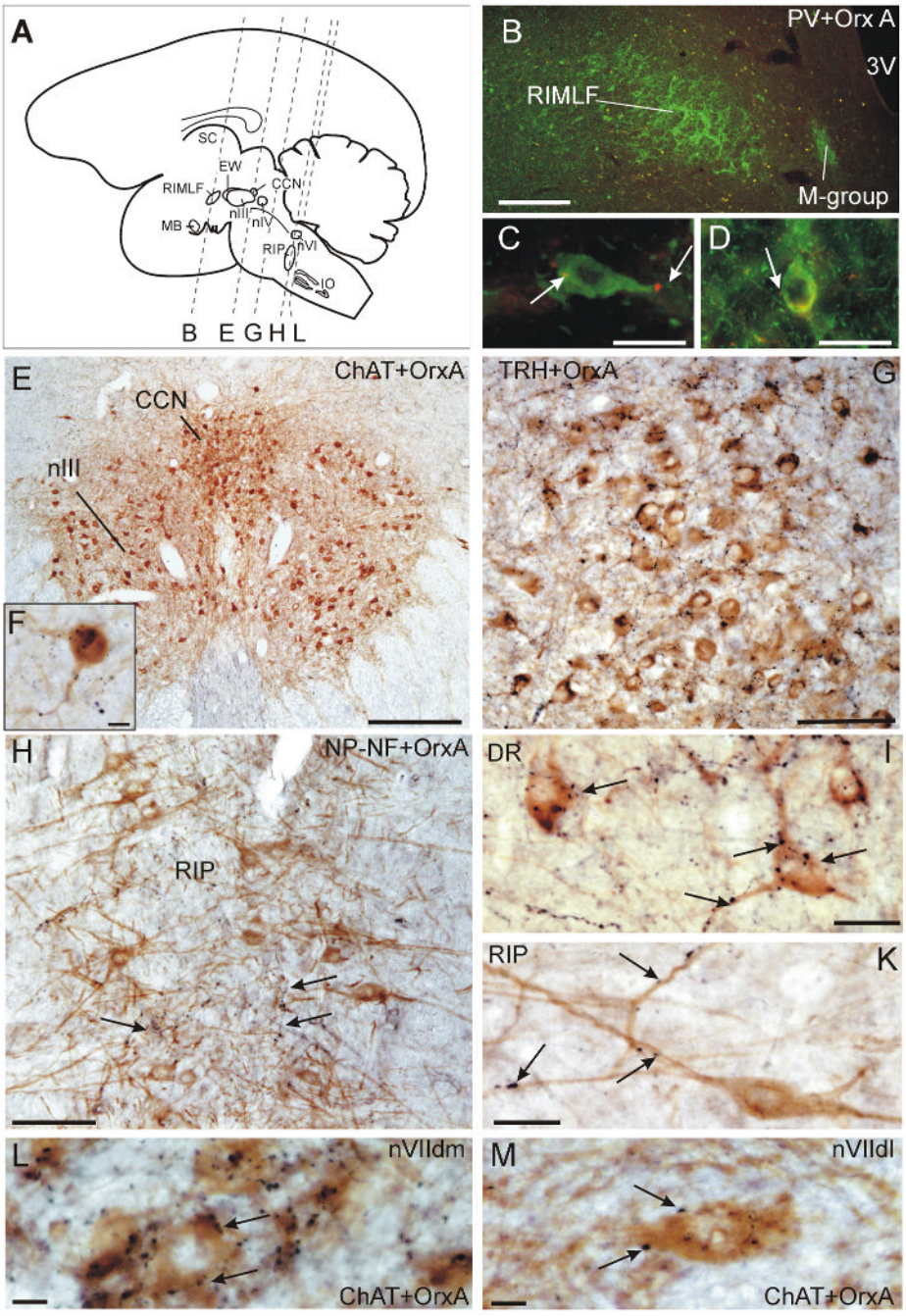

Figure 3.

Schematic sagittal view of a monkey brain (A) which indicates the frontal cutting planes of B, E, G, H, L. Frontal section through the RIMLF immunostained for parvalbumin (PV; green) and orexin A (Orx-A; red) in the overview (B) and at high magnification (C,D). Note the putative PV-positive burst neurons in RIMLF are contacted by only few Orx-A boutons (arrows, C, D). Overview of a frontal section through the central caudal nucleus (CCN) immunostained for choline acetyltransferase (ChAT; brown) and Orx-A (black) (E). Highpower magnification of ChAT-positive motoneurons of the levator palpebrae in CCN associated with numerous Orx-A punctae on the soma and dendrites (F). Overview of a frontal section through the dorsal raphe nucleus (DRN) immunostained for tryptophan hydroxylase

(TRH; brown) and Orx-A (black) (G). Highpower magnification of TRH-positive neurons in the DR covered by many Orx-A boutons (arrows) (I). Overview of a frontal section through the nucleus raphe interpositus (RIP) containing saccadic omnipause neurons immunostained for non-phosphorylated neurofilaments (NP-NF; brown) (H). Detailed view of a NP-NF-positive omnipause neuron associated with few Orx-A (black) boutons (arrows) (K). The ChAT-positive motoneurons in the dorsolateral subgroup in the facial nucleus (nVIIdl) innervating the orbicularis oculi muscle receive much less Orx-A boutons (M) than the motoneurons in the facial dorsomedial subgroup (nVIIdm) innervating the frontalis muscle (L). Scal bar: B, E = 500μm; C, D, I, K = 25μm; G, H = 100μm

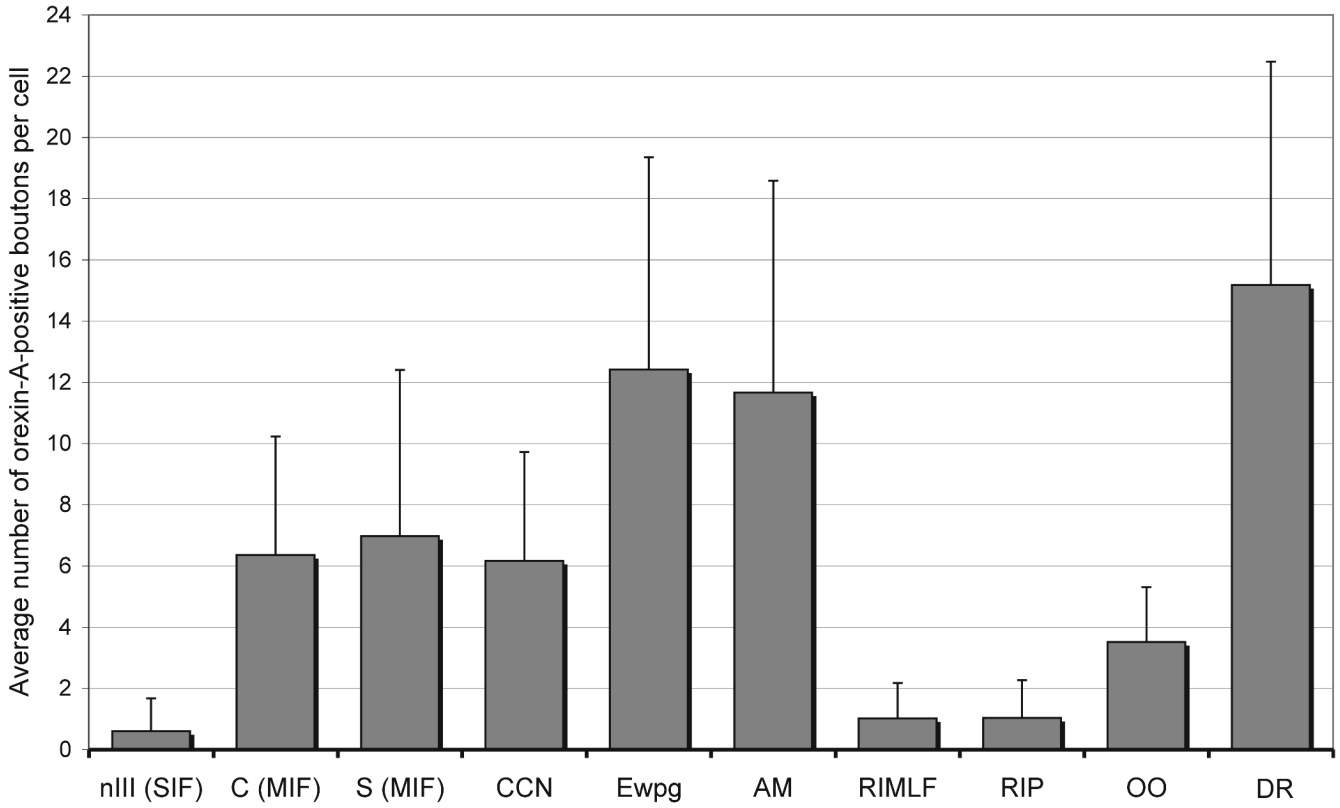

Figure 4.

Quantitative comparison of the average number of orexin-A positive boutons associated with single neurons of the visuomotor brain regions. The exact average of density values for orexin-A positive inputs and the standard deviations are written in the text. Note that the preganglionic neurons of the ciliary ganglion receive the strongest supply of orexin-A afferents compared to SIF-motoneurons and premotor neurons of saccades.

Table 1

List of antibodies and their sources with the applied methods used in this study.

Antibody	Host	Antigen	Manufacturer	Cat. No.	Dilution	
					Immunoperoxidase	Immunofluorescence
Orexin A	rabbit	orexin A	Chemicon, Temecula, CA	AB3098	1:6000	1:2000
ChAT	goat	choline acetyltransferase	Chemicon, Temecula, CA	AB144P	1: 1000	
PV	mouse	parvalbumin	Swant, Bellinzona, Switzerland	235	1:2500	1:2000
SMI32	mouse	non-phosphorylated neurofilaments	Sternberger Monoclonals, Lutherville, MD	SMI32	1:6000	1:1000
PH8-1	mouse	tryptophan hydroxylase	Chemicon, Temecula, CA	MAB5278	1:2000	