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Noradrenergic substrates sensing light within brainstem reticular formation as targets for light-induced behavioral and cardiovascular plasticity

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ABSTRACT

The occurrence of pure light exerts a variety of effects in the human body, which span from behavioral alterations, such as light-driven automatic motor activity, cognition and mood to more archaic vegetative functions, which encompass most organs of the body with remarkable effects on the cardiovascular system. Although empirical evidence clearly indicates occurrence of these widespread effects, the anatomical correlates and long-lasting changes within putatively specific neuronal circuitries remain largely unexplored. A specific role is supposed to take place for catecholamine containing neurons in the core of the brainstem reticular formation, which produces a widespread release of noradrenaline in the forebrain while controlling the vegetative nervous system. An indirect as well as a direct (mono-synaptic) retino-brainstem pathway is hypothesized to rise from a subtype of intrinsically photosensitive retinal ganglion cells (iPRGCs), subtype M1, which do stain for Brn3b, and project to the pre-tectal region (including the olivary pre-tectal nucleus). This pathway provides profuse axon collaterals, which spread to the periaqueductal gray and dorsal raphe nuclei. According to this evidence, a retino-reticular monosynaptic system occurs, which powerfully modulate the noradrenergic hub of reticular nuclei in the lateral column of the brainstem reticular formation. These nuclei, which are evidenced in the present study, provide the anatomical basis to induce behavioral and cardiovascular modulation. The occurrence of a highly interconnected network within these nuclei is responsible for light driven plastic effects, which may alter persistently behavior and vegetative functions as the consequence of long-lasting alterations in the environmental light stimulation of the retina. These changes, which occur within the core of an archaic circuitry such as the noradrenaline-containing neurons of the reticular formation, recapitulate, within the CNS, ancestral effects of light-driven changes, which can be detected already within the retina itself at the level of multipotent photic cells.

Key words

Photic retina • iPRGC, M1 Brn3b • circadian rhythm • brainstem reticular formation • locus coeruleus • rostral ventrolateral medulla • area postrema • A1 • A2

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Introduction

From the retina to extra-geniculate areas

The powerful plastic effects of light are evident already in the retina itself since pioneer papers during the mid'70s up to recent reports. This concept, is well established concerning intrinsic retinal circuitries dedicated to visual perception (Berry, 1976; Rose, 1977), and during retinal degeneration (Pinelli et al., 2020a; Pinelli et al., 2021a;b;c; Strettoi et al., 2022) including plastic changes in the retinal pigment epithelium (Pinelli et al., 2020b). This is now strengthened by evidence showing that, specific wavelengths increase proliferation rate of retinal stem cells over four-fold compared with that measured in baseline conditions (Wang et al., 2019). This phenomenon occurs within retinal stem cells, which do receive either blue or red stimulation. This phenomenon purely depends on the impact of light and it is highly dependent on the wavelength, which stimulates the retinal stem cells. In fact, in the anterior part of the retina a rich stem cell niche occurs, which promotes a wavelength-dependent cell proliferation and cell differentiation in the retina. In detail, following stimulation with blue wavelengths a preferential differentiation of stem cells towards a glial phenotype takes place, while infrared light stimulation produces the differentiation of the same stem cells into neurons (Wang et al., 2019). The significance of pure light in producing such an archaic vision-independent, photic plasticity is evident from the requirement of pure physical energy in the aspect of oscillatory phenomena of electromagnetic fields as the appropriate stimulus. This brings intriguing questions on the potential synergy in the form of synesthesia, which may act on retinal stem cells, when pulses of electromagnetic fields are coupled with pulses of acoustic energy. In fact, as recently reported, the training of patients suffering from central scotoma with acoustic biofeedback can progressively improve visual acuity, by moving fixation to eccentrically placed healthy area of the retina, which is primed to act as a "pseudo-fovea" (Tonti et al., 2021). In keeping with plasticity induced by pure light, which occurs depending on pulses of electromagnetic wavelengths, it is remarkable that both blue light, which stimulates stem cells to form glia, and red light, which stimulates the stem cells to form neurons need to be applied according to intermittent patterns. In fact, as originally demonstrated by Wang et al. (2019), when the photic stimulation is applied according to a specific timing (45 min for 5 consecutive days) both glial and neuronal phenotypes are induced just depending on the specific wave-length. Such a powerful effect of light on retinal regeneration shed a practical perspective in patients, who improved following photo-stimulation as reported in a recent manuscript (Pinelli et al., 2021c).

Transferring photic stimulation to the CNS

Since light, which feeds the retina, is able to transfer its effects within the whole CNS and from there to the whole body, the present manuscript analyzes which neuronal substrates may be involved beyond the retina and which plastic effect may occur to produce widespread changes in the human body.

The light, which enters the CNS through the retina, is transmitted to a variety of brain areas to be transduced within a variety of biochemical cascades. These extend way beyond what is currently defined as visual pathways. In fact, light is transferred along a number of non-visual extra-geniculate centers to impinge in a variety of brain circuitries, which were not primarily considered in the context of the visual system and are known to promote a number of behavioral and vegetative alterations. In fact, the remarkable evolution of the visual processing represents the ultimate step, which is triggered by light and photoreception. When studying archaic nervous systems the impact of light is rather represented by mere photic stimulation, which lacks the ability to provide information concerning the surrounding scenario, which is commonly defined as peripheral visual field. This implies neither shapes nor color detection, neither object nor movement perception but mere light, which variably contrasts and brakes in the shadow. In this way, light exposure produces activation of archaic brain circuitries, which are the final common pathways for different kind of stimuli. Within such a rudimental anatomical recipient, light merges with sounds, and pain and multiple sensory systems add on to adjust physiology and behavior in the human body.

Thus, light is the elementary stimulus, which appears at first in the visual system, when its evolutionary steps are reviewed. Accordingly, each living system, which possesses an external surface, gifted with light-sensitive structures is already definable as

gifted with photoreceptor in the general meaning. In fact, the etymology of the word "photoreceptor" it is not necessarily related to visual perception but merely indicates the elementary detection of light without any visual implication. In this context, even archaic systems such as plants possess photoreceptors, and they are powerfully modulated by light exposure. This is possible due to specific surface cells, which receive light and own specific biochemical species, light sensitive molecules, which structure and activity is modified by specific wave-lengths and light intensities.

When conceived within the frame of comparative biology the natural questions which rises up is the following: is such an ancestral photic role of light progressively lost in the evolution to be replaced by the visual system? Or, both photic and visual systems are rather preserved in the human retina? Are photic pathways preserved in their course from the retina to the CNS and within CNS itself? Again, is the presence of photosensitive molecules still relevant, aside from visual processing, in the photic effects produced by light exposure in the human CNS? Thus, the present article analyzes the relevance of light exposure in the human chemical neuroanatomy aside and beyond the classic neurotransmitters and pathways, which specifically define the visual perception. The effects of light within the retina, and along the pathways carrying non-image forming, photic stimuli within the CNS will be analyzed to define which plastic effects may derive from light exposure, independently from visual processing and perception, but merely fostered by light.

The main retinal source of photic pathways to the CNS

The maintenance of light-dependent responses in the retina, which occurs even in the absence of the CNS is a classic example of how conserved is the archaic organization of such a light sensitive epithelium. In fact, evidence indicates how, within the retina, a sub-class of neurons are able to sense light independently from image forming activities. This cell population corresponds to intrinsically photosensitive retinal ganglion cells (iPRGCs). These cells, which are identified for three decades (Freedman et al., 1999; Lucas et al., 2001), are currently conceived as seminal to send their input to brain areas where non-image forming centers are placed (Figure 1). This is supposed to sub-serve specific activities such as the simple photic reflex, which promotes the contraction of the smooth muscle working as the sphincter of the iris to impede an excess of light to impact the retina. Routinely such a reflex is considered to depend on the activation by iPRGCs axons of ganglionic cells projecting to extra-geniculate centers, which in turn activate the parasympathetic component of the oculomotor nerve to contract the sphincter iris. Indeed, mammalian retina preserves a CNS-independent mechanism, which allows a discrete group of small iPRGCs to promote miosis even in the absence of their projection to the CNS. This is demonstrated in mammals where miosis following light still occurs following denervation of the eyeball (Semo et al., 2014). In fact, a small part of iPRGCs, are able to act autonomously, keeping effective pupillary constriction when either the eye, or the iris itself are physically isolated from the brain. This phenomenon is named "intrinsic pupillary light reflex" (iPLR). The iPLR can be abolished only in the absence of iPRGCs. This phenomenon is thought to be induced by a small population of

iPRGCs placed in the retinal ciliary marginal zone (CMZ) (Semo et al., 2014), which corresponds to the classic definition of "ora terminalis". This intrinsic reflex is generated by melanopsin-positive, direct retino-ciliary projections, which appear to emanate from Brn3b negative, M1 type iPRGCs (Semo et al., 2014). These cells send fibers from the retina into the iris via the ciliary body. In a very recent paper these archaic cells were suggested to be involved also in the contraction of the ciliary muscle and to modulate also the dilatation of the iris. It is hypothesized that these small iPRGC, which act as sensor and effectors corresponds to the stem cell population, which are activated by specific wavelengths of photic energy. It is remarkable that, stem cells placed in the anterior ciliary zone described by Wang et al. (2019) similarly respond to melanopsin as a novel and specific modulator that directs light-induced stem cells to proliferate and differentiate, thus operating functional changes in the retina which can be transferred towards the CNS. Thus, the role of non-image forming cells in the retina is wide and it is preserved in mammals with a well-developed CNS (Semo et al., 2014). This concept indicates a preservation of archaic function within the mammalian retina itself, which are responsible

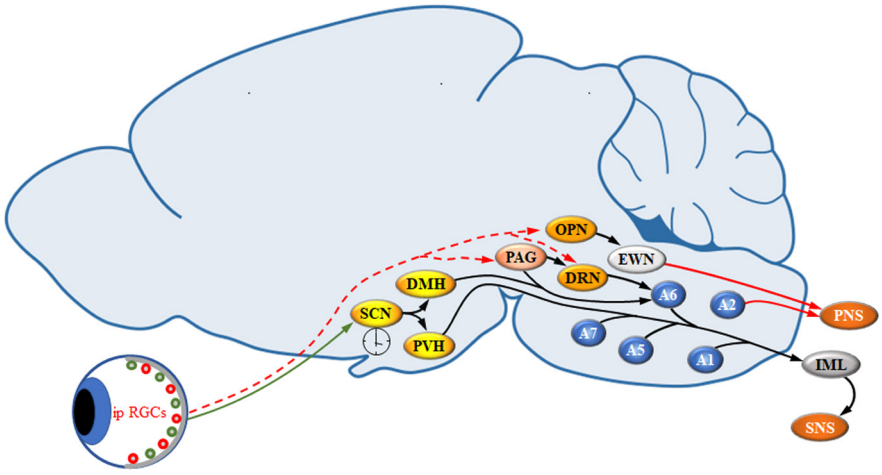

Fig. 1 - Occurrence of specific M1 iPRGC-dependent retinal extra-geniculate pathways.
In the retina, the presence of various iPRGCs generate different pathways. Among these, the M1 cells are responsible for projecting mainly to extra-geniculate brain areas. In the M1 group, two main streams can be distinguished. One is responsible for the activation of the suprachiasmatic nucleus, while the other projects mainly to pre-tectal neurons. These two streams are distinguished also based on the different subtypes of M1 iPRGC, which originate the pathway. In fact, the stream directed towards the suprachiasmatic nucleus mainly originates from Brn3b negative, M1 type ipRGC. In contrast, the retino-brain stream targeting the pre-tectal region outsources from Brn3b positive, M1 type ipRGC.
The figure shows the main targets for both streams. The solide red arrow refers to projections from Brn3b negative, M1 type ipRGCs, while the dashed arrow indicates projections from Brn3b positive, M1 type ipRGC.
Abbreviations. SNC: Suprachiasmatic nucleus; DMH: Dorso-medial hypothalamus; PVH: Paraventricular nucleus; PAG: Periaqueductal gray; OPN: Olivary pretectal nucleus; DRN: Dorsal raphe nucleus; EWN: Edinger-Westphal nucleus; A6: Locus coeruleus; A1-A2-A5-A7: Noradrenergic nuclei; IML: Intermedio-lateral column; SNS: Sympathetic Nervous System; PNS: Parasympathetic Nervous System.

for the occurrence of pure light-induced plasticity. This is likely to be preserved within CNS, where the preservation of non-image forming cells and centers is described. In fact, iPRGC were defined as the major conduits for retinal input to the brain (Semo et al., 2014). The present manuscript focuses on some of those archaic nuclei, which mediate light-induced plasticity in the CNS. These nuclei belong to a large group of extra-geniculate pathways. It is remarkable that, a number of these nuclei and neurons possess photosensitive molecules, which within the CNS lose their photo-sensitive function to act as neuromodulators. This is the case of melanin-containing melatonin cells in the pituitary gland, melanin-containing dopamine producing neurons in the mesencephalon and melanin-containing noradrenaline and adrenaline neurons in the reticular formation of the pons and the medulla oblongata, as well as serotonin neurons in the dorsal raphe nucleus. The light sensitive properties of these neurons suggest that the presence of the photo-pigment is preserved from the melanopsin-containing retinal ganglion cells to the CNS within monoaminergic extra-geniculate neurons, which are critical to sense light. In fact, the ability to react to light was seminal in identifying these neurons during pioneer studies. This was shown by Dahlström and Fuxe (1964), by exposing these neurons to the Falk-Hillarp luminescent histochemical method. It is remarkable that melanopsin can be detected within retina-raphe projections (Li et al., 2016), where it produces an intra-cellular cascade which induces marked recovery following axonal degeneration (Li et al., 2016). This suggests that the photo-pigment melanopsin and melanopsin-containing axons, which distribute within the CNS act as a sort of trophic guide for those areas connected with the photic retina. In fact, following axotomy, ipRGCs increase levels of a specific molecular complex named mechanistic target of rapamycin (mTOR), which induces axonal regeneration and it is produced by melanopsin itself (Li et al., 2016). The plastic effects of melanopsin on mTOR and axonal regeneration are induced by light exposure. Similarly the amount of melanopsin is correlated with the duration and intensity of response to light. This is why modern optogenetics

recapitulate the Falk-Hillarp method and uses light-induced activation of ectopic photo-pigment to promote selective and strong neuronal activation (Adamantidis et al., 2007). Thus, during evolution, the natural guide provided by photo-pigment-related neurotransmitters may be responsible for the development of highly interconnected neuronal networks such as the catecholamine cells in the extra-geniculate system, which form an anatomical and functional hub implicated in controlling alertness, mood, cortical plasticity as well as a number of vegetative functions.

This concept leads to the aim of the present study to detail, within the extra-geniculate systems, the chemical neuroanatomy of a subset of photo-pigment-containing extra-geniculate areas such as the catecholamine-containing neurons in the lateral column of the brainstem reticular formation (Bucci et al., 2017; Bucci et al., 2018).

In fact, while a number of studies address the plasticity induced by visual inputs during development and during adult re-arrangements of the visual system in authentic visual, image-forming areas such as the lateral geniculate body and the cerebral cortex, only a few reports are available concerning plasticity within extra-geniculate subcortical areas and plastic effects purely induced by light exposure. Among these, a seminal study analyzes the effects produced in the superior colliculus by retino-tectal projection in regulating plasticity in the developing CNS (Mu et al., 2006). This study demonstrates that, by pairing light stimuli with spiking of the tectal cells, a persistent enhancement of both excitatory and inhibitory responses induced by light occur within tectal neurons. Such a spike timing-dependent plasticity (STDP) strongly correlates with relative timing between light stimuli delivered to the tectum. In this way, light modifies the response of light-sensitive extra-geniculate neurons depending on the time-schedule of light exposure (Mu et al., 2006). This is reminiscent of the timing, which is key in pulsing light to induce plasticity within retinal stem cells. Here, plasticity takes place in shaping CNS tectal cell receptive fields. These changes are mediated by specific molecules, which are required to produce a persistent synaptic potentiation known as long term potentiation (LTP), such as brain-derived neurotrophic factor (BDNF). Conversely, nitric oxide (NO) is required to induce a persistent depression following light exposure known as long term depression (LTD, Mu et al., 2006).

The sudden cyclic effects of light on persistent alterations of brain excitability are evident in our daily life, as shown by the common evidence, which indicates how light regulates the sleep-waking cycle. The neural substrates sub-serving this process as part of circadian rhythms are profusely endowed within hypothalamus, and they are often identified in the suprachiasmatic nucleus (SCN). Nonetheless, evidence exists that catecholamine neurons of the brainstem reticular formation are involved in propagating photic stimuli (Munn et al., 2015, Bryère et al., 1986; Gasanova et al., 1985; Mager et al., 1984). In fact, a neural circuit directly connects what we call the photic retina represented by the intrinsically photosensitive retinal ganglion cells (iPRGCs) to a number of brain sites. The circadian biological rhythm, which is generated by light is a seminal and trivial example of many integrated effects and brain sites, which respond to pure light exposure. Among these, the photic retina is connected with corticotropin releasing hormone (CRH) containing neurons in optic pre-tectal nucleus (OPN, Zhang et al., 2021). This projection operates at sub-cortical level to generate non-rapid eye movement (NREM) sleep, without influencing REM sleep. The relevance of these light-responsive neurons within OPN is indicated by the evidence that, by inhibiting these neurons, light become non-effective in altering the NREM sleep (Zhang et al., 2021). Remarkably, these light-responsive neurons project to well-known wakefulness-promoting brain regions to inhibit arousal and driving light-induced NREM sleep (Zhang et al., 2021). This circuitry binding photic retina with the brain is neither necessary nor sufficient indeed for the acute effect of light on sleep.

Porcu et al 2018 reviewed how long-lasting changes produced by light may involve synaptic plasticity mainly focusing on the direct projection from iPRGCs to the SCN. In this work Authors emphasized the multiple connection of the SCN, which was considered as the pivot for all these effects. In detail, they examined how different photo-periods may affect alertness and cognition via the projection to the SCN. In this manuscript, various types of neuroplasticity in the SCN were described as potential mediators of long-lasting effects produced

by light in the CNS. Nonetheless, even in such a SCN-centered manuscript authors mentioned other sites, which may provide the target for the activity induced by retinal iPRGCs. These multiple areas involve the connection from the retina to the olivary pretectal nucleus (Figure 1, OPN also named pre-tectal area) and hence, the peri-aqueductal grey. Collaterals of this pathway generate a direct retinal projection to the dorsal raphe and possibly, the pontine nucleus locus coeruleus (LC, A6). At first LC was the only cell group of the brainstem reticular formation being involved in these plastic effects induced by timing of light exposure. However, in a very recent manuscript profuse evidence was added on and multiple nuclei of the reticular formation also including DA-containing and 5HT-containing cells were analyzed. In their elegant article Maruani and Geoffroy (2022) emphasize how the photic stimulation from the retina may regulate the mood via acting on different pathways. As they indicate, while a classic pathway engages the SCN, another is independent from SCN and it recruits a number of extra-geniculate areas but the SCN (Figure 1). These additional nuclei include the specific object of interest of the present manuscript represented by catecholamine noradrenaline-containing cells of the brainstem reticular formation. The central areas, which are targeted by these photic pathways depend on the specific iPRGC placed in the retina. In fact, the M1 sub-type of iPRGC is seminal to the projection from the retina to extra-geniculate centers. This is seminal to understand degeneration of extra-geniculate pathways, which occurs in Huntigton's disease. In fact, in such a disorder a specific apoptosis of M1 type of iPRGCs takes place, while other (M2-M5) iPRGCs are spared (Lin et al., 2019). Among the M1 isotype of iPRGC, different types can be distinguished as expressing or non-expressing the transcription factor Brn3b. In fact, Brn3b-positive M1 ipRGCs project to the olivary pretectal nucleus, while Brn3b-negative M1 ipRGCs project to the suprachiasmatic nucleus (SCN) and are connected to circadian rhythms (Li and Schmidt, 2018) (Figure 2). Aside from a projection to the pre-tectal area, (olivary pretectal nucleus), the Brn3b-positive M1 ipRGCs are seminal for midbrain projection including the PAG, dorsal raphe and noradrenaline cell groups. This indicate that, depending on which specific subtype of iPRGC is activated, a variety of behavior and vegetative functions can be regulated.

It is remarkable that, in order to produce these iPRGC-triggered effects on sleep, neurons from pre-tectal area send collaterals to a number of nuclei of the brainstem reticular formation. Thus sleep regulation can be operated as part of circadian rhythms by the SCN which is innervated by Brn3b-negative M1 iPRGCs, while the powerful reticular input to the sleep-waking cycle is generated in the retina by another subset of iPRGCs, the Brn3b-positive M1 ipRGCs. This cell project to the

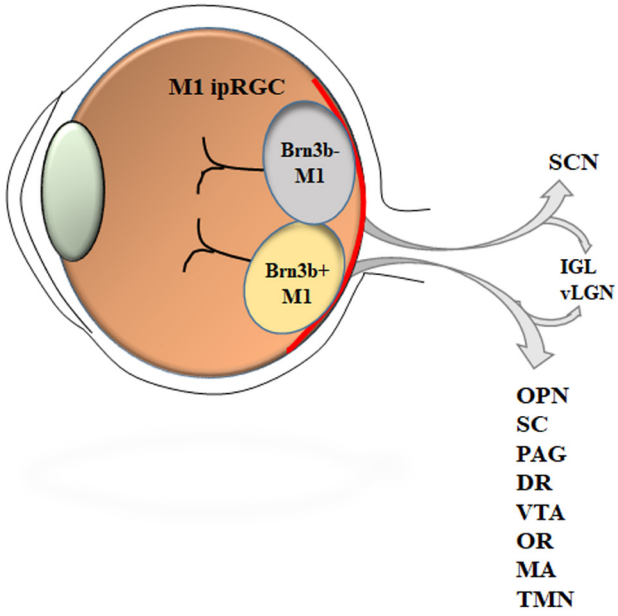

Fig. 2 - Divergency and convergency of two types of M1 iPRGC axons (and streams).
In the retina two main pathways can be distinguished concerning the innervation of extra-geniculate areas. These two streams originate from different subtypes of M1 iPRGC, which originate the pathway. In fact, the stream directed towards the suprachiasmatic nucleus mainly originates from Brn3b negative, M1 type ipRGC. In contrast, the retino-brain stream targeting the pre-tectal region outsources from Brn3b positive, M1 type ipRGC. The M1 retinal streams are shown specifically with their origin in the retinal iPRGCs, and their target areas. There is an overlapping between the two streams since the inter-geniculate leaflet and the ventral part of the lateral geniculate body receive retinal afferents from both streams. Origins and Targets of each stream are explained in the Figure.
Abbreviations. ipRGC: intrinsically photosensitive retinal ganglion cells; SCN: Suprachiasmatic nucleus; IGL: intergeniculate leaflet; vLGN: ventral lateral geniculate nucleus; OPN: Olivary pretectal nucleus; SC: superior colliculus; VTA: ventral tegmental area; OR: orexin nuclei; MA: medial amygdala; PAG: Peri-Aqueductal grey; DR: dorsal raphe; TMN tuberomammillary nucleus.

olivary pretectal nucleus and a number of nuclei, which include the monoamine-containing cells of the reticular formation (Figure 1 and Figure 2). There are some nuclei where the projection of Brn3b-negative M1 ipRGCs and Brn3b-positive M1 ipRGCs may overlap. These nuclei correspond to the inter-geniculate leaflet and the ventral aspect of the lateral geniculate body (Figure 2, Li and Schmidt, 2018). These nuclei were analyzed in the study of Zhang et al., (2021). In fact, according to the plastic effects of light, these nuclei can be tracked by persistent changes in gene expression, which occur following specific patterns of light pulses according to specific protocols, which produce epigenetic effects. All these nuclei are recipient of monosynaptic connections with pre-tectal axons. These include a posterior hypothalamic cell group containing histamine neurons, which is the tubero-mammillary nucleus (TMN) and a number of nuclei of the brainstem reticular formation such as the dopamine-containing ventral tegmental area (VTA) of Tsai (A10), and the dorsal raphe nucleus (DRN). These neurons promote wakefulness (Li et al., 2021; Moriya et al., 2021). However, light-responsive neurons within POA lack a direct connection with other wake-promoting nuclei such as the pedunculopontine nucleus (PPT), the laterodorsal tegmental nucleus (LDT), the parabrachial nucleus (PB) and the LC, such an effect appears to be mediated via the activation of PAG. The lack of OPN neurons to directly inhibit LC neurons is not surprising. In fact, a key role of LC in the sleep-waking cycle consists in suppressing REM sleep (Lu et al., 2006; Scammel et al., 2017). Consistently, the hegemonic role of light-responsive neurons within OPN merely concerns the NREM sleep. On the other hand, the effects of LC in suppressing REM sleep are not mandatory. In fact, cortical states are regulated in a redundant fashion by sub-cortical nuclei. Thus, although LC is key in suppressing REM sleep such a suppression can be overcome by LC destruction. This suggests alternative presumably noradrenergic pathways, which may take over to suppress REM sleep (Figure 3). The effects of light variations on catecholamine cells of the brainstem reticular formation were specifically investigated by Matsumura et al., (2015). In detail, they explored the effects of artificial light/dark cycle on the level of various catecholamine within different brain areas. Surprisingly, they could not demonstrate any alteration of DA within DA neurons and their projection sites. In contrast, serotonin levels were suppressed when dark/light exposure was halved (6h:6h compared with natural 12h:12h). This was reciprocated by NA levels, which were found to be significantly increased. This study indicates that, among catecholamine neurons an increase of the noradrenergic component, but not the dopaminergic, is affected by prolonged abnormal light exposure in brain regions that control several neural and physiological functions. These include brain areas involved in the regulation of physiological circadian rhythms, stress responses and behavior (Figure 3).

Disruption to a half of light exposure can be produced chronically for 1 month in order to assess whether plastic persistent changes in catecholamine neurons occur. This leads to a significant increase in NA levels within the LC and its projection areas. Although the increase in NA is mostly evident in the lateral hypothalamus. The NA innervation of the hypothalamus is only partially provided by the LC (Fornai et al., 1995; Fornai et al., 1996a; Fornai et al., 1996b). The role of other small NA cell groups of the brainstem contribute significantly to the innervation of the hypothalamus. Similarly, disrupted light exposure led to behavioral and cardiovascular abnormalities. While behavioral alterations are compatible with the hegemonic projections of the LC in the telencephalon, cardiovascular effects are generated in complex circuitry, where the LC contributes but the leading role is exerted by other small NA-containing neurons of the reticular formation (Figure 3, Guyenet et al., 2013). In detail, behavioral disturbances consist of disruption of circadian rhythm and the onset of anxious behaviours (which is consistent with the role of LC in sleep waking cycle, anxiety and alert). However, alterations of core body temperature, and heart rate are likely to be due to additional nuclei. In fact, in this latter case the LC is not necessarily a main regulator but it rather plays a secondary effect (cardiovascular function) by acting on other NA nuclei which clusters in the NA brainstem reticular formation as small cell group (Figure 3, Guyenet et al., 2013). In the context of this manuscript we provide anatomical evidence for additional NA nuclei which are connected with the LC and are known to play a key role in

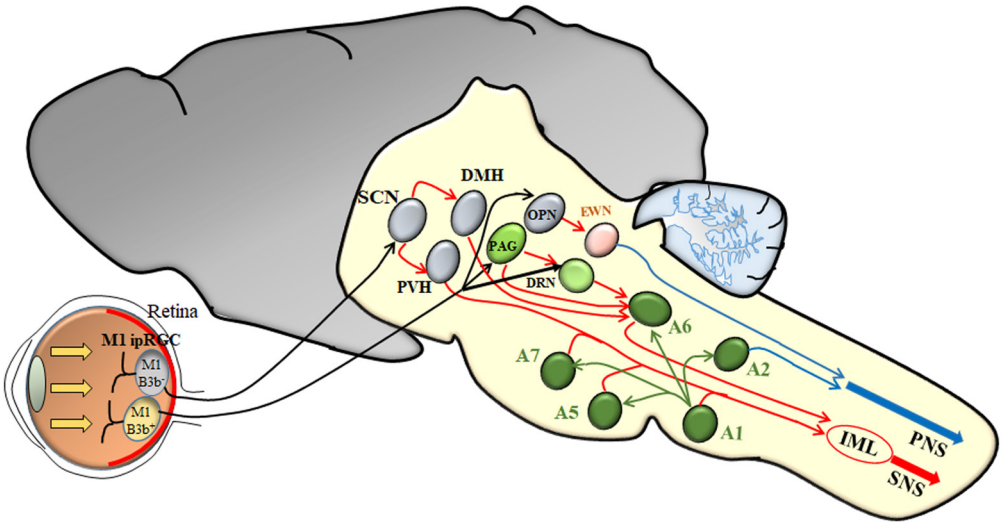

Fig. 3 - The conundrum of brainstem light-sensing noradrenergic nuclei.
In the brainstem, visual extra-geniculate nuclei are identified within the core of the noradrenaline-containing neurons of the reticular formation. Activation of the noradrenaline cell group by Brn3b positive, M1 type iPRGC mainly occurs via the PAG and the pre-tectal region, although the dorsal raphe provides a contribution and some evidence exists concerning a direct retino-coeruleus pathway. Once activated these NA nuclei generate a highly interconnected network, where each nucleus provides a specific contribution to various projecting pathways. For instance the pre-motor control of the sympathetic nervous system is exerted by a number of NA nuclei. These include the A6, A6sc A5, A7 areas with a powerful contribution of the rostral ventrolateral medulla containing the A1/C1 area. The parasympathetic outflow is mainly controlled by the A2/C2 areas. Relevant ascending projection mainly rise from the LC and provide a widespread innervation of the pro-encephalon. Abbreviations. SCN: Suprachiasmatic nucleus; DMH: Dorso-medial hypothalamus; PVH: Paraventricular nucleus; PAG: Periaqueductal gray; OPN: Olivary pretectal nucleus; DRN: Dorsal raphe nucleus; EWN: Edinger-Westphal nucleus; A6: Locus coeruleus; A1-A2-A5-A7: Noradrenergic nuclei; IML: Intermedio-lateral column; SNS: Sympathetic Nervous System; PNS: Parasympathetic Nervous System.

vegetative functions as indicated in Figure 3. These are hypothesized to transfer the effects of variations in light exposure to the vegetative nervous system to alter a number of peripheral organs including the cardiovascular system, the kidney the liver the gut and even the skin. In fact, among these small NA clusters of neurons include the pre-motor nuclei, which innervate the preganglionic neurons of the orthosympatetic system. These neurons are described in the rostral ventrolateral medulla (Guyenet et al., 2013). The effects of light on these small NA nuclei is often neglected, while they are likely to provide a substantial contribution concerning the vegetative effects produced by the extra-geniculate system to sustain vegetative changes induced by a persistent alteration of light exposure. The experimental section of this article provides detailed evidence of these small NA nuclei, which sense light and are interspersed along with LC within the lateral column of the brainstem reticular formation.

Material and Methods

Animals

These experiments were carried out in 12 weeks old C57BL6/J male mice. Mice were kept under environmentally controlled conditions 12-h light/dark cycle with food and water ad libitum. Environmental stress was reduced to a minimum in order not to alter the catecholamine synthesis and release and to keep steady the stimuli acting on the brainstem catecholamine reticular formation. Four C57/BL6 male mice (Harlan, S. Pietro al Natisone, UD, Italy), weighting 22-25g were used in the present study. Mice were housed for one week, four per cage, under the observance of adequate measures to minimize animal pain and discomfort. The European rules (CEE 86/609) were followed concerning animal housing, health and experimentation. The brainstem samples used for this study were dissected out from controls mice in the course of experiments carried out between years 2011 and 2013. The slices from various levels of the brainstem were harvested in our bank of mouse

tissues previously used under a project approved by the Italian Ministry of Health (authorization number 267/2011-B).

Immuno-Histochemical Analysis

Brains were dissected, fixed in ethanol (60%), acetic acid (10%), and chloroform (30%), and included in paraffin. De-waxed tissue sections (20 mm) were incubated with 0.1% Triton X-100 (Sigma Aldrich, Cat# 93443; lot n_: BCBN7646V) for 15 min and then with hydrogen peroxide (3%) for 10 min. Slices were incubated for 1 h with 10% Normal Horse Serum (Sigma Aldrich, Cat# S-2000; lot n_: ZB0929), and for 30 min, with monoclonal mouse anti-TH antibody in 2% Normal Horse Serum (1:100; Sigma Aldrich, Cat# T1299 RRID:AB_477560; lot n_: 015M4759V). Then these specimen were incubated for 10 min with secondary biotin-coupled anti-mouse antibody (1:400; Vector Laboratories, Cat# BA-2000; lot n_: Y0907), followed by exposure to Horseradish Peroxidase Streptavidin for 5 min (1:100; Vector Laboratories, Cat# SA-5004; lot n_: ZC1115). 3,3-Diaminobenzidine tetrachloride (Sigma–Aldrich, Cat# D4293; lot n_: SLBJ3609V) was used for detection. Negative controls were not incubated with primary antibody.

TH-positive cells in the brainstem were identified within specific nuclei involved in behavioral and cardiovascular function. These catecholamine nuclei were identified by TH immunostaining in 20 mm coronal mouse brain sections regularly collected every 320 mm from Bregma –2.7 to –3.66 according to the atlas of Paxinos and Franklin (2001). For ach representative slide and nucleus, images are taken at low (5X) and high (63X) magnifications.

In detail, VTA and SNpc were present at low magnification in the same slide, A6 (locus coeruleus) and nucleus subcoeruleus were present in the same slice along with the the rostral part of the LC (often named nucleus epicoeruleus). In the picture collecting, the TH positive rostral part of dorsal raphe, also the TH positive area in the periaqueductal gray (PAG) and the A8 retro-rubral field were included. Caudally, the A1 cell group in the rostral ventrolateral medulla was visible in the ventral aspect of the medulla while towards the dorsal medulla the A2 was visible in the context of the nucleus tractus solitaries, while on the midline at this level, the A3 area was present. More caudally, the area postrema was evident as clusters of TH positive neurons at the causal border of the fourth ventricle.

Results

Anatomical placement of rostral and caudal photo-responsive TH-Positive Nuclei in the Mouse Brainstem

Immuno-histochemical analysis of TH-positive neurons of the mouse brainstem allow us to draw a systematic detailed anatomical representation of those catecholamine-containing nuclei in the reticular formation starting from mesencephalic DA cell bodies (Figure 4). These belong to the Substantia Nigra pars Compacta (SNpc, Figure 5) and Ventral Tegmental Area (VTA, Figure 6) which are the projection of a subset of extra-geniculate pathways via the pre-tectal area, the perifornical nuclei, and the periaqueductal grey (PAG). In fact, the occurrence of NA-containing, TH positive neurons is already present in the ventrolateral extent of the PAG as shown in Figure 7. These neurons continue ventro-laterally within epicoeruleus nucleus (Figure 7), which continues up in the lower mesencephalon the area of the LC complex. On the midline, these neurons are contiguous to the rostral extent of the dorsal raphe nucleus which contain catecholamine neurons, which exert a powerful effect on the sleep-waking cycle (Figure 7). In the same image the placement of the retro-rubral field placed in the caudal mesencephalon is evident just caudal to the presence of the red nucleus (Figure 7). This latter nucleus is mostly composed of DA-containing neurons and continues downward the DA-containing cell group, which is placed in the mesencephalic tegmentum (Figure 4) such as the TH positive A9 (Substantia Nigra pars compacta, SNpc) and the A10 (Ventral Tegmental Area, VTA), which appear as the most rostral catecholamine nuclei in the brainstem. VTA and SNpc are large and long with a rostro-caudal extent approaching 1,300 mm. In Figure 4, VTA and SNpc catecholamine nuclei are visible in the mouse brain at Bregma level –3.02, which according to Paxinos and Franklin (2001) corresponds to the full localization of the VTA and SNpc. Representative images of TH immune-reactive cells of the VTA (Figure 5) and SNpc (Figure 6) are reported also at higher magnification

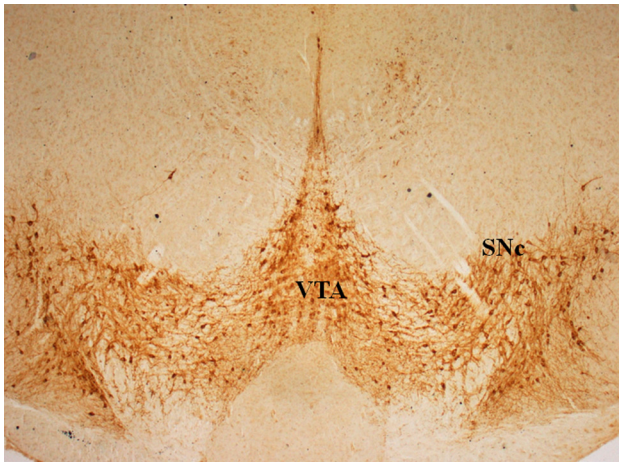

Fig. 4 - Tyrosine-Hydroxylase staining of VTA and SNpc at low magnification.
Representative picture showing the mesencephalic tegmentum, where TH-positive neurons are stained within substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA). The image is taken from a C57 Black mouse injected with saline. TH staining prolongs in the mid-line to reach the periaqueductal gray (PAG). The magnification is 5X.

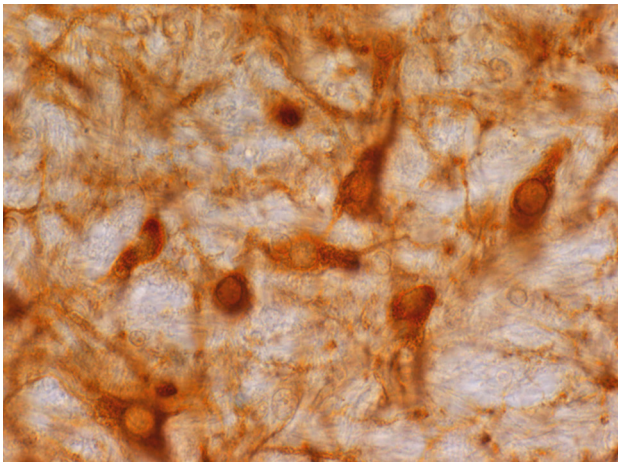

Fig. 5 - Tyrosine-Hydroxylase staining of VTA at high magnification.
The representative picture shows TH positive neurons clustered in the ventral tegmental area of the mesencephalon in the ventral extent of the medial tegmentum. TH positive neurons are mainly multipolar, receive direct retinal projection and send their axons in the meso-limbic and meso-cortical areas. The image is taken from a C57 Black mouse injected with saline. The magnification is 63X.

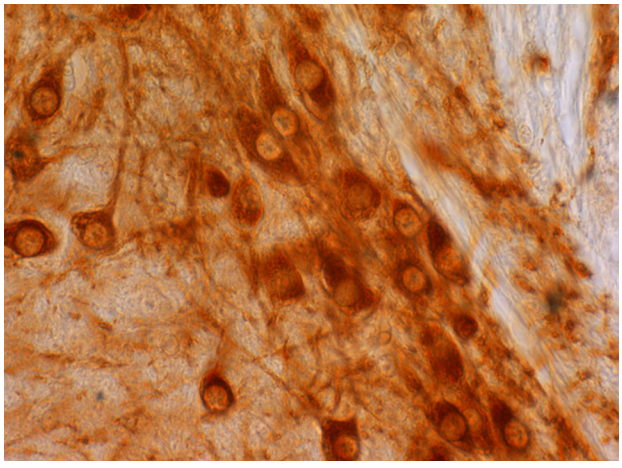

Fig. 6 - Tyrosine-Hydroxylase staining of SNpc at high magnification.
The representative picture shows densely-packed TH positive neurons clustered in the substantia nigra pars compacta of the mesencephalon in the ventral extent of the tegmentum. Two types of neurons are evident, the dorsal ones in the SNpc dorsal tier, which are smaller and mainly bipolar and those within the SNpc ventral tier, which are bigger pyramidal-like and multipolar. The image is taken from a C57 Black mouse injected with saline. The magnification is 63X.

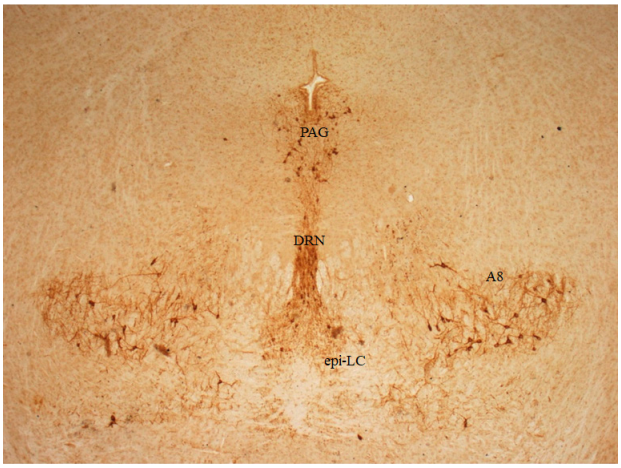

Fig. 7 - Tyrosine-Hydroxylase staining in the pons-mesencephalon transition area.
Representative picture shows the tegmentum at the level of pons-mesencephalon transition. At this level, three TH-positive nuclei are evident. The retrorubral field (RRF) is placed on each side, while neurons of the rostral extent of the dorsal raphe are placed at midline. At each side, this column merges with the rostral extent of the locus coeruleus, so-called epi-coeruleus nucleus. While dorsally these neurons merge with the ventral aspect of the periaqueductal grey (PAG) neurons. The image is taken from a C57 Black mouse injected with saline. The magnification is 5X.

(63X), which shows the morphological features of TH-positive cells of the VTA and the dorsal (dt) and ventral (vt) tier of the SNpc. These slices come from brain sections regularly collected every 160 mm from –3.8 to –4.12 Bregma levels.

The level of Figure 7 corresponds to Bregma level –3.96 (Paxinos and Franklin, 2001). This is the site where the A8 catecholaminergic nucleus is well evident. This nucleus which is also known as retro-rubral field (RRF), is placed in the tegmentum of the mesencephalon and it lies caudally to the red nucleus and, as it appears from the picture of Figure 7 it extends lateral to the PAG formation while it is dorsal to the midline raphe nucleus. This nucleus contain catecholamine-neurons, mainly producing DA, it extends for a short length compared with other DA-containing mesencephalic cell groups. In fact, the RRF has a rostro-caudal extension of roughly 0.5 mm. The A8 cell group possesses the highest immunostaining for dopamine beta-hydroxylase among DA neuron areas, which means a remarkable content of NA, with scattered cells stained for the epinephrine marker, phenylethanolamine N-methyl transferase. Injection of the retrograde tracers indicate that noradrenergic innervation of A8 arise primarily from A1, A2, A5, and locus ceruleus (A6) neurons (Mejías-Aponte et al., 2009). Among catecholamine cell groups, the most prominent innervation derive from medullary A1 and A2 areas and adrenaline fibers from the C1 area. This is key to bring the visceral regulation at midbrain levels on A8 neurons, which receive a significant projection from extra-geniculate visual pathways (Horowitz et al., 2004). In fact, as mentioned in the introduction a significant amount of light sensing neurons are placed within the ventral mesencephalon; according to Mejías-Aponte et al. (2009), these projections are provided by the the intergeniculate leaflet (IGL). At this level, neurons of the geniculate complex send collaterals, which provide a profuse ascending innervation to basal forebrain along with a routine influence on the suprachiasmatic nucleus (SCN). Apart from ascending pathways, the IGL send descending fibers to the visual mesencephalon and hindbrain, which influence visuomotor function. These include the retino-recipient medial, lateral and dorsal terminal nuclei, the nucleus of Darkschewitsch, the oculomotor central gray in the PAG, the cuneiform, and the lateral dorsal, pedunculopontine, and subpeduncular pontine tegmental nuclei. Also direct retinal projection to the brainstem may occur since intraocular retrograde tracing marks retinal terminal fields within Barrington's nucleus, the dorsal raphe, locus coeruleus, and retro-rubral field. Thus, among catecholamine, DA containing neurons of A8 is highly connected with NA nuclei and takes part in the visual midbrain (Mejías-Aponte et al., 2009).

As shown in figure 7 at the same rostro-caudal level of A8, one can appreciate the TH positive portion of the peri-aqueductal gray (high magnification in Figure 8), which is influenced by visual pathways. Again, at this level a median TH-positive nucleus which reaches up the PAG is evident. The placement of these TH-positive cells appears to correspond to TH-containing cells which are described in the rostral part of the dorsal raphe nucleus (Figure 7, high magnification in Figure 9, Cho et al., 2017). Dorsal raphe is another region known to receive collaterals from the extra-geniculate visual pathways (Pickard et al., 2015). In fact, a specific subset of retinal ganglion cells named Y cells send direct retino-raphe projection (Huang et al., 2017). These cells generate extra-geniculate axons, which collateralize both in the superior colliculus and dorsal raphe neurons (Huang et al., 2017). These Y cells are defined by electrophysiology and correspond to the alpha ganglion cells which send their axon in the optic nerve, where retinal afferent fibers emerge from the optic tract at the border between the pretectal area and superior colliculus. This pathway corresponds to the one outsourcing from Brn3b positive M1 iPRGC of Figure 2 and it proceeds by descending into the periaqueductal gray (PAG) to form a plexus in the dorsal raphe nucleus (Pickard et al., 2015). This projection produces light-induced alertness and fosters escape behavior. In fact, TH positive neurons in the dorsal raphe are key in promoting alert (Lin et al., 2021) and discern stimulus salience. At this level TH positive neurons can be detected ventrally in the PAG (Figure 7 and Figure 8); at this level a significant modulation by visual pathways occurs. The presence of NA neurons in the PAG is decribed also in previous studies, (Bucci et al., 2017), it is likely that these cells are related to the effects of stress and psychostimulants (Ferrucci et al., 2019). The role of PAG on NA activity is considered to depend on projections from

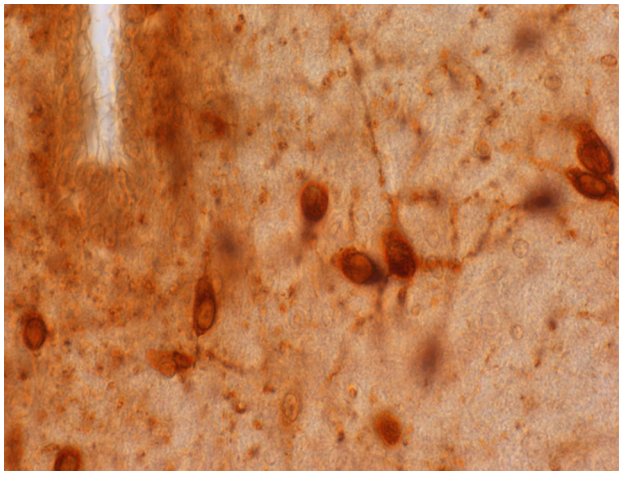

Fig. 8 - Tyrosine-Hydroxylase staining in the periaqueductal grey (PAG).
The representative picture shows a few TH positive neurons densely clustered within the PAG area, in the lower mesencephalon, showing catecholamine cells in the ventral contour of the central aqueduct at high magnification. These small and scattered neurons are distinct from the pale TH-immune-positive cells which are visible on the wall of the central aqueduct. The image is taken from a C57 Black mouse injected with saline. The magnification is 63X.

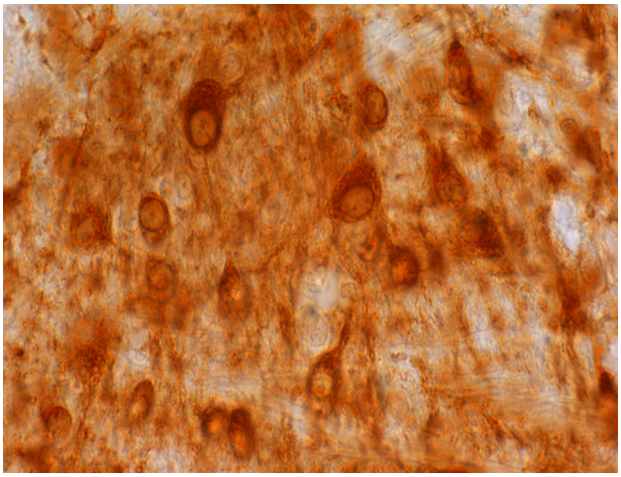

Fig. 9 - Tyrosine-Hydroxylase staining in the rostral dorsal raphe nucleus.
The representative picture shows a densely packed TH positive neurons across the midline (raphe) of the pons. The catecholamine cells are intermingled in a reticulum of densely crossing TH positive fibers where varicosities can be appreciated. These neurons seem to play a fundamental role in awakening and alertness and are directly innervated by a retino-raphe pathway which arises from M1 Bnl3b positive iPRGC and project to the olivary pre-tectal nucleus to branch off collaterals to these cells. The image is taken from a C57 Black mouse injected with saline. The magnification is 63X.

PAG to catecholamine containing neurons of the brainstem (Bajic et al., 1999). However, a direct NA projection from PAG needs to be considered as well as a retino-NA neurons direct projection (Mejías-Aponte et al., 2009). The activation of PAG during light exposure is produced. This is based on the profuse collateralization, which derive from the specific subset of iPRGC, which descend in the brainstem (Hattar et al., 2006).

The ventral PAG contains specifically TH positive neurons. Remarkably, the ventral aspect of PAG is connected to cardiovascular control via the autonomic nervous system (Pereira et al., 2010). The projection from the retina to PAG neurons were described carefully by Hattar et al. (2006), by showing that iPRGC may target via profuse collateralization the superior colliculus, the suprachiasmatic nucleus and the olivary pretectal nucleus within the pre-tectal regions. Nonetheless, some fibers projection are more widespread than previously including the superior colliculus, and periaqueductal gray. Most retinal afferents to PAG contain melanopsin (Hattar et al., 2006) and produce a strong mTOR mediated plasticity upon reiterated stimulation, which leads to synapse consolidation for light-induced primordial responses.

When proceeding caudally in the brainstem, the occurrence of TH positive neurons occur in the medial parabrachial nucleus (PB), and immediately caudal to the rostral pole of PB, on the lateral aspect of the pons, the nucleus of lateral lemniscus (A7 nucleus) for a length of roughly 320 mm.

At this level, ventral to PB, and when PB is still present in the dorso-medial aspect, A7 can still be fully appreciated in the lateral extent of the pons. Here also appears the A6sc, with an approximate length of 640 mm. These nuclei, such as A5 and A7 are highly interconnected with RRF, VTA and receive a dense NE and E innervations originating from caudal medullary catecholamine nuclei such as the A1, A2, C1 area, respectively (Figure 3, Mejías-Aponte et al., 2009).

At a slightly caudal level, the big pontine NE nucleus A6 (locus coeruleus, LC) appears (Figure 10), extending rostro-caudally for a length of about 480 mm. At this level, Figure 6 shows the highest amount of NA-containing cells in the brainstem. The A6 along with nucleus sub-coeruleus and the

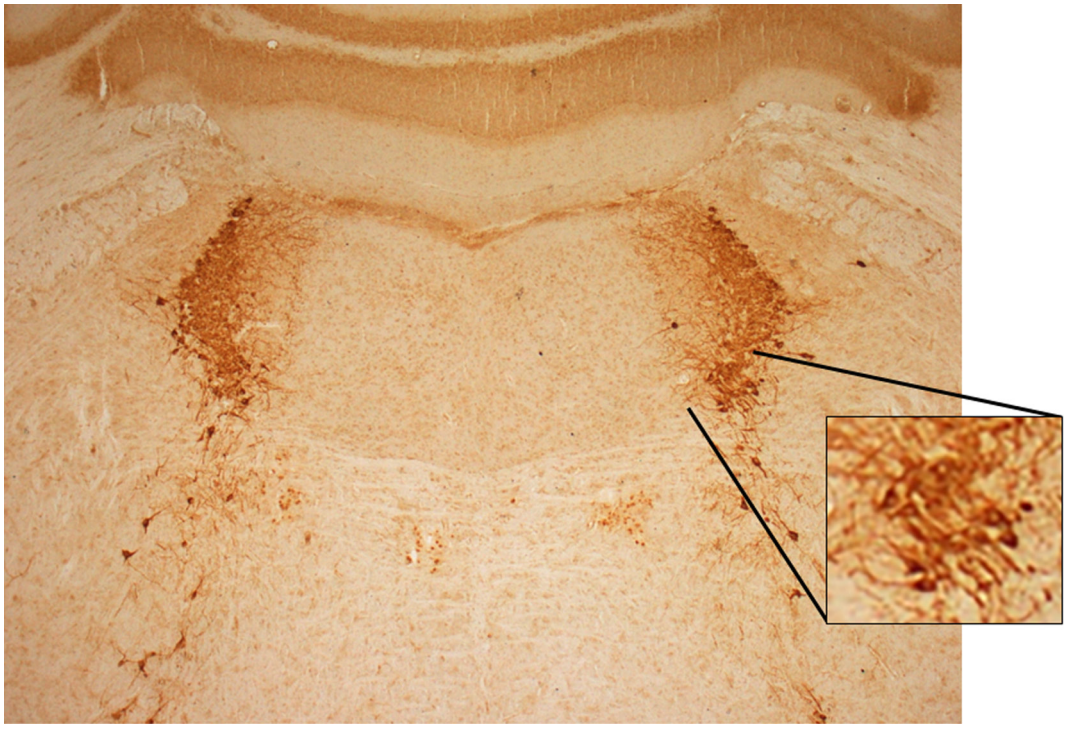

Fig. 10 - Tyrosine Hydroxylase staining in the pons showing the LC complex.
Representative picture show the dorsal aspect of the pons. At this level, the LC complex is evident. This consist of the main LC nucleus, which extends ventrally in the division named nucleus sub-coeruleus. Lateral and ventral to the LC, the A5 nucleus appears. The image is taken from a C57 Black mouse injected with saline. The magnification is 5X. In the insert LC neurons are shown at high magnification (63X).

A5 cell group possess a remarkable anatomical and functional overlapping, which generated the definition of LC complex. The LC (A6) is the best-characterized among NE-containing nuclei and it is calculated that 50% of all brain NE is produced at this level (Moore and Bloom, 1979; Foote et al., 1983).

LC is placed slightly medial beneath the floor of fourth ventricle and it continues ventrally in the nucleus sub-coueruleus (A6sc area, Figure 10), which laterally and ventrally borders the A5 region. Due to its wide area and densely packed cell population (insert of Figure 10), the involvement of LC in visual pathways is routinely over-emphasized compared with other nuclei belonging to the LC complex and additional NA nuclei. In fact, the big size of the nucleus and densely packed neurons (insert of Figure 10) allow easy electrophysiological and anatomical investigations making it hegemonic in the study of afferents and efferent connections. Again, many studies aimed at establishing visual connections with noradrenaline-containing cells in the brainstem reticular formation led to *negligee* the role of satellite NA cell group. This leaves largely unexplored the collateralization of some extra-geniculate pathways to LC satellite nuclei. The response of LC neurons to visual stimuli concerning the photic message is well-demonstrated. In fact, the occurrence of depression during light deprivation is commonly interpreted as the consequence of a reduced NA activation of LC neurons (Bowrey et al., 2017). Visual pathways reach the locus coeruleus mainly through polysynaptic networks (as shown in Figure 1/2) which arise from extra-SCN, extra-geniculate areas. In turn, LC innervation is seminal for the activity of multiple visual cortical areas, where it exerts a powerful control concerning a number of effects including brain plasticity (Zhang et al., 2016). The light-driven effect on LC are related to alertness, attention, novelty orienting, and it produce a powerful effect on the sleep-waking cycle. The circuitries, which produces an activation of the LC following light stimulation are mostly provided by projections which pass through extra-geniculate, non-image forming sites driven by retinal cells of the iPRGC type. A typical connection

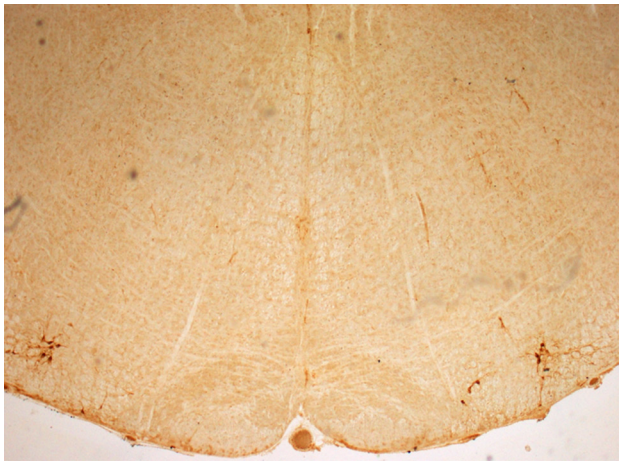

Fig. 11 - Tyrosine-Hydroxylase staining in the ventral upper medulla.
The A1/C1 area is shown as TH positive neurons placed at sub-pial level. This corresponds to the so-called rostral ventrolateral medulla (RVLM). At this level neurons key for cardiovascular and breathing control are placed along with pre-motor sympathetic neurons, which modulate a number of metabolic functions. The image is taken from a C57 Black mouse injected with saline. The magnification is 5X.

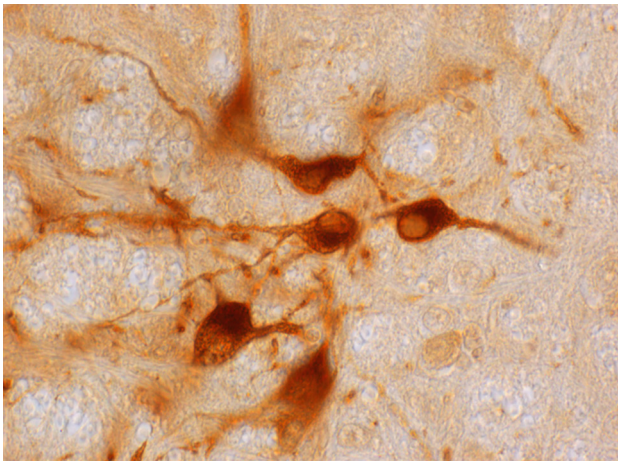

Fig. 12 - Tyrosine-Hydroxylase staining in the ventral upper medulla at high magnification.
The representative picture shows a few TH positive neurons densely clustered in the A1 area, in the rostral ventrolateral medulla at high magnification. These small neurons possess multipolar shape. The image is taken from a C57 Black mouse injected with saline. The magnification is 63X.

being described for the LC neurons is the one, which comes from hypothalamic peri-fornical neurons which sends off excitatory orexin-fibers to the LC. This is demonstrated also for satellite nuclei such as the A6sc area and the A5 nucleus. This input critical to produce the effects of orexin since the loss of LC occludes most of the effects of orexin on sleep-waking cycle (Hagan et al., 1999; Gompf and Aston-Jones, 2008; Kohlmeier et al., 2013; Sears et al., 2013). In line with the LC-centered investigation of photic NA nuclei, it should be kept in mind that, even caudal medullary noradrenaline neurons are shown to be targeted similarly by these orexin neurons. This is the case of the nucleus of the solitary tract and its intermingled neurons within the A2 area (Peyron et al., 1998; Date et al., 1999).
In the caudal part of the mouse brainstem we found other TH-positive cell groups. A lateral group corresponds to the sub-pial rostral ventro-lateral medulla (Figure 11). This is key when considering how light sensitive neurons in the hypothalamus exert powerful effects on those noradrenaline neurons, which are pivot to regulate cardiovascular and vegetative functions (Figure 3). In fact, as shown in Figure 11 and Figure 12 neurons belonging to the rostral ventrolateral medulla are evident at subpial level to form the so-called C1/A1 complex, which is implicated in the control of blood pressure, breathing activity and the physiology of a number of peripheral organs (Figure 3). Neurons of A1 area appear as a small group of medium-sized cells (Figure 11 and Figure 12), which were recently shown to undergo plastic changes (Busceti et al., 2019). In dorsal position and more caudal to the A1 group a dorso-medial group, containing TH positive cells, is evident (Figure 13). This is specifically known as nucleus of ala cinerea C2/A2, which overlaps with the dorsal nucleus of the vagus (DMV) and the nucleus of the solitary tract (NTS) (Figures 13, and Figure 14). This cell group is involved in the activation of the parasympathetic nervous system and at the same level it corresponds to a median group of cells, which is defined as C3 (Figure 13 and Figure 15). The C3 group is placed just on the midline and it probably overlaps with the definition of nucleus raphe obscurus (Figure 13), which seems to be key in the breathing control. At its caudal pole the ala cinerea continues downwards along the midline to cover the end of the fourth ventricle in the so-called area postrema (AP) where a conspicuous number of TH positive cells are present (Figure 16). These appear as scattered bipolar or multipolar small-sized neurons (Figure 17) which clusters at the obex of the fourth ventricle.

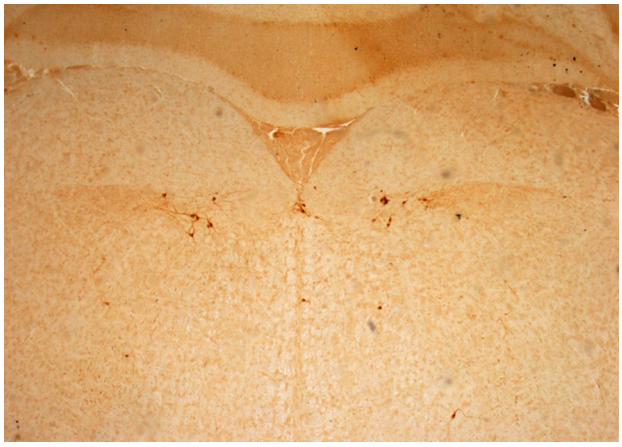

Fig. 13 - Tyrosine-Hydroxylase staining in the dorsal upper medulla.
The representative picture shows TH positive neurons clustered in two spots. The more lateral corresponds to the A2/C2 area, while the C3 area is present on the mid-line. These nuclei possess a different role, since the A2/C2 area, which is placed within the vagal trigone takes close synaptic contacts with the dorsal motor nucleus of the vagal nerve and the nucleus tractus solitarius. This A2/C2 area is also known as ala cinerea and it prolongs to the lower medulla. The mid-line C3 nucleus which overlaps with nucleus raphe obscurus and it is limited to this level. The A2/C2 area control vagal afferents and the parasympathetic nervous system. The image is taken from a C57 Black mouse injected with saline. The magnification is 5X.

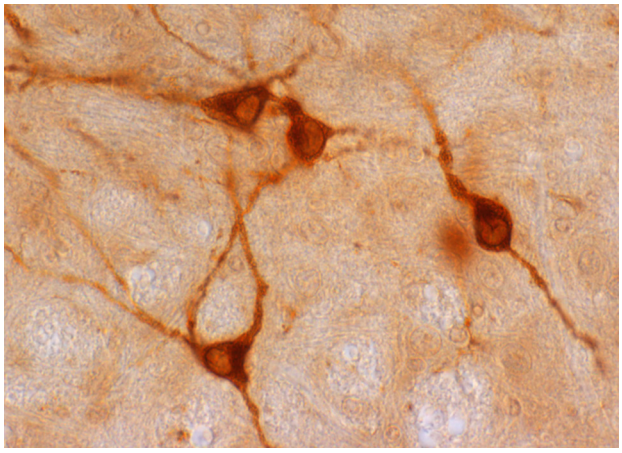

Fig. 14 - Tyrosine-Hydroxylase staining in the dorsal upper medulla at high magnification.
The representative picture shows a few TH positive neurons clustered in the A2 area, ala cinerea, in the medulla at high magnification. These small neurons are scattered and possess either a multipolar or bipolar shape. The image is taken from a C57 Black mouse injected with saline. The magnification is 63X.

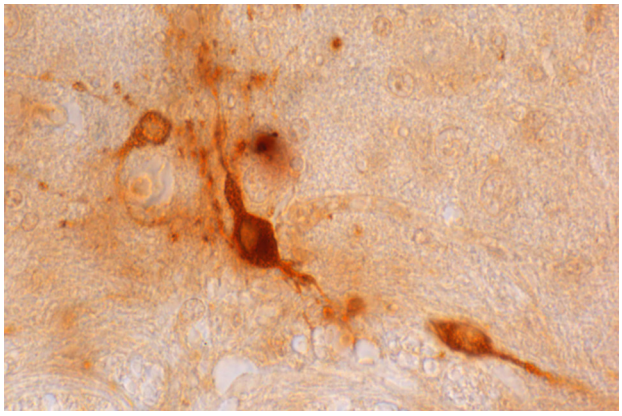

Fig. 15 - Tyrosine-Hydroxylase staining in the mid-line of dorsal upper medulla.
The representative picture shows a few TH positive neurons clustered in the C3 area at high magnification. These neurons are clustered on the mid-line and are very scattered. Some varicosities along the course of a TH positive axon are evident. The image is taken from a C57 Black mouse injected with saline. The magnification is 63X.

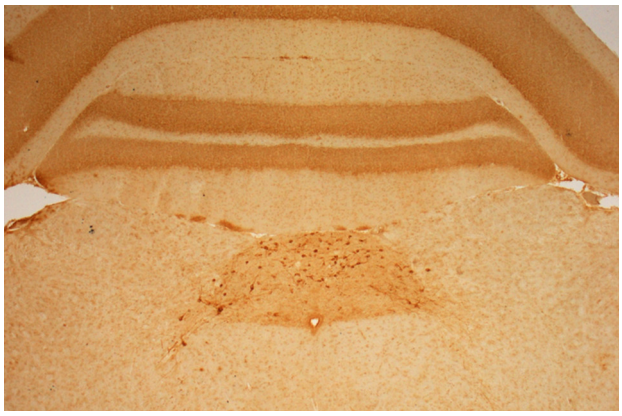

Fig. 16 - Tyrosine- Hydroxylase staining in the dorsal lower medulla.
The representative picture shows TH positive neurons clustered in in the ala cinerea, which invades the midline to cover the area postrema at the lower end of the fourth ventricle. The area postrema continues downward on the mid-line the bilateral spots of ala cinerea. The image is taken from a C57 Black mouse injected with saline. The magnification is 5X.

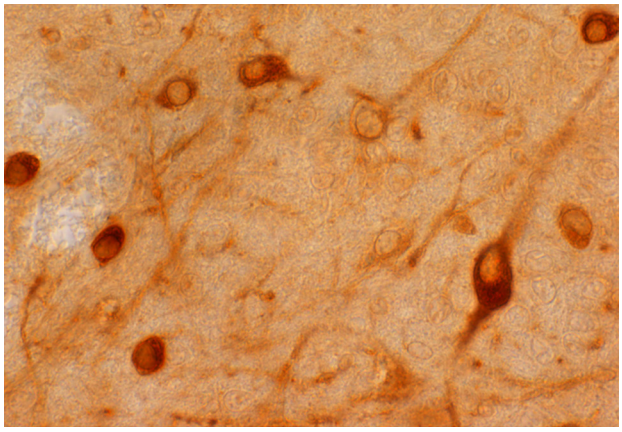

Fig. 17 - Tyrosine-Hydroxylase staining in the area postrema. The representative picture shows a few TH positive neurons clustered in the area postrema, in the lower medulla at high magnification. These small neurons are abundantly represented. The image is taken from a C57 Black mouse injected with saline. The magnification is 63X.

In this section it appears mandatory to mention the discrepancy we contantly find at experimental level (present study, Bucci et al., 2017; Busceti et al., 2019) between the definition of adrenaline and noradrenaline containing neurons. In fact, since we use TH as catecholamine marker, we could not discriminate between adrenaline (C) and noradrenaline (A) containing neurons when considering the medullary catecholamine nuclei. Thus, the current definition of the groups C1/A1 and C2/A2 remain confined to a general population, which stains with TH primary antibodies. Indeed, when the specific marker of adrenaline containing neurons, which is represented by the enzyme phenyl-ethanolamine-N-methyl-transferase (PNMT) is investigated, by using specific primary antibodies, no signal is detected at the level of the presumed C1, C2 and C3 regions of the medulla. In these very same experimental conditions, the primary antibody against PNMT works very well to stain the adrenaline containing cells in the adrenal medulla. This may be due to a very low amount of the enzyme PNMT within C1-C3 regions. However, no signal is detected even increasing the amount of the primary antibody. Again, when looking at the literature, studies which used PNMT antibodies to stain C neurons are only a few manuscripts. In addition, a very low specific staining compared with a high staining background is present. This rises up the question whether the presence of adrenaline-containing cells within the medullary reticular formation should be further investigated and better established. Only when assessing mRNA level for PNMT by quantitative RT-PCR a signal is detected at the level of the medulla oblongata (Busceti et al., 2019). This leaves the question untwisted and calls for additional evidence, which may allow to distinguish between medullary adrenaline and noradrenaline cell groups (C and A, respectively). This is likely to explain why, when referenced in the literature, these cell groups are defined as C1/A1 or C2/A2 without any further specific distinction. These nuclei, when considered following a TH staining, possess a rostro-caudal length exceeding 1.4 mm being placed at opposite side of the medullary contour. In particular, C1/A1 is placed in sub-pial position ventrally and laterally in the medulla (Figure 11), while C2/A2 is located in the dorsal and medial border of the medulla oblongata (Figure 13), overlying neurons of the dorsal motor nucleus of the vagus nerve (DMV) and the nucleus of the solitary tract (NTS). Thus, the A2/C2 area, is interspersed within the DMV and NTS to provide an ash-like appearance, which explains why the *nomina anatomica* used the term *ala cinerea* to identify this region. In fact, the latin term *cinerea* refers to the ash-like appearance in the fresh *ex vivo* aspect of the dorsal medulla in the low floor of the fourth ventricle. This ash-like region is defined *ala* since it is shaped like a wing. Thus, *ala cinerea* refers to an ash-like stained, wing-like shaped region. This region is chromatically reminiscent of the ash for the occurrence of black spots (melanin-containing catecholamine cells) in the grey matter (radicular and sensory neurons within the DMV and NTS, respectively). The occurrence of the lowest group of catecholamine containing neurons extends caudally to the border of the rostral spinal cord. At this level, when the fourth ventricle is no longer present, a dorsal area just beneath the cerebellum is visible

and it is known as *area postrema* (Figure 16). This region continues down the row of black spots being described in the *ala cinerea* and owns clusters of small sized TH positive cells as reported in Figure 17. *Area postrema* in our mice extends for roughly 0.6 mm rostro-caudally and it corresponds to what reported in the atlas by Paxinos and Franklin, (2001). Among these nuclei, the densest neuronal placement occurs for the nucleus LC (A6, Insert of Figure 10), which matches what counted in our previous work (Bucci et al., 2017) reporting a cell density in the LC which surpasses even the densely packed TH positive neurons occurring in the VTA (Figure 5) and SNpc (Figure 6). The cell density in the *area postrema* is also remarkable (Figure 17). The significance of *area postrema* relates to a number of vegetative activities. Among these, *area postrema* exerts a powerful control on the subdivision of the vagal DMN, which innervates the pancreas. There is a sharing between area postrema, some A1 neurons along with A5 and A2 neurons of *ala cinerea,* to control the vagal outflow to the pancreas. In this way, the metabolic effects of light are thought to exert an influence on pancreas through specific monoamine-containing nuclei of the lowest medullary reticular formation (Loewy et al., 1994).

Discussion

The present results indicate the occurrence of multiple extra-geniculate light-sensing TH positive nuclei and their specific placement within the lateral column of the brainstem reticular formation. All these nuclei are, directly or indirectly, impacted by light exposure. Evidence is reported that these nuclei are strongly innervated by the photic retina (Szabadi et al., 2018, Figure 3). Such an effect is mediated via extra-geniculate pathways and relay both on posterior orexin containing neurons of the hypothalamus and PAG. Although much of the evidence emphasizes the role of LC, which is considered to be hegemonic in the regulation of the orthosympathetic nervous system (Szabadi et al., 2018), these medullary centers may prevail over the LC in vegetative control (Figure 3). In fact, even considering the occurrence of a coeruleus-spinal projection to preganglionic sympathetic neurons, NA projection to the spinal cord are formed also by other pontine NA nuclei such as the A5 and A7. Some authors emphasize the medullary A1 area in the rostral ventrolateral medulla as the main pre-motor center to control the preganglionic sympathetic neurons of the spinal cord through descending projection (Guyenet et al., 2013). In contrast the A2 area would exert an inhibitory control on the parasympathetic nervous system (Figure 3), which according to Szebadi (2018) would still be mediated by an inhibitory projection from the LC. In any case, a strong connection exist between A6, A5, and A7 with A1 and A2 (Figure 3). According to this scenario, the major role of LC would be related to its telencephalic projection to work on alertness, cognition and the sleep waking cycle.

These NA containing nuclei, and extensively the LC, would receive indirect projection from the photic retina via extra geniculate pathways, mostly the pre-tectal region and other indirect circuitry including the suprachiasmatic nucleus. The main role in the activation of these brainstem nuclei is attributed to peri-fornical pre-tectal and tubero-mammillary areas and the PAG formation.

At any rate, the noradrenergic hub of the reticular formation needs to be considered as being a part of the photic system, since it receives abundant projection from the photic retina (mostly from the pathway generated from Brn3b positive M1 iPRGC, Figure 1 and Figure 2), and it is involved in a number of light induced alterations, which affect behavior and body homeostasis. Light exposure exerts plastic effects on these nuclei, which are witnessed by a light-induced expression of c-fos (Shuboni et al., 2015). This may explain the long-lasting effect produced by light exposure on a number of NA-dependent activities such as metabolism, alertness, mood, sleep-waking cycle, stress modulation, aggressiveness. Considering the plethora of effects induced by persistent alterations of noradrenergic activity and their plastic over-activation induced by light exposure, one may infer on a number of body functions, which are now more and more evident. In line with these, a persistent effect of light exposure on metabolism and body fluid homeostasis as well as the dynamic changes in the amount of adipose tissue are considered.

Although it belongs to everybody's life the empirical knowledge about how much the environmental light modifies our behavior and well-being, the specific

connections remain far to be firmly established. The profuse collateralization and widespread effects of the noradrenergic nuclei belonging to the brainstem reticular formation are likely to be the mainstream through which light exposure produces its persistent alterations. Thus, it is not surprising that, activities of these nuclei and mainly the LC impacts also on the amount of light which enters the eyeball. In fact, acting as a sort of feed forward mechanism the over-activity of LC, which is induced by light, in turn, promotes an increase in light exposure to the photic retina. As reported in the comprehensive manuscript by Szebadi (2018) the increase in the activity of the LC produces a concomitant increase in pupil diameter in primates (Aston-Jones et al., 2005). This can be produced also via manipulating the circuitry via micro-stimulation of the LC, which induces dilation of the pupils both in primates and rodents (Joshi et al., 2016; Reimer et al., 2016; Liu et al., 2017). Analogous effects are documented by functional MRI in humans, where an activation of a pontine spot compatible with the LC occurs upon stimuli, which concomitantly induce pupil dilation (Murphy et al., 2014; de Gee et al., 2017).

The effects of light exposure on plasticity of vegetative and cardiovascular functions

The classic definition of visual plasticity deals with retinal plasticity as well as plastic phenomena in the lateral geniculate nucleus or the primary and associated visual cortex. For such a reason the study of subcortical areas involved in visual plasticity was not deeply investigated. Even considering the role of subcortical areas in inducing visual plasticity the analysis is confined to those subcortical nuclei, which relay visual (image-forming) information. This is the case of the comprehensive manuscript by Duménieu et al., (2021), which focuses on the dorsal part of the lateral geniculate body (dLGB) and superior colliculus to compare the plasticity of these nuclei when related, either to visual acuity, and to vision-driven behavior. Despite a knowledgeable investigation, authors did not address the issue of non-image forming sub-cortical visual system. If, from one hand, this is partly included in the non-visual aspect of the superior colliculus, all subcortical areas involved in photic plasticity are not addressed. Therefore, the specific plastic effects, which occur in a further specific subset of extra-geniculate projections from the photic retina (M1 iPRGCs) to brainstem noradrenergic nuclei need mostly to be inferred in the context of studies involved in the specific functions promoted by the ascending and descending reticular formation. Thus, the plasticity related to cardiovascular function driven by the effects of light needs to be considered along with changes induced by light within number of vegetative centers along with the effects on the sleep-waking cycle, and the state of attention, anxiety, pupil dilation, and mood.

Recent evidence indicates that exposure to various pattern of light produces plastic changes in the cardio-vascular system (Alaasamet al., 2021). This is evident already at 10 days following exposure to artificial light photo-periods, which is mimicking the intensity, which is present in the environmental light pollution (<5 lux). This evidence was generated by specific experiments designed to elucidate the mechanisms, which determine the increased prevalence of cardiovascular diseases (along with the onset of sleep disruption and physiological stress) in people exposed to abnormal light stimulation. These effects appear to depend on both altered natural photoperiod and the specific wavelength, at which light emission occurs.

These effects are evident along phylogenesis since they are already detectable in zebra finches (Taeniopygia gutatta), where exposure to abnormal light photoperiods generates plastic effects such as cardiac hypertrophy which are concomitant with epigenetic alteration. It is remarkable that these effects are independent from alterations in the plasticity and activity of the suprachiasmatic nucleus and brain clock centers. Thus, it is likely that, according to the pathway which preferentially activates NA-containing neurons of the reticular formation, non-SCN extra-geniculate pathways, as those generated in retinal Brn3b positive M1 iPRGCs are promoting these effects (Figure 1). These retinal afferents are largely independent from the activity of the SCN and relay their activity through a profuse projection to monoamine nuclei. In keeping with this, it is hypothesized that, brainstem catecholamine reticular nuclei may mediate these effects. In fact, within such a hub of highly interconnected brain catecholamine centers the kernel of cardiovascular control takes place (Figure 3, Guyenet et al., 2013). According to the profound effects concerning

the control of ortho-sympathetic neurons of the spinal cord, the pre-motor center in the medullary noradrenaline reticular formation are expected to sort significant metabolic effects. If this is the case we should expect that abnormal light exposure determines the onset of metabolic alterations. In fact, recent literature leads mounting evidence about light-related metabolic alterations (Rumanova et al., 2020). These include the metabolic effects induced in the liver and pancreas (Harmsen et al., 2022). The deleterious effects of light exposure during night-time at an intensity of 100 lux were recently demonstrated by Mason et al., 2022 as a consequence of abnormal light-induced activation of the ortho-sympathetic nervous system, inappropriately, during night time (Mason et al., 2022). Such an effect was associated to long-lasting metabolic alterations such as insulin resistance. Among wavelengths, short waves, perceived as blue were the most powerful to induce synchronization concerning circadian system. Recent evidence indicates that, both arterial pressure and heart rate are affected by exposure to short wavelengths and similarly, the kidney is affected concerning fluid and electrolytes excretion (Bryk et al., 2022). In contrast, the near infrared wavelengths produce plastic effects on the specific myocardium by altering the development of the cardiac rhythm (Niehoff et al., 2022). The intimate relationship between light exposure and cardiovascular activity was introduced in 2017 by Chellappa et al. (2017), who based their analysis on the brainstem connection of the photic retina through its projection to the SCN. Indeed, it is likely that, despite contributing to the function of the retina on the heart, such an extra-geniculate area does not represent the primary center, which translate photic information to the heart. In fact, based on the anatomical connections reported in this manuscript the mesencephalic projections of extra-geniculate pathways from Brn3b positive M1 iPRGCs to the pre-tectal region and the PAG are likely to activate the noradrenergic nuclei of the brainstem reticular formation controlling the activity of the heart. In fact, these cells modulate and/or represent the premotor centers of sympathetic preganglionic neurons which innervate the heart and blood vessels. The key role of the brainstem noradrenergic system in mediating the vegetative effects of light exposure emerges from everybody's experience. The classic inhibition of micturition, which occurs during night is likely to be related to the inhibition of both the PAG and LC. In fact, LC represents the main activator of the Barrington's nucleus in the brainstem reticular formation, which act as a trigger of micturition (Verstegen et al., 2017; Garcia Dubar et al., 2021). In contrast, during the transition from sleep to wake a powerful activation of the LC takes place and the act of micturition characterizes early time interval following awakening. The relevant effects of light-induced modulation on medullary noradrenergic neurons forming the premotor centers of the ortho-sympathetic innervation of the heart is provided by the interesting study of Wang et al., (2021). These authors indicate that plastic disruption of sleep-waking cycle produce a maladaptive cardiac remodeling, which in turn, is mediated by the effects of light, which activate the sympathetic nervous system. In fact, the suppression of sympathetic activity prevents inappropriate light-induced cardiac remodeling. Since noradrenergic neurons receive a powerful impact from peri-fornical orexin neurons of the lateral hypothalamus, which are activated by blue light, it is not surprising that the increase in blood pressure is also attenuated by orexin antagonists. The sites of action of orexin antagonists are placed both in the LC (where they mainly affect sleep) but mostly in the rostral ventrolateral medulla (A1 area of the present study) where the effects on blood pressure are supposed to occur (Li et al., 2013, Figure 3). The powerful effects of light exposure on the physiology and pathophysiology of vegetative functions with an emphasis on the cardiovascular system was pioneer discussed by Weil et al., 2009 who observed the impact of specific patterns of photoperiod on heart activity. The specific wavelength is critical. In fact, while blue light increases blood pressure, infrared light promotes vasodilation (Weihrauch et al., 2021).

As evident in the present manuscript, the PAG itself does possess a considerable amount of TH positive NA neurons, which merge caudo-laterally with LC and, on the midline, it continues with the dorsal raphe nucleus in its catecholamine component. As drawn in Figure 3, a remarkable interconnection exists between these NA nuclei in the brainstem reticular formation, which is supposed to translate the effects of light to multiple domains under the control of these nuclei. In turn, these nuclei

project to various brain areas, where they may translate a variety of functions (regulating the sleep-waking cycle, alertness, anxiety, mood) including profound effects on the vegetative system with an emphasis on cardiovascular activity and also breathing. As previously reported, the impact of an altered regulation of the vegetative nervous system may impact innumerous organs of the human body. These include effects on liver metabolism (Seydoux et al., 1979; Hartmann et al., 1982; Häussinger and Kordes, 2019; Imai et al., 2022), pancreatic activity (Jansen et al., 1997), kidney function (Becker et al., 2019; Osborn et al., 2021), adipose tissue (Straat et al., 2020) and spleen (Hirooka et al., 2020).

Although over-activity of the noradrenergic nervous system is currently interpreted as the result of a specific over-activity of neurons belonging to the locus coeruleus, a number of nuclei shown in the present study need to be considered. In fact, when referring specifically to a direct impact on cardiovascular control the role of the small group of TH positive neurons within the rostral ventrolateral medulla (A1) takes a leading role. This nucleus is involved both in the regulation of cardiac rhythm and inotropism as well as altering the excitability which governs the modulation of potential arrhythmia. The role of the A1 noradrenaline group is seminal since it impacts directly on the ortho-sympathetic neurons of the spinal cord, where it may alter directly the sympathetic tone profusely in the body. The reticular nature of such a widespread afferent and efferent connections may explain why light produces and increased alertness to an auditory odd-ball task (Kiehl and Liddle, 2003; Kiehl et al., 2001; Stevens et al., 2010; Halgren et al., 1998).

The role of LC in sleep is rather involved in brain plasticity, which is induced by the sleep waking cycle. In fact, as shown by Cirelli et al., (1996) and Tononi et al., (1995) the expression of wake-dependent early inducible genes in the cortex is lost upon destruction of LC. The plasticity induced by LC activity may involve the whole telencephalon, even the visual cortex. The powerful effects of LC in the plasticity of the visual system is described by Kasamatsu (1987; 1991). In these studies a specific role of the main NA nucleus was examined directly in the visual cortex. In detail, the LC exerts a powerful plastic effect through the activation of beta-receptors on priming the visual cortex concerning ocular dominance. The plastic effects of LC activation are activity-dependent and produce a synergism with ionotropic glutamate receptor excitation (Kasamatsu et al., 1991). The plasticity-inducing effects of LC are extended to a variety of physiological and pathological conditions (Fornai et al., 2011; Galgani et al., 2020; Gesi et al., 2000; Giorgi et al., 2003; Giorgi et al., 2006; Giorgi et al., 2008; Giorgi et al., 2017; Giorgi et al., 2019; Giorgi et al., 2020a; Giorgi et al., 2020b; Giorgi et al., 2021a; Giorgi et al., 2021b; Giorgi et al., 2022; Ruffoli et al., 2011), which include learning and memory and seizures. In all these cases a prominent role is exerted by beta receptors, which modulate specific intracellular signaling pathways (Lazzeri et al., 2021; Biagioni et al., 2022). The role of LC in photic plasticity emerged in the study by Watabe et al., (1982), who showed activation of the reticular nucleus LC following the stimulation of both geniculate and extra-geniculate pathways including: the optic chiasm (OX), the dorsal lateral geniculate nucleus (LGN), the superior colliculus (SC), and the visual cortex (VC). It is worth to be mentioned that the very same LC neurons were activated following auditory and painful stimuli, which indicates the reticular nature of these neurons and the convergence of multiple stimuli to modulate the plasticity of light-induced response. This is the case of plasticity induced by LC within allo-cortical areas following photic stimulation as reported by Hansen (2017). In detail, LC activation exerts a powerful effects on memory consolidation which depends on the activation of beta receptors. This effect is not related with visual tasks but it is rather widespread to multiple domains and it is triggered by multi-modal stimuli including the photic retina, which impinge on reticular noradrenergic nuclei of the brainstem.

The photic regulation of mood

The profound effects of light therapy on mood are known for over 3,000 years and they were empirically used in all kind of human cultures. The neuronal basis of these effects are complex and still poorly elucidated, though representing a hot topic in neuroscience research. Light may affect mood via different pathways. These are mostly independent from visual pathways or image forming retinal afferents. In fact, as summarized by

Maruani and Geoffroy (2022) light may activate mood-related circuitries either in the process of regulating circadian rhythms through the classic extra-geniculate system which project to the SCN, or independently via other centers, which are impacted by light independently from circadian rhythms, ruling out the monosynaptic impact on SCN. Among these alternative SCN-independent pathways, which are involved in modulating the sleep-waking cycle, alertness, anxiety, and general emotion we find a variety of retino-brain pathways: there is a direct inhibitory projection to the ventrolateral preoptic nucleus, and to perifornical orexin producing cells in the hypothalamus. The pathway relevant to this study involves monoamine catecholamine-containing neurons in the reticular formation. All these pathways may converge to cortical and corticoid areas of the limbic system where mood processing occurs, such as the amygdala, the olfactory tubercle, the nucleus accumbens, the hippocampus to complete the neural circuitry which acts as a light-driven mood regulator center. According to the present study, the role of monoamine brain system needs to be considered also considering that NA pathways from the locus coeruleus may be activated directly from light as shown by Aston Jones (2005) and may specifically receive a direct projection from retinal iPRGCs (VandeWalle et al., 2006).

A glimpse on evolution

The occurrence of behavioral and vegetative changes under the effects of light-induced brain plasticity is strongly mediated by the impact of light on the brainstem reticular formation. This complex of nuclei represents an archaic sensory system, which also regulate primordial behavior and vegetative functions. The preservation of such a fundamental light-driven system owing an archaic origin suggests that light may induce fundamental behaviors across different species. This is evident when light sensitive cells may coincide with a whole organism as it occurs in cyanobacteria. In fact, these organisms are able to sense external light and move according to the direction of a light source. This process is defined as phototaxis, which occurs concomitantly with photosynthesis (Menon et al., 2021). These movements occur through type IV pili (T4P) which binds and spend mechanical energy consisting in “twitching” or “gliding” motility (Menon et al., 2021). Being cyanobacteria often occurring in group, intercellular communication though exocytosis of polysaccharides grants coordination of movements among bacteria in the colony. This archaic community behavior is similar to what occurs during chemotaxis or quorum sensing. The ability to sense light in archaic systems allow to detect the source, direction, wavelength, and intensity. For instance, Fungi may sense light of different wavelengths through specific blue-, green-, and red-light photoreceptors, similarly to the human retina. Each color possess a specific phyto-chrome, where blue sensing requires flavin as chromophore, and red light is sensed through phytochrome (Yu et al., 2021). It should be emphasized how old is the process which connects light exposure with cell plasticity. In fact, in Aspergillus, blue-or red- or far-red light alter the expression of at least 1100 genes (Yu et al., 2021), with persistent changes in the organism. As in the human retina, fungal occurrence of light sensing crucially relies on mitochondria (Mu et al., 2006). In fact, mitochondria are involved in phytochrome-dependent light sensing, which extends the relevance of these organelles to all phytochrome-dependent photoreceptors including plants, bacteria, and fungi (Mu et al., 2006). All phytochrome contain a linear, heme-derived tetrapyrrole as chromophore. Linearization of heme requires heme-oxygenases (HOs), which reside inside chloroplasts in planta.

The effects of light as a plastic drive in whole organisms are substantiated by powerful epigenetic effects induced by light exposure. This is reflected by the amount of DNA methylation, histone acetylation, exosome modulation allowing the transmission of specific gene promoters or suppressors (Becker et al., 2016). In fact, light stimulation is a powerful environmental factor, which triggers widespread neuronal plasticity encompassing various animal species from insects to mammals

(Rybak and Meinertzhagen, 1997; Kiorpes, 2015; Barth et al., 1997; Stieb et al., 2010). Light-induced plasticity may impact the whole organism, such as in the bee, where the behavior of bee workers is modulated persistently by light-induced plasticity occurring within a number of neuronal networks (Becker et al., 2016).

Conclusions

Light is a primordial and essential stimulus, which governs behavior and homeostasis. The role of light is preserved during evolution and it sub-serves basic vegetative functions, while evolving to modulate complex behaviors. The present manuscript is dedicated to provide an anatomical representation and discussing primordial light-sensing nuclei within the noradrenergic brainstem reticular formation. This is a part of the most archaic core of the extra-geniculate pathways, which modulate essential behaviors and vegetative activity such as cardiovascular control. These brainstem centers, similarly to other related nuclei receive a preferential innervation from a subtype of retinal ganglion cells. In fact, within the retina at least 5 subtypes (M1-M5) of ganglion cells possess a photo-pigment (melanopsin) which allows light-sensing, independently of rod/cone photoreceptors. The M1 subtype of these iPRGCs project preferentially to light-sensitive, non-image forming brain areas. Among these, the Brn3b positive M1 phenotype owns a preferential projection to the pre-tectal region and distributes mono-synaptically to the rostral reticular formation including the PAG and the dorsal raphe nuclei. The activation of most noradrenergic reticular nuclei is thought to be operated by this specific extra-geniculate pathway. In fact a recent manuscript by vanderWalle et al., 2007 indicates that humans exposed to various selective wavelength owns a marked activation of the brainstem reticular formation at the level of LC when the wavelength corresponds to the one perceived by melanopsin-expressing retinal ganglion cells. Once the specific nuclei reported in the manuscript are activated by a photic stimulus a number of archaic phenomena take place. Upon reiterated pattern of light pulses plastic effects take place, which alter human behavior, mood and vegetative functions such as the cardiovascular system. This extends to influence a number of organs, which affect metabolism and homeostasis. The role of melanopsin in the vessels is also evident locally within the retina, where melanopsin itself, once activated by blue specific wavelengths produces vasodilation (Stachurska and Sarna, 2019). This manuscript discusses the evidence available to decipher the innumerous effects of light and focusing on the noradrenergic nuclei of the brainstem as a site to be further analysed to comprehend the connections between the intensity, patterns, wavelengths and timing inherent to light exposure and multiple behavioral and vegetative functions. This is further strengthened by the occurrence of additional non-visual photo-pigments in the inner retina including Müller glial cells, such as the photo-isomerase retinal G protein-coupled receptor, encephalopsin, and neuropsin (Guido et al., 2022). These photo-pigments all detect a wavelength similar to melanopsin, in the blue spectrum, which calls for specific investigations also concerning light-induced non-image forming effects within the retina and from the retina to archaic light-sensing areas within the CNS.

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