THE FUNCTIONAL ANATOMY OF THE RETICULAR FORMATION

EDITED BY: Ugo Faraguna, Michela Ferrucci, Filippo S. Giorgi and Francesco Fornai

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THE FUNCTIONAL ANATOMY OF THE RETICULAR FORMATION

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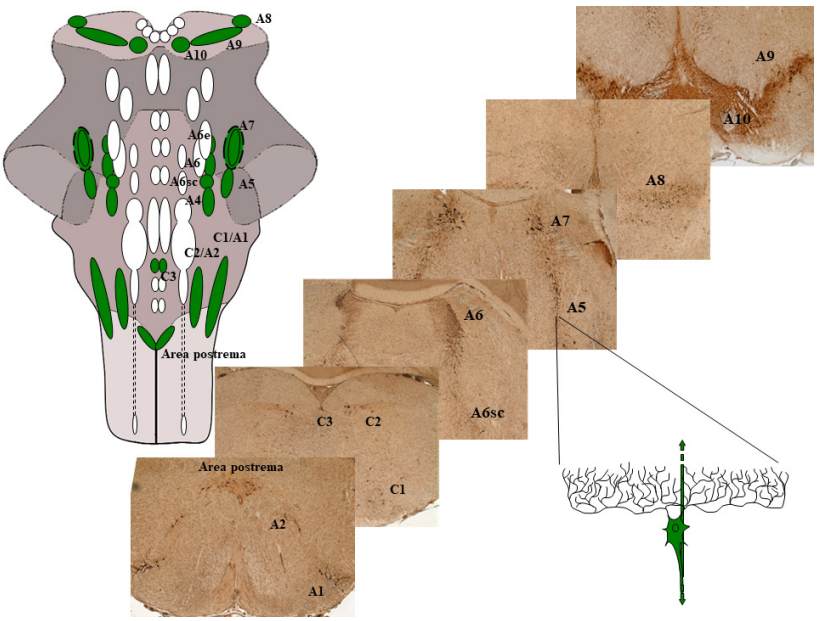

Rostro-caudal pictures from the brainstem report some catecholamine-containing nuclei of the mouse reticular formation (tyrosine-hydroxylase immunostaining). The cartoon represents these nuclei as green spots of the brainstem. A schematic view shows a typical isodendritic reticular neuron.

Image modified from: Francesco Fornai and Michela Ferrucci, "Anatomia funzionale della formazione reticolare nel tronco encefalico dell'uomo" (2016), Pisa University Press, Pisa (IT), ISBN 978-88-6741-740-7.

The brainstem reticular formation is the archaic core of ascending and descending pathways connecting the brain with spinal cord. After the pioneer description of the activating role of the ascending reticular activating system by Moruzzi and Magoun in 1949, an increasing number of studies have contributed to disclose the multifaceted roles of this brain area. In fact, the brainstem reticular formation sub-serves a variety of brain activities such as the modulation of the sleep-waking cycle, the level of arousal and attention, the drive for novelty seeking behaviors and mood. Meanwhile, descending pathways play a key role in posture modulation, extrapyramidal movements, and autonomic functions such as breathing and blood pressure. Moreover, both descending and ascending fibers of the reticular formation are critical in gating the sensory inputs and play a critical role in pain modulation and gaze control.

All these activities are impaired when a damage affects critical nuclei of the reticular formation. Remarkably, in neurodegenerative diseases involving reticular nuclei, the rich collaterals interconnecting reticular isodendritic neurons represent a gateway for disease spreading placing the role of the reticular nuclei as a pivot in a variety of brain disorders.

The present Research Topic is an updated collection of recent studies, which contribute to define the systematic anatomy of the reticular formation, its physiological and pharmacological features, as well as its involvement in neurodegenerative disorders and neuroprotection.

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EDITORIAL
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Editorial: The Functional Anatomy of the Reticular Formation

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Keywords: sleep-wake cycle, arousal, emotional brainstem, locus coereleus, pain, iso-dendritic neurons, catecholamine, drug addiction

Editorial on the Research Topic

The Functional Anatomy of the Reticular Formation

The brainstem reticular formation (RF) represents the archaic core of those pathways connecting the spinal cord and the encephalon. It subserves autonomic, motor, sensory, behavioral, cognitive, and mood-related functions. Its activity extensively modulates cortical excitability, both in physiological conditions (i.e., sleep-wake cycle and arousal) and in disease (i.e., epilepsies). Such a wide variety of effects arises from the long course and profuse axonal branching of isodendritic reticular neurons, which allows the neuronal message to travel toward the entire cerebral cortex and downstream to the spinal cord. On the other hand, the isodendritic architecture featuring a monoplanar branching allows most RF neurons to cover roughly half of the brainstem and to be impinged by ascending and descending pathways. In parallel, such a generalized influence on CNS activity occurs in combination with highly focused tasks, such as those involved in the coordination of gaze.

Thus, this special issue necessarily encompasses such a multi-faceted nature of the RF. In fact, the integration of multiple activities within the brainstem reticular circuitries may explain why alterations of each of these domains may affect the emotional sphere, paving the way to the concept of emotional brainstem (Venkatraman et al.). This brainstem region was explored in pioneer electrophysiological studies carried out by Moruzzi and Magoun (1949), who first demonstrated a crucial role of this wide area in activating and deactivating cortical EEG background amplitude and frequency. Interestingly, they demonstrated that there is a direct diffuse connection of different levels of RF (ranging from medulla to midbrain) with the whole cortex. At that time, however, the anatomical substrates responsible for such effects were largely ignored, and even the systematic definition of the RF as a complex of specific nuclei was still to be defined. Moreover, also the neurochemical substrates responsible for such effects were still to be discovered. In the following decades the main neurons constituting different areas of RF; and their neuro- and co-transmitters mediators have been characterized. Nevertheless, some biochemical and neuroanatomical features of specific RF neurons still need to be better defined, in different species, including humans. Therefore, a contribution of the present issue is entirely dedicated to a systematic analysis of all catecholamine-containing nuclei within the mouse RF (Bucci et al.). This paper, while confirming classic morphological studies on the isodendritic core of the RF (Brodal, 1957; Ramón-Moliner and Nauta, 1966), sheds new light on a few previously undefined reticular neurons. In fact this study showed that some neurons located in the area postrema are indeed catecholamine cells, placed continuously and downstream to the A2 area (Area Cinerea).

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The high connectivity of reticular nuclei may explain why a variety of different sensory information (i.e., visceral, trigeminal, and vestibular) may impact cognitive functions through ascending reticular neurons, pertaining to the catecholamine nucleus Locus Coeruleus (LC) (De Cicco et al.). Consistently, this issue includes an original investigation on how proprioceptive trigeminal afferents may affect attention and arousal via a tight neuroanatomical interaction between the proprioceptive trigeminal mesencephalic nucleus and the LC (Tramonti Fantozzi et al.). The specific role of LC in sustaining cognitive functions is substantiated by its diffuse branching (Brodal, 1957, 1981) and noradrenaline volume transmission (Fuxe et al., 1988, 2015; Agnati et al., 1995; Agnati and Fuxe, 2000) which produces widespread extrasynaptic paracrine effects. In this way LC, apart from a monosynaptic influence on cortical neurons, may affect the neurovascular unit as well (Giorgi et al.; Petit and Magistretti, 2016; Iadecola, 2017). It is well knows that LC activity exerts a powerful modulation of astrocytes, pericytes and microglia (Heneka et al., 2010; O'Donnell et al., 2012; Iravani et al., 2014). These extraneuronal effects might explain the role of microglial phagocytosis in sleep disorders (Nadjar et al.). Glial cells are also critical for releasing cytokines and chemokines messengers with both proinflammatory and neuroprotective actions. This may lead to an endogenous neuroprotective effect mediated by P27R receptors, as demonstrated by Lim et al.

Within this framework, Giorgi et al. stress the role of LC in modulating the neurovascular unit as a possible mechanism counteracting neurodegeneration in Alzheimer's Disease. This may add on novel cell-to-cell-based pathogenic effects in which misfolded proteins may spread monosynaptically from reticular axons to cortical neurons, according to a prion-like pattern (Giorgi et al.).

For instance, specific patterns of neuronal loss affecting catecholamine-containing reticular nuclei may produce a constellation of phenotypes in Parkinson Disease (PD). In fact, depending on which reticular nucleus is affected, a variety of both motor and non-motor (autonomic, sleep and mood-related, behavioral, and cognitive) symptoms, may occur. This mostly applies to non-motor symptoms, which appear to underlie different PD subtypes, each one owing a specific pattern of brainstem involvement (Gambardella et al.). Frequently, the onset of PD, instead of consisting of motor disturbances, coincides with autonomic alterations and pain. In this regard, the role of the RF in driving painful stimuli, and controlling pain-related circuitries, was reviewed by Martins and Tavares. These authors centered brainstem pain control in a reticular loop, which includes the periaqueductal gray, the rostro-ventro-medial medulla and the ventro-lateral medulla (Martins and Tavares).

The key role of the brainstem RF in mediating those activities relevant to species survival, such as pain and reward, sets the ground for these brain regions as preferential targets for drugs of abuse as reported by Ferrucci et al. In particular, while most of the literature on the effects of amphetamines has focused on their effects on dopaminergic neurons, there are several reports indicating a key role of the effects of amphetamines on LC in mediating many of their behavioral effects, including reward. Furthermore, interesting data indicate that the interaction of RF pontine cholinergic neurons (Ch5 and Ch6) with midbrain DA neurons might be crucial for the hyperlocomotion induced by amphetamines (Ferrucci et al.).

So far, the RF has been viewed mainly as an archaic collection of ascending and descending systems, and interconnected nuclei, which play only a rough and ancestral role in interlacing various CNS areas. Nevertheless, specific nuclei of the RF act as premotor centers, involved in the fine-tuning of the gaze, both along the vertical and horizontal plane. This latter function was investigated by Wang et al. who defined the central mesencephalic reticular formation as a conduit for the collicular saccadic signals in the horizontal gaze (Wang et al.).

All these features are covered by specific contributions of the research topic, which offers an updated view to define the anatomical correlates of the multiple and interconnected roles played by the brainstem reticular formation in health and disease.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Systematic Morphometry of Catecholamine Nuclei in the Brainstem

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Catecholamine nuclei within the brainstem reticular formation (RF) play a pivotal role in a variety of brain functions. However, a systematic characterization of these nuclei in the very same experimental conditions is missing so far. Tyrosine hydroxylase (TH) immune-positive cells of the brainstem correspond to dopamine (DA)-, norepinephrine (NE)-, and epinephrine (E)-containing cells. Here, we report a systematic count of TH-positive neurons in the RF of the mouse brainstem by using stereological morphometry. All these nuclei were analyzed for anatomical localization, rostro-caudal extension, volume, neuron number, neuron density, and mean neuronal area for each nucleus. The present data apart from inherent informative value wish to represent a reference for neuronal mapping in those studies investigating the functional anatomy of the brainstem RF. These include: the sleep-wake cycle, movement control, muscle tone modulation, mood control, novelty orienting stimuli, attention, archaic responses to internal and external stressful stimuli, anxiety, breathing, blood pressure, and innumerable activities modulated by the archaic iso-dendritic hard core of the brainstem RF. Most TH-immune-positive cells fill the lateral part of the RF, which indeed possesses a high catecholamine content. A few nuclei are medial, although conventional nosography considers all these nuclei as part of the lateral column of the RF. Despite the key role of these nuclei in psychiatric and neurological disorders, only a few of them aspired a great attention in biomedical investigation, while most of them remain largely obscure although intense research is currently in progress. A simultaneous description of all these nuclei is not simply key to comprehend the variety of brainstem catecholamine reticular neurons, but probably represents an intrinsically key base for understanding brain physiology and physiopathology.

Keywords: catecholamine, dopamine, norepinephrine, epinephrine, tyrosine hydroxylase, reticular formation, brainstem, stereology

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INTRODUCTION

Catecholamine-containing nuclei in the brainstem represent the main source of catecholamine in the CNS. Neurons belonging to these nuclei produce and release either norepinephrine (NE), dopamine (DA), or epinephrine (E) (Falck et al., 1962; Anden et al., 1964, 1965; Dahlström and Fuxe, 1964a,b, 1965; Hökfelt et al., 1974, 1984; Paxinos et al., 1995; Fuxe et al., 2010). When they contain catecholamines the neuronal phenotype is labeled with letter "A" (Dahlström and Fuxe, 1964a,b; Fuxe, 1965), and this is currently the case for NE or DA, while E-releasing neurons were later distinguished by the letter "C" (Hökfelt et al., 1974).

These catecholamine-containing nuclei are nowadays conventionally included and classified within the so-called lateral zone of the brainstem according to Nieuwenhuys et al. (1988). This is widely accepted as reported in most pivotal publications (Nieuwenhuys et al., 1988, 2007; Martin et al., 1990; Standring, 2008). In fact, it combines neurochemical, topographical, and functional approaches, thus overcoming single anatomical or neurochemical or functional criteria (Moruzzi and Magoun, 1949; Brodal, 1957; Conrad and Pfaff, 1976; Paxinos and Watson, 1986; Jones, 1995; Koutcherov et al., 2004). Accordingly, the lateral column can be further subdivided into an internal (more medial) and an external (more lateral) zone. From a phylogenic perspective, the mesencephalic DA system, represented by A8 [retrorubral field (RRF)], A9 [substantia nigra pars compacta (SNpc)] and A10 [ventral tegmental area of Tsai (VTA)] nuclei, is probably the most ancient component of the reticular formation (RF) (Dahlström and Fuxe, 1964a; Understedt, 1971; Lindvall and Björklund, 1978; Brodal, 1981; German et al., 1983; Björklund and Lindvall, 1984; Oades and Halliday, 1987). All these highly conserved DA nuclei, are conventionally classified in the lateral zone (Nobin and Bjorklund, 1973; Blessing et al., 1978; Saper and Petito, 1982; Tanaka et al., 1982; Pearson et al., 1983; Hökfelt et al., 1984; Nieuwenhuys et al., 1988, 2007; Björkund and Dunnett, 2007a,b; Standring, 2008; Cavalcanti et al., 2016; Medeiros et al., 2016). These nuclei provide key anatomical circuitries with great relevance in clinical settings (Fallon and Moore, 1978; François et al., 1999; Smith and Kieval, 2000; Medeiros et al., 2016).

Brainstem catecholamine nuclei represent the core of highly conserved structures along the evolution of CNS (Yamamoto and Vernier, 2011). In fact they are involved in the regulation of basic activities such as breathing, blood circulation, sleep-waking cycle, motor control (Barrington, 1925; Seiger and Olson, 1973; Demirjian et al., 1976; Pasquier et al., 1980; Brodal, 1981; Woulfe et al., 1988; Ellenberger et al., 1990; Delagrange et al., 1993; Guyenet et al., 1993; Valentino et al., 1993; Erickson and Millhorn, 1994; Dick et al., 1995; Smith et al., 1995; Fields et al., 2007; Li et al., 2008; Brown et al., 2012; Guyenet et al., 2013; Medeiros et al., 2016).

Despite the existence of a great number of papers concerning the mesencephalic DA-containing nuclei and some NE only a few contrasting reports deal with NE A4 nucleus (Paxinos and Watson, 1986; Paxinos and Franklin, 2001; Bux et al., 2010). This appears as a layer of TH positive neurons under the floor of the fourth ventricle which sends axons to the cochlear nuclei (Thompson, 2003). Similarly, scanty observations are available concerning A3 and C3 nuclei (Howe et al., 1980; Vincent, 1988; Kitahama et al., 1994; Paxinos and Franklin, 2001; Menuet et al., 2014). On the other hand, the presence of E-nuclei C1 and C2 is constant among human and animal species. C1 and C2 represent the rostral extent of A1 and A2 nuclei, respectively. In particular, the caudal extent of A2/C2 area is also known as ala cinerea nucleus which continues caudally to form the so-called area postrema (AP) (Potes et al., 2010; Rinaman, 2011; Mangano et al., 2012). The constellation of catecholamine nuclei depicted above, corresponds to a few nuclei placed within a small brain region but exerting a widespread influence in the CNS both via descending (Mason and Fibiger, 1979; Hammar et al., 2004) and ascending (Everitt et al., 1983) fibers. In fact, the iso-dendritic nature of these nuclei generates a high collateralization which in some cases enables just a single neuron to project to the entire forebrain. In fact, TH-immune-positive axons (i) course for long distances along the CNS; (ii) produce innumerable collaterals; (iii) each collateral possesses innumerable varicosities; (iv) each varicosity releases catecholamine in addition to other neurotransmitters; (v) catecholamines diffuse way beyond the synaptic cleft to reach extra-synaptic sites, thereby affecting neurons, glia and brain vessels. It is not surprising that a dysfunction of these nuclei produces a variety of brain disorders which fall into different domains of medical practice, way beyond neurology and psychiatry (Hoffmann et al., 2003; Furukawa et al., 2004; Willemsen et al., 2010). Despite playing such a critical role in fundamental activities, to date only some TH-immune-positive nuclei have been characterized in great detail. In fact, no systematic description of all these nuclei by using unbiased stereology in the very same experimental condition has ever been provided so far. In the present study we performed in depth stereological and morphometric analyses of all TH-immune-positive nuclei in the mouse brainstem in order to provide an overall description of these catecholamine neurons.

MATERIALS AND METHODS

Animals

Experiments were carried out in 12 weeks old C57BL6/J male mice (28 ± 2 g) ($N = 9$) (Charles River, Calco, LC, Italy). All mice were kept under environmentally controlled conditions (room temperature = 22°C; humidity = 40%) on a 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 RF.

Immune-Histochemical Analysis

Brains were dissected out, fixed in ethanol (60%), acetic acid (10%), and chloroform (30%), and included in paraffin. Deparaffinized tissue sections (20 μm) 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),

TABLE 1 | Technical features applied to each area under investigation.

Region	Number of slides	Dissector size	Counting frame
A9	8	50 × 50	120 × 120
A10	7	50 × 50	150 × 150
A8	3	40 × 40	120 × 120
PB	3	40 × 40	100 × 100
A7	2	40 × 40	110 × 110
A6sc	4	40 × 40	110 × 110
A6	3	35 × 35	120 × 120
A5	4	30 × 30	80 × 80
C1/A1	9	40 × 40	100 × 100
C2/A2	9	35 × 35	90 × 90
AP	4	50 × 50	120 × 120

and successively 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). Samples were then 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 control was performed without incubation with primary antibody.

Stereological Analysis

The number of TH-positive cells in the brainstem was assessed by stereological technique and optical fractionator using a Zeiss Axio Imager M1 microscope equipped with a motorized stage, a focus control system (Zeta axis), and a digital video camera. The software Image-Pro Plus 6.2 for Windows (Media Cybernetics, Inc.) equipped with a Macro was used for the analysis of digital images. Macro was obtained by Immagine and Computer (Italy, MI), and the characteristics of this Macro are reported by Gundersen and Jensen (1987). The analysis was performed on 26 sections of 20 μm, sampled every 160 μm on the horizontal plan of the brainstem, in which all the areas of interest were identified and outlined at 2.5X magnification. TH-positive cells

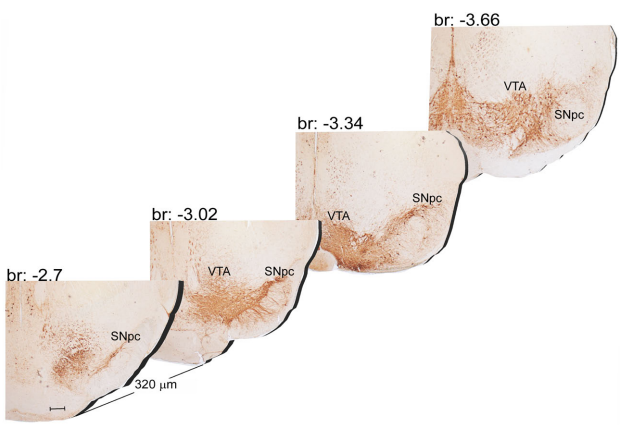
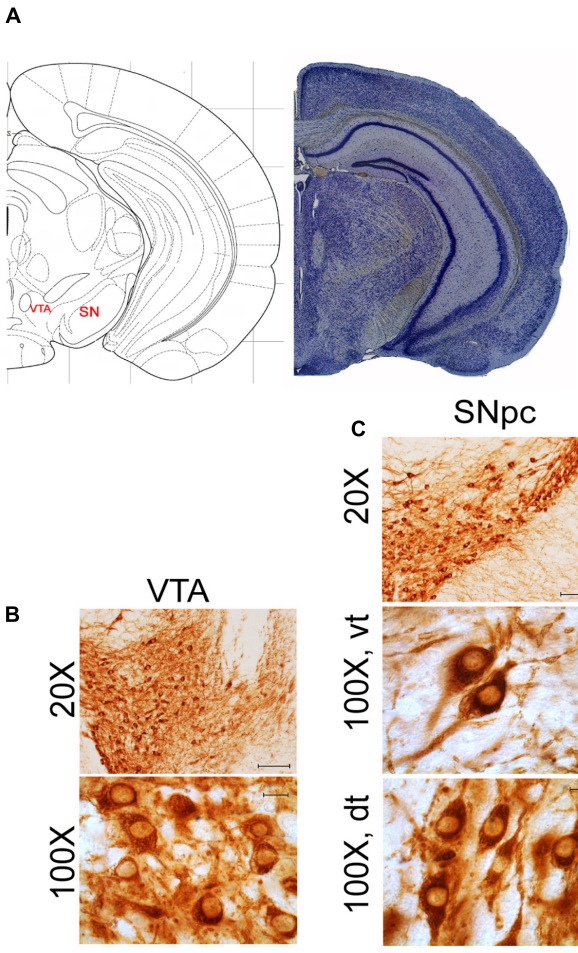


FIGURE 1 | Rostro-caudal reconstruction of the VTA and SNpc catecholamine nuclei. TH immunostaining in 20 μm coronal mouse brain sections regularly collected every 320 μm from –2.7 to –3.66 Bregma levels. The figure shows a 3D-like antero-posterior reconstruction of the VTA and SNpc nuclei. Scale bar: 200 μm.



D

	TH+ cells (total number)	Volume (mm^3)	TH+ cells density ($cells/mm^3$)	TH+ cell body area (μm^2)
SNpc	15694 +/- 587	0.171 +/- 0.006	91859 +/- 6164	vt: 220 +/- 12 dt: 139 +/- 5
VTA	17493 +/- 553	0.241 +/- 0.029	75273 +/- 10492	192 +/- 8

FIGURE 2 | Anatomical and morphometric analyses of TH-positive cell of the VTA and SNpc catecholamine nuclei. **(A)** Nissl staining of the mouse brain at the Bregma level –3.02 (Paxinos and Franklin, 2001) showing the anatomical localization of the VTA and SNpc. **(B,C)** Representative images of TH immunoreactive cells of the VTA and SN. Images at higher magnification (100X) show the morphological features of TH-positive cells of the VTA and the dorsal (dt) and ventral (vt) tier of the SNpc. Scale bar: 100 μm for 20X and 10 μm for 100X. The corresponding morphometric analyses are shown in **(D)**. Values are means ± SEM.

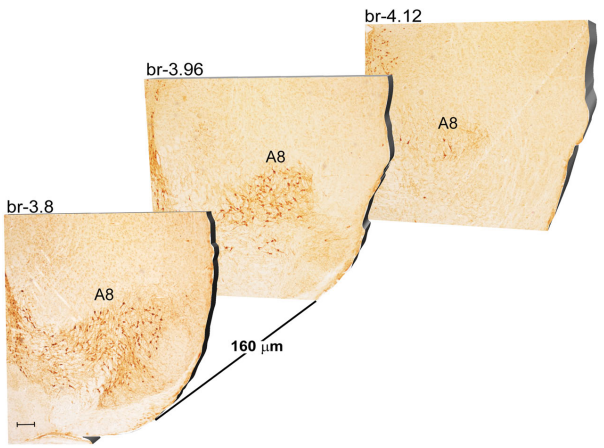
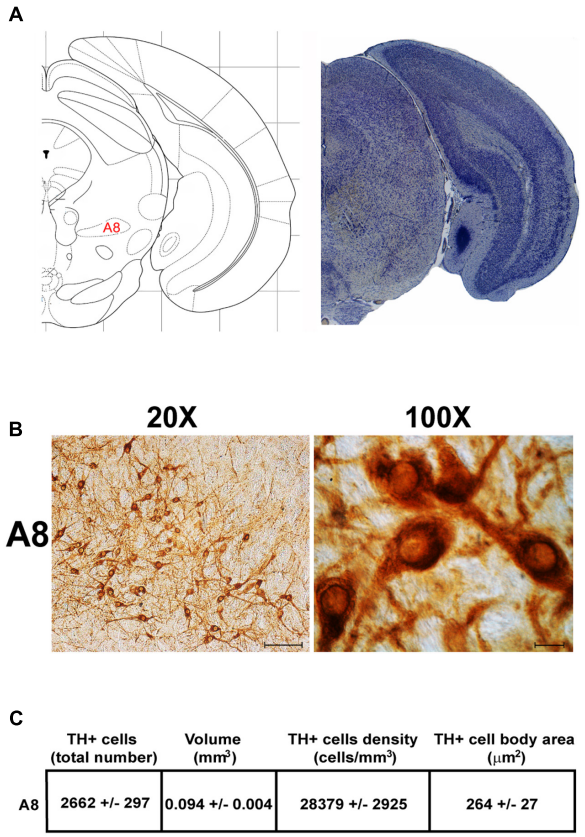


FIGURE 3 | Rostro-caudal reconstruction of the A8 catecholaminergic nucleus. TH immunostaining in 20 μm coronal mouse brain sections regularly collected every 160 μm from –3.8 to –4.12 Bregma levels. The figure shows a 3D-like antero-posterior reconstruction of the A8 catecholaminergic nucleus. Scale bar: 200 μm.



	TH+ cells (total number)	Volume (mm^3)	TH+ cells density ($cells/mm^3$)	TH+ cell body area (μm^2)
A8	2662 +/- 297	0.094 +/- 0.004	28379 +/- 2925	264 +/- 27

FIGURE 4 | Anatomical and morphometric analyses of TH-positive cell of the A8 catecholaminergic nucleus. **(A)** Nissl staining of the mouse brain at the Bregma level –3.96 (Paxinos and Franklin, 2001) showing the anatomical localization of the A8. **(B)** Representative images at higher magnification (20 and 100X) of TH immunoreactive cells of the A8. Scale bar: 100 μm for 20X and 10 μm for 100X. The corresponding morphometric analyses are shown in **(C)**. Values are means ± SEM.

were counted at 100X magnification (NA 1.3) as previously described (King et al., 2002). For stereological analysis, we used a different grid of dissectors depending on the area that we analyzed. The parameters used for the stereological evaluation are summarized in **Table 1**.

The total number of TH-positive cells was computed according to the formula: $N = \Sigma(n) \times 1/SSF \times 1/ASF \times 1/TSF$, where "n" is the total number of cells counted on each dissector; "SSF" (fraction of sections sampled) is the number of regularly spaced sections used for counts divided by the total number of sections across the areas; "ASF" (area sampling frequency) is the dissector area divided by the area between dissectors (dissector area × dissector number/region area); and "TSF" (thickness sampling frequency) is the dissector thickness divided by the section thickness.

The Cavalieri estimator method was used to evaluate the volume of each area examined by stereological cell count. In brief, volume analysis was conducted throughout the rostro-caudal extent of our regions of interest. In order to obtain the area of the region of interest, its contour was drawn by the operator. We applied the formula: $V = A \times t \times S$, where "A" is the area of the region of interest; "t" is the thickness of the section and "S" is the space between sections.

Cell Body Area

To evaluate cell body area, we captured 20 images for each region at 100X magnification. We analyzed each image, outlining the cell body present in our images. Image Pro Plus 6.2 software was used to asses the precise extent of the outlined area.

Statistics

Descriptive statistics were obtained by expressing the mean+SEM for each count in each nucleus. In detail, no significant difference for each specific feature (cell area, cell number, cell density, nuclear volume) concerning the same nucleus, was detected among all animals. After such a validation the mean measurements were the results of the mean values obtained from nine mice being stereologically evaluated. Therefore, inferential statistics, carried out by ANOVA, was used to compare different parameters between different nuclei to assess how cell number, cell body area, nuclear volume, and cell density vary between various catecholamine-containing nuclei. One-way ANOVA was applied using the Bonferroni *post hoc* test. The null hypothesis was rejected when $p < 0.05$.

RESULTS

Anatomical Mapping of TH-Positive Nuclei in the Mouse Brainstem

Immune-histochemical analysis of TH-positive neurons of the mouse brainstem allowed us to obtain a systematic detailed anatomical characterization of all catecholamine-containing nuclei.

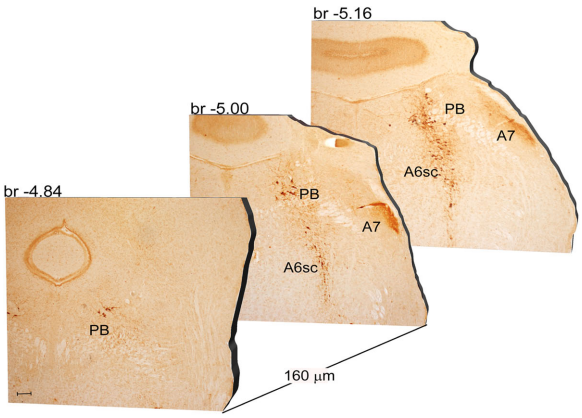
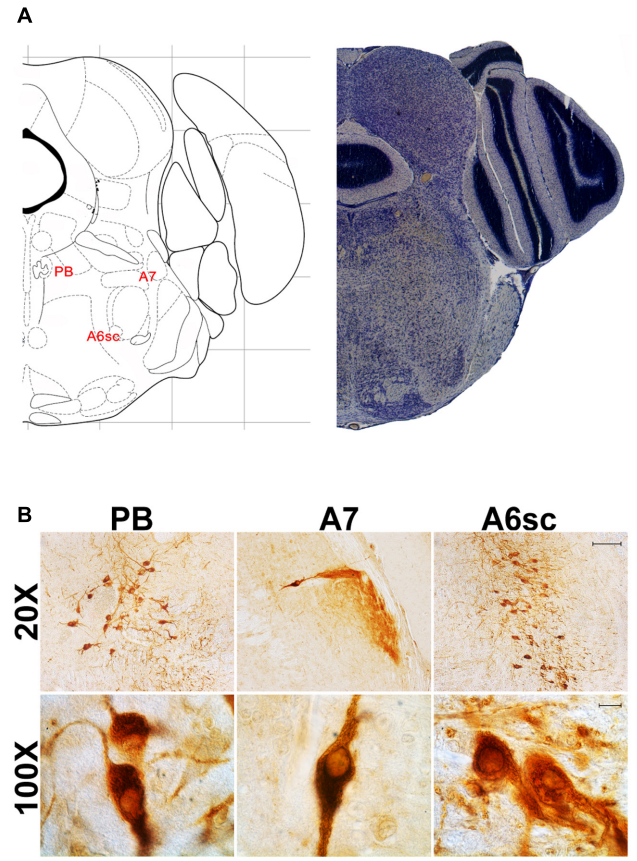


FIGURE 5 | Rostro-caudal reconstruction of the PB, A7 and A6sc catecholaminergic nuclei. TH immunostaining in 20 μm coronal mouse brain sections regularly collected every 160 μm from –4.84 to –5.16 Bregma levels. The figure shows a 3D-like antero-posterior reconstruction of the PB, A7 and A6sc catecholaminergic nuclei. Scale bar: 200 μm.



C

	TH+ cells (total number)	Volume (mm^3)	TH+ cells density ($cells/mm^3$)	TH+ cell body area (μm^2)
PB	1257 +/- 143	0.033 +/- 0.002	40044 +/- 3692	299 +/- 19
A7	814 +/- 103	0.030 +/- 0.003	28763 +/- 6828	340 +/- 30
A6sc	1015 +/- 157	0.089 +/- 0.007	11572 +/- 1748	277 +/- 27

FIGURE 6 | Anatomical and morphometric analyses of TH-positive cell of the PB, A7, and A6sc catecholaminergic nuclei. **(A)** Nissl staining of the mouse brain at the Bregma level –5 (Paxinos and Franklin, 2001) showing the anathomical localization of the PB, A7 noradrenergic group and the A6sc. **(B)** Representative images of TH immunoreactive cells of the PB, A7, and A6sc. Images at higher magnification (100X) show the morphological features of these TH positive cells. Scale bar: 100 μm for 20X and 10 μm for 100X. The corresponding morphometric analyses are shown in **(C)**. Values are means ± SEM.

The A9 (SNpc) and the A10 (VTA) appear as the most rostral catecholamine nuclei in the brainstem RF being entirely placed in the mesencephalon. The rostro-caudal extent for the SNpc corresponds roughly to 1280 μm (Bregma −2.7/Bregma −3.98), while it roughly measures 1120 μm for VTA (Bregma −2.86/Bregma −3.98) (**Figures 1**, **2A**).

Another DA-containing nucleus called A8 (also known as RRF), is placed in the tegmentum of the mesencephalon and it lies caudal and dorsal to the level of SNpc. This nucleus extends for a shorter length compared with other DA-containing mesencephalic cell groups. In fact, the RRF has a rostro-caudal extension of 480 μm (Bregma −3.8/Bregma −4.28) (**Figures 3**, **4A**). It is remarkable that, at the same rostro-caudal level of A8, it can be described a median TH-positive nucleus which adjoins dorsally the peri-acqueductal gray (PAG). The placement of these TH-positive cells appears to correspond to DA-containing cells which are described in the rostral part of the dorsal raphe nucleus (Ikemoto, 2007; Cho et al., 2017). When proceeding along the rostral-caudal axis of the mouse brainstem from the mesencephalon to the rostral pons there are a number of TH-positive nuclei (**Figures 5**, **6A**, **7**, **8A**). The most rostral among these nuclei corresponds to the medial parabrachial nucleus (PB), measuring 480 μm in length (Bregma −4.84/Bregma −5.32) (**Figures 5**, **6A**). Immediately caudal to the rostral pole of PB, on the lateral aspect of the pons, it appears the A7 nucleus (nucleus of lateral lemniscus) for a length of roughly 320 μm (Bregma −5.00/Bregma −5.32) (**Figures 5**, **6A**). At this level, ventral to PB, and when PB is still present in the dorso-medial aspect and A7 can still be fully appreciated in the lateral extent of the pons, it also appears the A6sc, with an approximate length of 640 μm (Bregma −5.00/Bregma −5.64) (**Figures 5**, **6A**). At a slightly caudal level, PB region is filled by the presence of the big pontine NE nucleus A6 [locus coeruleus (LC)], extending rostro-caudally for a length of about 480 μm (Bregma −5.34/Bregma −5.82) (**Figures 7**, **8A**). At this level, the A6sc is well-evident in the ventral extent of A6 (**Figures 7**, **8A**). This is why the PB, A6, and A6sc nuclei are often recognized as a nuclear complex, named LC complex (**Figures 5**, **6A**, **7**, **8A**). At the same level of A6 and A6sc, it can be appreciated the A5 nucleus, which is placed more ventral in the lateral band of the catecholamine RF and extends for a length of 640 μm (Bregma −5.34/Bregma −5.98) (**Figures 7**, **8A**).

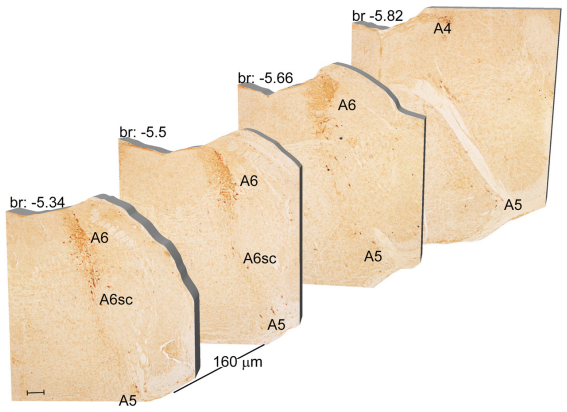
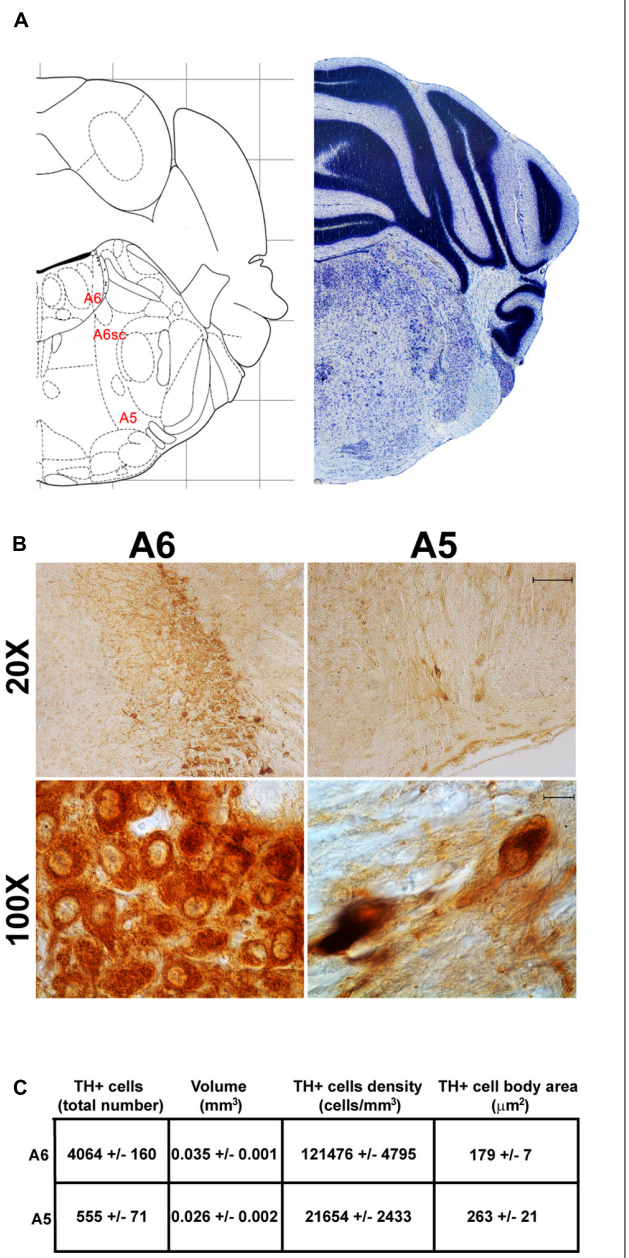


FIGURE 7 | Rostro-caudal reconstruction of the A6, A6sc, and A5 catecholaminergic nuclei. TH immunostaining in 20 µm coronal mouse brain sections regularly collected every 160 µm from –5.34 to –5.82 Bregma levels. The figure shows a 3D-like antero-posterior reconstruction of the A6, A6sc, and A5 catecholaminergic nuclei. Scale bar: 200 µm.



	TH+ cells (total number)	Volume (mm^3)	TH+ cells density ($cells/mm^3$)	TH+ cell body area (μm^2)
A6	4064 +/- 160	0.035 +/- 0.001	121476 +/- 4795	179 +/- 7
A5	555 +/- 71	0.026 +/- 0.002	21654 +/- 2433	263 +/- 21

FIGURE 8 | Anatomical and morphometric analyses of TH-positive cell of the A6 and A5 catecholaminergic nuclei. **(A)** Nissl staining of the mouse brain at the Bregma level –5.34 (Paxinos and Franklin, 2001) showing the anathomical localization of A6, A6sc, and A5 noradrenergic group. **(B)** Representative images of TH immunoreactive cells of the A6 and A5. Images at higher magnification (100X) show the morphological features of these TH positive cells. Scale bar: 100 µm for 20X and 10 µm for 100X. The corresponding morphometric analyses are shown in **(C)**. Values are means ± SEM.

The nucleus LC (A6) is probably the best-characterized among NE-containing nuclei of the brainstem RF. This nucleus is the main site for the synthesis of the whole brain NE. It is assumed that 50% of all brain NE is produced by LC neurons (Moore and Bloom, 1979; Foote et al., 1983). LC is placed dorso-caudally with respect to PB and slightly medial beneath the floor of fourth ventricle (**Figures 7**, **8A**). As we mentioned, the LC continues ventrally in the A6sc area which indeed continues slightly lateral and more ventral in the A5 region. At this level, A5 is placed toward the pial surface of the pons close to the roots of the facial nerve (**Figures 7**, **8A**). Remarkably, at the level of the facial nerve roots, in a dorsal position, we succeeded to find a small catecholamine nucleus which corresponds to the A4 cell groups (**Figure 7**).

In the caudal part of the mouse brainstem we found the following TH-positive cell groups: a lateral group corresponding to the sub-pial rostral ventro-lateral medulla C1/A1 (**Figures 9**, **10A**); a dorso-medial group, specifically known as nucleus of ala cinerea C2/A2, which is intermingled between the dorsal nucleus of the vagus (DMV) and the nucleus of the solitary tract (NTS) (**Figures 9**, **10A**). Its posterior extent toward the obex of the medulla corresponds to area postrema (AP) (**Figures 9**, **10A,B**). In this work, since we use TH as catecholamine marker, we could not discriminate between epinephrine- (C) and norepinephrine- (A) containing components of the C1/A1 and C2/A2. Both C1/A1 and C2/A2 possess a conventional rostro-caudal length of 1440 µm (Bregma −6.36/Bregma −7.8) (**Figures 9**, **10A,B**). In particular, C1/A1 is located in the rostral-ventro-lateral medulla (RVLM), whereas C2/A2 is located into the DMV running laterally and caudally over the solitary tract (**Figures 9**, **10A,B**). The last catecholamine nucleus we found in the brainstem was AP, having a rostro-caudal extension of 640 µm (Bregma −7.32/Bregma −7.96) (**Figures 9**, **10A,B**). AP is placed immediately beneath the cerebellum, extending caudally and medially to the NTS. Here the floor of the fourth ventricle is barely detectable; it is caudally defined by the obex, while rostrally continues toward the ala cinerea, where the C2/A2 cell groups are routinely defined (**Figures 9**, **10A,B**).

Counts of TH-Positive Cell Number, Cell Area, Nuclear Volume, and Cell Density Within All Catecholamine Nuclei of Mouse Brainstem

Stereological analysis demonstrated VTA (A10), SNpc (A9), RRF (A8), and LC (A6) as the catecholamine nuclei containing the highest number of TH-positive cells (17,493 ± 553 for VTA; 15,694 ± 587 for SNpc; 2,662 ± 297 for A8; 4,064 ± 160 for A6) (**Figures 2B–D**, **4B,C**, **8B,C**, **11A,B**).

In keeping with data reporting the cell number, VTA and SNpc also possess the biggest region volume with a slight prevalence (0.241 ± 0.029 mm^3) for VTA compared with SNpc (0.171 ± 0.006 mm^3) (**Figures 2D**, **11C,D**). This number again is consistent with data expressing the cell count in each of these DA nuclei of the mesencephalon (**Figures 2D**, **11A,B**). The region volume exceeds the results for cell number as shown by the cell density which again is high both in the VTA and SNpc (75,273 ± 10492 cells/mm^3 for VTA; 91,859 ± 6164 cells/mm^3 for SNpc) (**Figures 2B–D**, **11E,F**). It is interesting to note that A6, although representing a catecholamine nucleus with a high number of TH positive cells, possesses a relatively small volume (0.035 ± 0.001 mm^3) (**Figures 8C**, **11C,D**), which leads to the highest value of cell density in the LC (121,476 ± 4795 cells/mm^3) (**Figures 8C**, **11E,F**).

In contrast, the catecholamine nuclei with the lowest cell density are A6sc and C1/A1 (11,572 ± 1748 and 11,540 ± 992 cells/mm^3 respectively; **Figures 6B,C**, **10C,D**, **11E,F**). This is likely to be due to the small number of TH positive cells (1,015 ± 157 and 1,027 ± 52 respectively; **Figures 6B,C**, **10C,D**, **11A,B**) present within these high volume nuclei (0.089 ± 0.007 and 0.091 ± 0.004 mm^3 respectively; **Figures 6B,C**, **10C,D**, **11C,D**).

This substantiates the LC nucleus as being the richest norepinephrine containing nucleus of the entire CNS, at large.

Cell Body Area Assessment

The cell body area of TH-positive cells was investigated for each catecholamine brainstem nucleus. Our data show that neurons with the highest cell body area are located in the central part of the brainstem as evident from the Gaussian-like shape of the graph in **Figure 11G**. In fact, the highest cell body area was measured in TH positive neurons of PB (299 ± 19 μm^2), A7 (340 ± 30 μm^2), and A6sc (277 ± 27 μm^2) (**Figures 6B,C**, **11G,H**). In contrast, TH positive neurons with a smaller surface are placed in SNpc(dt) (139 ± 5 μm^2, **Figures 2C,D**, **11G,H**) and AP (86 ± 5 μm^2, **Figures 10C,D**, **11G,H**).

DISCUSSION

The brainstem RF represents an ancestral part of the brain hosting evolutionary preserved catecholamine nuclei. In particular, the lateral part of the brainstem RF is mostly characterized by archaic NE- and DA-containing nuclei, which were described back to the Tasmanian devil. An exception among these well-preserved nuclei can be made for C3 and A4 nuclei, which often lack in some species (Patzke et al., 2014).

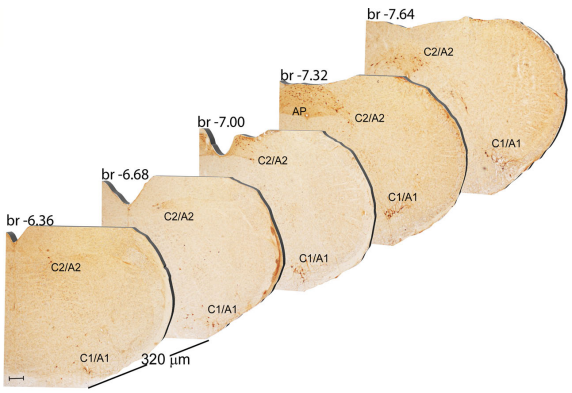
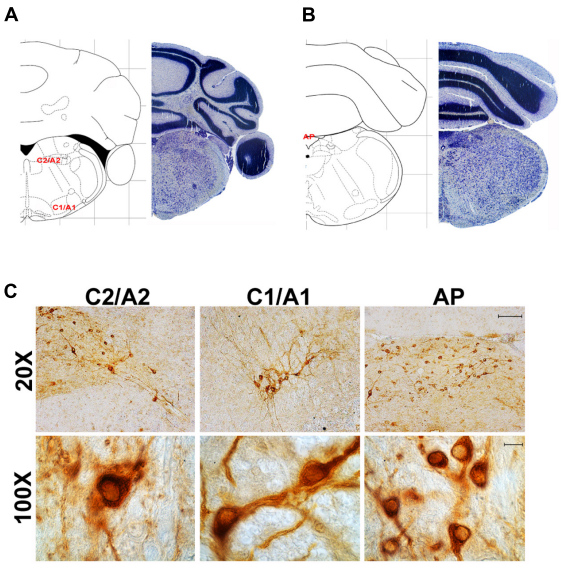


FIGURE 9 | Rostro-caudal reconstruction of the C1/A1 C2/A2 and AP catecholaminergic nuclei. TH immunostaining in 20 µm coronal mouse brain sections regularly collected every 320 µm from –6.36 to –7.64 Bregma levels. The figure shows a 3D-like antero-posterior reconstruction of the C1/A1 C2/A2 and AP catecholaminergic nuclei. Scale bar: 200 µm.

Our analysis and characterization of the mouse brainstem emphasizes DA-containing nuclei as the most populated catecholamine nuclei of the brainstem. Among these nuclei, the highest neuronal number is present within mesencephalic A10 (VTA), followed by A9 (SNpc). On the other hand, A8 (RRF) possesses a much lower neuron number, which is comparable to that of NE nuclei. The high number of TH immune-positive neurons within mesencephalic DA-containing nuclei is partly related to the big size of these nuclei, which are the largest of all catecholamine nuclei of the brainstem. In fact, considering the density of TH-immune-positive neurons, the highest value belongs to the pontine NE nucleus A6 (LC), followed by A9 (SNpc) and A10 (VTA) nuclei. These three nuclei possess at large the highest TH cell density, since all of them surpass by more than twofold the density of neurons counted in all other catecholamine cell nuclei. Both cell density and nuclear volume express the anatomical magnitude of a given catecholamine nucleus, although this is completely non-related with the cytological measurements which occur within the nucleus itself. The big size and high neuron number measured in these nuclei is likely to depend on the early appearance of these nuclei in the phylogeny. In fact, as reported in the introduction the A9 and A10 area represent the ancestral spot in the brainstem isodendritic RF (Dahlström and Fuxe, 1964a; Understedt, 1971; Lindvall and Björklund, 1978; Brodal, 1981; German et al., 1983; Björklund and Lindvall, 1984; Oades and Halliday, 1987). In contrast, mean neuronal area follows a different pattern, with peaks in the center of the brainstem RF with A7 expressing the highest neuronal area with an average cell body area surpassing 300 μm^2. This pattern indicates a magno-cellular neuronal size, which is centered at about mid-brainstem level, where indeed the A7 nucleus is placed. This neuronal size progressively diminishes both caudally and rostrally. This generates a sort of bell-shaped, Gauss-like distribution with the lowest neuronal area being



D

	TH+ cells (total number)	Volume (mm^3)	TH+ cells density ($cells/mm^3$)	TH+ cell body area (μm^2)
C1/A1	1027 +/- 52	0.091 +/- 0.004	11540 +/- 992	173 +/- 8
C2/A2	673 +/- 59	0.044 +/- 0.002	15146 +/- 1219	154 +/- 6
AP	1822 +/- 153	0.054 +/- 0.002	34207 +/- 2750	86 +/- 5

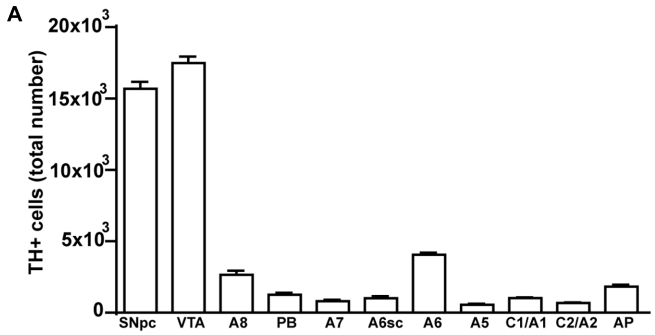
FIGURE 10 | Anatomical and morphometric analyses of TH-positive cell of the C1/A1, C2/A2, and AP catecholaminergic nuclei. Nissl staining of the mouse brain at the Bregma level –6.36 **(A)** and –7.32 **(B)** (Paxinos and Franklin, 2001) showing the anatomical localization of C1/A1, C2/A2, and AP catecholaminergic groups. **(C)** Representative images of TH immunoreactive cells of the C1/A1, C2/A2, and AP. Images at higher magnification (100X) show the morphological features of these TH positive cells. Scale bar: 100 μm for 20X and 10 μm for 100X. The corresponding morphometric analyses are shown in **(D)**. Values are means ± SEM.

measured in the dorsal tier of the A9 (SNpc) and in AP, where the mean neuronal area measures about 100 μm^2 (threefold less). This is in agreement with the presence of a parvi-cellular zone in the lateral extent of the caudal RF (Ter Horst et al., 1991). Although the RF is often described to cover the central tegmentum of the brainstem, several areas we describe here as being part of catecholamine nuclei of the brainstem, are placed either in the sub-ventricular zone (see for instance the medio-dorsal nucleus, and AP), or at the opposite, they can be found in sub-pial position ventrally in the lateral medulla (such as the C1/A1 region). This authentic topography of the RF is often missed out when generally describing these neurons as the core of the brainstem tegmentum. Likewise, despite being described as distinct catecholamine nuclei, a detailed neuro-anatomical tracking demonstrates the occurrence of scattered TH-positive cells joining dorso-medial with ventro-lateral aspects of these catecholamine nuclei. This is mostly evident in the area of LC, where scattered TH-immune-positive cells connect the LC nucleus, the A6sc area with the medial parabrachial (PB) region A5. In this latter case, the concept is so evident that the name LC complex can be used to define such a TH-immune-positive region. The A4 area, when present, can be considered within this complex as well. Moreover, as evidenced in the present study, scattered cells proceed ventro-laterally to join the A5 area. Likewise, in the caudal medulla interspersed TH-immune-positive cells can be described aligning between the A1/C1 and the A2/C2 regions. This confirms what already described in a previous study by Kitahama et al. (2009). Thus, it seems that most of these catecholamine-containing nuclei keep a sort of continuity, which is in line with the high synaptic connectivity and commonalities of neuronal circuitries and with the functions they are involved in.

In fact, considering the synaptology of the catecholamine nuclei of the brainstem RF, a specific network can be appreciated starting from the most caudal aspects (Madden and Sved, 2003) and extending to catecholamine nuclei (Guyenet et al., 2013).

In fact, a recent study shows that mesencephalic RRF, VTA and to a lesser extent SNpc receive a dense NE and E innervations originating from A1, A2, A5, LC (A4 and A6) and from C1 area, respectively (Mejías-Aponte et al., 2009). The projection of catecholamine fibers to mesencephalic DA neuron areas implies a functional connection between these nuclei in providing homeostatic regulation. For instance, A1 neurons are involved in hemodynamic regulation (Blessing and Willoughby, 1985); A2 neurons in the regulation of cardiovascular activity and food intake (Rinaman, 2003); A5 neurons regulate the respiratory rhythm generator of the rostral ventro-lateral medulla (Hilaire et al., 2004), whereas C1 neurons are barosensitive and also regulate sodium and water balance (Guyenet, 2006). In turn, strong visceral information form the ventro-lateral and dorso-medial medulla is conveyed toward the LC (A6) (Aston-Jones et al., 1986, 1991; Guyenet, 1991). Noteworthy, such visceral inputs may reach and thus trigger the response of DA midbrain neurons. For instance, cardiovascular afferent inputs originating from homeostatic centers of the lower brainstem have been shown to modulate DA neural responses (Kirouac and Ciriello, 1997). Inputs from the LC may also provide information about the arousal state or about ongoing behavioral performance. In fact, interactions between DA and LC systems have been suggested to exist during learning and motivated behavior (Aston-Jones and Cohen, 2005). Thus, it is likely that the flow of information carried by NE/E fibers to mesencephalic DA areas translates into a shared role between catecholamine nuclei in maintaining organisms' physiology.

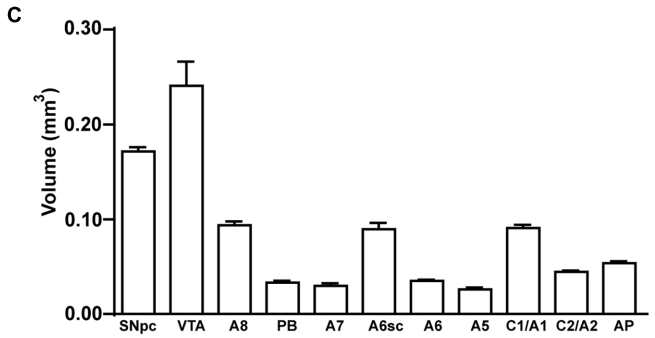
It is remarkable that this catecholamine-based inter-reticular network connects each other the brainstem reticular nuclei and altogether reticular regions with hypothalamus for establishing autonomic functions, sleep-waking cycle and other archaic physiological events controlled by the RF. For instance, the "catecholamine connection" is key for the sleep control played by orexin-containing neurons in the hypothalamus. In fact, most of the neurons represent the target of orexin-containing axons coming from the hypothalamus (Puskás et al., 2010). It is believed that NE cell groups of the lower RF placed in the brainstem are



B

	SNpc	VTA	A8	PB	A7	A6sc	A6	A5	C1/A1	C2/A2
SNpc										
VTA	***									
A8	****	****								
PB	****	****	****							
A7	****	****	****	ns						
A6sc	****	****	****	ns	ns					
A6	****	****	****	****	****	****				
A5	****	****	****	ns	ns	ns	****			
C1/A1	****	****	****	ns	ns	ns	****	ns		
C2/A2	****	****	****	ns	ns	ns	****	ns	ns	
AP	****	****	*	ns	*	*	****	****	ns	***

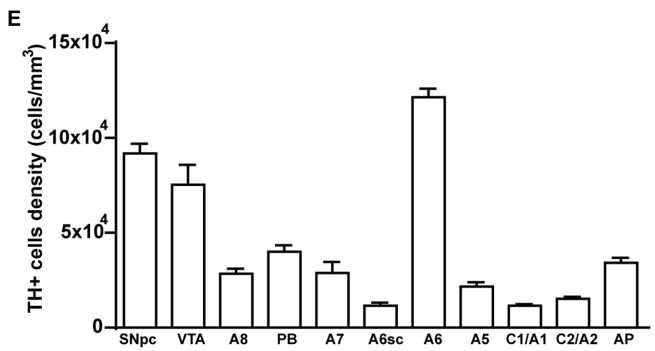
OneWay ANOVA+Bonferroni Multiple comparison test



D

	SNpc	VTA	A8	PB	A7	A6sc	A6	A5	C1/A1	C2/A2
SNpc										
VTA	****									
A8	****	****								
PB	****	****	****							
A7	****	****	****	ns						
A6sc	****	****	ns	****	****					
A6	****	****	****	ns	ns	****				
A5	****	****	****	ns	ns	****	ns			
C1/A1	****	****	ns	****	****	ns	****	****		
C2/A2	****	****	****	ns	ns	****	ns	ns	****	
AP	****	****	****	ns	ns	***	ns	**	***	ns

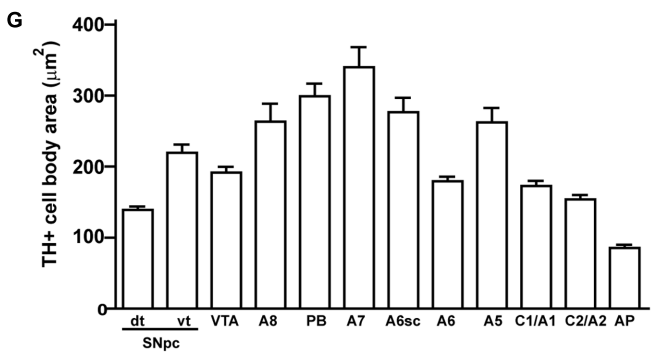
OneWay ANOVA+Bonferroni Multiple comparison test



F

	SNpc	VTA	A8	PB	A7	A6sc	A6	A5	C1/A1	C2/A2
SNpc										
VTA	ns									
A8	****	****								
PB	****	****	ns							
A7	****	****	ns	ns						
A6sc	****	****	**	****	ns					
A6	***	****	****	****	****	****				
A5	****	****	ns	**	ns	ns	****			
C1/A1	****	****	*	****	ns	ns	****	ns		
C2/A2	****	****	ns	****	ns	ns	****	ns	ns	
AP	****	****	ns	ns	ns	****	****	ns	***	**

OneWay ANOVA+Bonferroni Multiple comparison test



H

		SNpc dt	SNpc vt	VTA	A8	PB	A7	A6sc	A6	A5	C1/A1	C2/A2
SNpc	dt											
	vt	ns										
VTA		ns	ns									
A8		****	ns	*								
PB		****	ns	****	ns							
A7		****	***	****	ns	ns						
A6sc		****	ns	****	ns	ns	ns					
A6		ns	ns	ns	**	****	****	****				
A5		****	ns	*	ns	ns	ns	ns	**			
C1/A1		ns	ns	ns	**	****	****	****	ns	**		
C2/A2		ns	ns	ns	****	****	****	****	ns	****	ns	
AP		ns	****	****	****	****	****	****	****	****	****	**

OneWay ANOVA+Bonferroni Multiple comparison test

FIGURE 11 | Total number, volume, density, and cell body area of TH positive nuclei. **(A)** Stereological analysis of TH-positive cells. **(B)** Statistical analysis (one-way ANOVA plus Bonferroni); $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. **(C)** Volumetric quantification of each cathecholaminergic nucleus obtained with the Cavalieri's method. **(D)** Statistical analysis (one-way ANOVA plus Bonferroni); $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. **(E)** TH-positive cell density (cells/mm^3). **(F)** Statistical analysis (one-way ANOVA plus Bonferroni); $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. **(G)** TH-positive cell body area (μm^2). **(H)** Statistical analysis (one-way ANOVA plus Bonferroni); $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

the main targets of orexin projections. This is reported for each E- (C1, C2, C3) and NE- (A6, A1, A2, A4, A5, and A7) cell group. Nonetheless, the LC complex, including the LC sensu stricto (A6) and the A6sc area, remains the greatest target of orexin fibers (Hagan et al., 1999; Horvath et al., 1999; van den Pol et al., 2002; Saper et al., 2005; Gompf and Aston-Jones, 2008; Kohlmeier et al., 2013; Sears et al., 2013). Similarly, a conspicuous number of orexin fibers is found in the so-called nucleus of the solitary tract, which indeed corresponds to the A2 area (Peyron et al., 1998; Date et al., 1999).

Some of these nuclei send conspicuous descending projections to the spinal cord (Blessing et al., 1981). In particular, NE nuclei, which project to the cervical and thoracic spinal cord are found at the level of C1/A1, C2/A2, A5, A6, A6sc, and A7, but these also include the small and inconstantly described A4 group.

The concept of area postrema (AP) is quite complex since it has been poorly characterized in pure anatomical studies. AP is placed across the midline of the lower part of the dorsal medulla down toward the obex. This obliges the anatomists to move aside the inferior cerebellar peduncle and tuberculus gracilis to access the area, which ends at the level of the obex. The AP is functionally well-known for its emetic effects [so-called chemosensitive trigger zone (CTZ)] and as a circumventricular organ (Shapiro and Miselis, 1985; Borison, 1989; Miller and Leslie, 1994; Price et al., 2008; Rinaman, 2011). Again, NE neurons of the AP seem to play an important role in anorexia induced by the pancreatic hormone amylin, which provides a message of satiation (Potes and Lutz, 2010). In fact, these neurons possess amylin receptors which modulate projections from AP to lateral parabrachial (PB) nucleus and to the solitary tract (ala cinerea, A2). Being a circumventricular organ AP develops as a richly vascularized area containing both TH- and DBH- immune-staining close to blood vessels. This suggests an additional non-synaptic effect of AP catecholamines, which may act to modify the neurovascular unit and/or being released as hormones within liquor (Pangestiningsih et al., 2009). The population of AP catecholamine neurons appears as an appendix of the A2 cell group (ala cinerea) which extends caudally toward the obex. The synaptic control of AP neurons is directed toward the dorsal motor nucleus of vagus (still intermingled within A2), selectively to those parasympathetic neurons which innervate the fundus but not the antrum of the stomach (Pearson et al., 2007).

Despite the AP is analyzed in a scattered way concerning the nature of its cells and its position, there are TH-positive, catecholamine-containing nuclei, medially and caudally to the A2 complex (Armstrong et al., 1981, 1982; Miceli et al., 1987). The vagal trigone, which is composed by the DMV (dorsal motor nucleus of vagus) and the NTS (nucleus of the solitary tract), is often called ala cinerea considering these terms as equivalent. The term ala cinerea refers to the occurrence of a gray area where dark/black points are visible macroscopically. This is due to the fact that there are iso-dendritic CA-containing cells intermingled between radicular parasympathetic sensory neurons of the DMV and viscero-sensory neurons of the NTS. Thus, just like the ash (*ciner*, in latin), this area is gray with black dots. When referring to the concept of ala cinerea, the presence of reticular neurons within non-reticular nuclei is key. In fact, if one wishes to indicate the A2/C2 (roughly corresponding to the dorso-medial nucleus) CA-containing nuclei, only TH-containing reticular neurons should be considered independently by their presence in the DMV or NTS. Conversely, when referring to the NTS and the DMV, the black dots of neuromelanin-containing neurons of the A2 area should be mentally erased since they are neither radicular parasympathetic nor visceral sensory neurons, but authentic catecholamine-producing, neuromelanin-containing iso-dendritic reticular neurons. Ala cinerea refers to a concept which encompasses the radicular, the sensory and the reticular nature of this dorso-medial area of the caudal medulla.

Although the anatomical continuity between the A2 area and the caudal AP is often neglected, in the present study we report the catecholamine nature of AP neurons and the density of its catecholamine-containing cells. Thus, it is not surprising that catecholamine-modulating drugs by acting on this catecholamine area produce significant effect on emesis (Peroutka and Snyder, 1982; Smith et al., 2012). Our study is the first, which clearly classifies the AP as a catecholamine-containing reticular nucleus, which continues backward the extent of the ala cinerea or, even better, its reticular component (i.e., the A2 area).

All these concepts and the essence of the anatomical connections of reticular nuclei witness for a merging between morphological, neurochemical, and functional similarities. This merging does not derive from pure serendipitous binding between randomly connected areas, but it rather expresses how neurochemical similarities drive the formation of synapses in the early development, when the genesis of functionally connected neural networks does occur. In conclusion, this is a unique study, since it documents the quantitative stereology encompassing all catecholamine-containing nuclei within the same brainstem, from the rostral VTA of Tsai up to caudal AP. Hereby, apart from providing the simultaneous description of all catecholamine-containing nuclei we also report evidence concerning A4 and AP nuclei, which are poorly and contrastingly described.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of 'European guidelines, OPBA IRCCS Neuromed'. The protocol was approved by the 'OPBA IRCCS Neuromed'.

AUTHOR CONTRIBUTIONS

DB performed stereological analysis and wrote the manuscript; CB performed immunohistochemical analysis; MC and PDP performed histological analysis; FB, FL, MM, and LR revised manuscript; FN and FF supervised research and revised manuscript.

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Reticular Formation Connections Underlying Horizontal Gaze: The Central Mesencephalic Reticular Formation (cMRF) as a Conduit for the Collicular Saccade Signal

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The central mesencephalic reticular formation (cMRF) occupies much of the core of the midbrain tegmentum. Physiological studies indicate that it is involved in controlling gaze changes, particularly horizontal saccades. Anatomically, it receives input from the ipsilateral superior colliculus (SC) and it has downstream projections to the brainstem, including the horizontal gaze center located in the paramedian pontine reticular formation (PPRF). Consequently, it has been hypothesized that the cMRF plays a role in the spatiotemporal transformation needed to convert spatially coded collicular saccade signals into the temporally coded signals utilized by the premotor neurons of the horizontal gaze center. In this study, we used neuroanatomical tracers to examine the patterns of connectivity of the cMRF in macaque monkeys in order to determine whether the circuit organization supports this hypothesis. Since stimulation of the cMRF produces contraversive horizontal saccades and stimulation of the horizontal gaze center produces ipsiversive saccades, this would require an excitatory cMRF projection to the contralateral PPRF. Injections of anterograde tracers into the cMRF did produce labeled terminals within the PPRF. However, the terminations were denser ipsilaterally. Since the PPRF located contralateral to the movement direction is generally considered to be silent during a horizontal saccade, we

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Abbreviations: 6n, abducens nerve; 7n, facial nerve; III, oculomotor nucleus; IV, trochlear nucleus; VI, abducens nucleus; VII, facial nucleus; At, axon terminal; BC, brachium conjuntivum; BDA, biotinylated dextran amine; CG, central gray; cMRF, central MRF; Cun, cuneiform nucleus; Den, dendrite; GABA, gamma amino butyric acid; HRP, horseradish peroxidase; IC, inferior colliculus; InC, interstitial nucleus of Cajal; IO, inferior olive; LC, locus ceruleus; LL, lateral lemniscus; MD, medial dorsal nucleus; MdRF, medullary reticular formation; MG, medial geniculate nucleus; ML, medial lemniscus; MLF, medial longitudinal fasciculus; MVes, medial vestibular nucleus; MRF, mesencephalic reticular formation; nPC, nucleus of the posterior commissure; nRTP, nucleus reticularis tegmenti pontis; OPt, olivary pretectal nucleus; P, pyramid; PAG, periaqueductal gray; PB, parabrachial nuclei; PH, nucleus prepositis hypoglossi; PhaL, *Phaseoulus vulgaris* leucoagglutinin; piMRF, peri-InC portion of the MRF; PN, pontine nuclei; PRF, pontine reticular formation; PPRF, paramedian pontine reticular formation; Pul, pulvinar; Pt, pretectum; PPt, posterior pretectal nucleus; R, red nucleus; RIP, nucleus raphe interpositus; SC, superior colliculus; SG, supragenual region; SGI, intermediate gray layer; SGS, superficial gray layer; SN, substantia nigra; SO, superior olive; SOA, supraoculomotor area; Ves, vestibular nucleus; Vm, trigeminal motor nucleus; Vp, principal trigeminal nucleus; Vs, spinal trigeminal nucleus; WGA-HRP, wheat germ agglutinin conjugated to horseradish peroxidase.

then tested the hypothesis that this ipsilateral reticuloreticular pathway might be inhibitory. The ultrastructure of ipsilateral terminals was heterogeneous, with some displaying more extensive postsynaptic densities than others. Postembedding immunohistochemistry for gamma-aminobutyric acid (GABA) indicated that only a portion (35%) of these cMRF terminals are GABAergic. Dual tracer experiments were undertaken to determine whether the SC provides input to cMRF reticuloreticular neurons projecting to the ipsilateral pons. Retrogradely labeled reticuloreticular neurons were predominantly distributed in the ipsilateral cMRF. Anterogradely labeled tectal terminals were observed in close association with a portion of these retrogradely labeled reticuloreticular neurons. Taken together, these results suggest that the SC does have connections with reticuloreticular neurons in the cMRF. However, the predominantly excitatory nature of the ipsilateral reticuloreticular projection argues against the hypothesis that this cMRF pathway is solely responsible for producing a spatiotemporal transformation of the collicular saccade signal.

Keywords: oculomotor, eye movement, saccade, superior colliculus, PPRF, gaze

INTRODUCTION

Humans continuously examine their environment through a series of gaze changes involving saccadic eye movements and, in some cases, accompanying head movements that require accurate and precise coordination. Consequently, when neurological disorders or deficits interfere with gaze, the outcome can be debilitating (Leigh and Zee, 2015). Numerous physiological studies have demonstrated that the superior colliculus (SC) plays an important role in selecting gaze targets (Wurtz and Goldberg, 1972; Munoz and Guitton, 1986; Paré et al., 1994; Freedman and Sparks, 1997; Sparks et al., 2001). The connections of the SC with pontine gaze centers are by way of the crossed tectobulbospinal tract or predorsal bundle, which targets the contralateral paramedian pontine reticular formation (PPRF; Harting, 1977; May, 2006; Basso and May, 2017). The PPRF contains premotor neurons that initiate horizontal saccades through their inputs to motoneurons and internuclear neurons found in the ipsilateral abducens nucleus (Hepp et al., 1989; Horn, 2006). The PPRF has been hypothesized to generate a command for horizontal saccades by extracting it from saccadic signals provided by the SC (Fuchs et al., 1985; Moschovakis et al., 1996). It contains premotor neurons that confer a code for the size and speed of the movement upon the abducens nucleus (Luschei and Fuchs, 1972; Keller, 1979; Horn, 2006). These premotor cells are referred to as medium lead saccadic burst neurons due to their high rate of firing in conjunction with saccades and the fact they begin firing before the short lead burst of the motoneurons (Fuchs et al., 1985; Moschovakis et al., 1996). They specifically fire for ipsiversive saccades and are silent for contraversive ones. This role for the PPRF is supported by stimulation studies and evidence that lesions in this region produced horizontal gaze palsies (Goebel et al., 1971; Cohen and Komatsuzaki, 1972; Kato et al., 2006).

The pontine portion of the horizontal gaze center consists of the medial region of the nucleus reticularis pontis oralis (NRPO) and nucleus reticularis pontis caudalis (NRPC), which houses medium lead excitatory burst neurons (EBNs). The EBNs activate the ipsilateral abducens motoneurons causing the ipsilateral eye to abduct (for review Henn and Cohen, 1976; Hepp and Henn, 1983; Strassman et al., 1986a; Hepp et al., 1989). They also contact internuclear neurons in the abducens nucleus that activate medial rectus motoneurons in the contralateral oculomotor nucleus, so that a comparable movement is made by the opposite eye, producing conjugate eye movements. The horizontal gaze center also includes the medial region of the nucleus paragigantocellularis dorsalis of the rostral medulla, as it houses inhibitory burst neurons (IBNs). IBN firing is similar in timing and intensity to that of EBNs during saccades and fixation (Hikosaka and Kawakami, 1977; Hikosaka et al., 1978; Yoshida et al., 1982). IBNs suppress the activity of antagonist muscles (the contralateral lateral rectus and ipsilateral medial rectus) through a glycinergic, crossed inhibitory projection to the motoneurons and internuclear neurons in the abducens nucleus (Yoshida et al., 1982; Strassman et al., 1986b).

While this circuitry is well worked out, the precise pathway(s) whereby the SC, which chooses saccade targets, sends this information to the EBNs and IBNs is still a matter of argument. Furthermore, the manner in which collicular signals are converted into the necessary burst neuron firing patterns is still obscure (see Moschovakis et al., 1998). For example, there is conflicting evidence with respect to whether the SC directly targets EBNs and IBNs. Raybourn and Keller (1977) could not find action potentials in monkey EBNs whose latencies were short enough following electrical stimulation of the SC to suggest monosynaptic input. Evidence from cats suggests monosynaptic tectal projections are supplied to PPRF premotor neurons (Grantyn et al., 1980, 1987; Grantyn and Berthoz, 1987; Izawa et al., 1999), and more specifically to IBNs (Hikosaka and Kawakami, 1977; Grantyn et al., 1979; Takahashi et al., 2005). Furthermore, Chimoto et al. (1996) found evidence for

monosynaptic tectal inputs to cat EBNs, when the omnipause inhibition was gated. However, Keller was not able to reproduce this effect in monkeys (Keller et al., 2000). There is evidence that long lead burst neurons (LLBNs) in the rostral brainstem receive direct inputs from the SC (Luschei and Fuchs, 1972; Hepp and Henn, 1983; Scudder et al., 1996a), and send efferents to medium lead burst neurons. Therefore, it has been suggested that these cells may serve as interneurons between the SC and premotor neurons in primates (Scudder et al., 1996b).

The central mesencephalic reticular formation (cMRF) is one of the structures that contains LLBNs (Waitzman et al., 1996; Handel and Glimcher, 1997). While earlier reports of a saccade-related area within the midbrain reticular formation (MRF) exist (cat: Szentagothai, 1943; Bender and Shanzer, 1964), the cMRF was first described in detail and named by Cohen and Büttner-Ennever (1984) and Cohen et al. (1985). They defined it as an area in the midbrain tegmentum of primates that produces horizontally directed contraversive saccades following electrical stimulation. They suggested three possible roles for the cMRF: saccade triggering, feedback control of saccadic activity and feed forward control of saccade-related activity. A similar area has been identified in goldfish, suggesting this structure is a common vertebrate feature (Angeles Luque et al., 2005; Luque et al., 2006), although its stimulation in fish did not just produce saccades; the animals turned their head and realigned their bodies.

Waitzman et al. (1996) recorded from individual cMRF neurons in awake, behaving monkeys and showed that about three quarters of cMRF neurons discharge before and/or during contraversive, visually guided rapid eye movements, and during contraversive spontaneous saccades in the dark. The number of spikes appeared to correlate with the size of the horizontal component of the saccade. In later reports, they made reversible lesions in this structure (Waitzman et al., 2000a,b). Based on their findings, they subdivided the MRF into a caudal region, the cMRF, where inactivation affects the horizontal component of saccades, and a rostral region, the peri-interstitial nucleus of Cajal portion of the MRF (piMRF), in which inactivation affects the vertical component of saccades. They suggested that a group of LLBNs located in the piMRF play a role in vertical saccadic eye movements, in contrast to the cMRF, whose LLBN activity is more related to the horizontal eye movements.

The targets of the various cell types described by these studies were not antidromically identified. Only the cells that project back upon the SC have been described physiologically (Moschovakis et al., 1988b). The activity of these reticulotectal neurons resembles, in most respects, the activity of the collicular cells providing them input. More recent studies identified separate classes of cMRF neurons in monkey that are associated with saccade metrics, including amplitude, velocity and duration (Cromer and Waitzman, 2006, 2007). These authors proposed that cMRF cells whose firing is most tightly coupled to saccade velocity may represent an intermediate step in the spatiotemporal transformation needed to convert the firing of output cells within the motor map present in the SC into the temporally coded firing of premotor neurons in the PPRF. Thus, these cMRF reticuloreticular cells would receive collicular input and project to the contralateral horizontal gaze center.

There are anatomical reasons why the cMRF is a good candidate for this role. The cMRF receives an extensive, topographically organized input from the SC (Edwards, 1975; Harting, 1977; Cohen and Büttner-Ennever, 1984; Moschovakis et al., 1988a,b; Chen and May, 2000; May et al., 2002). In fact, predorsal bundle axons supply axon collaterals to the cMRF prior to crossing in the dorsal tegmental decussation to supply the PPRF (Grantyn and Grantyn, 1982; Moschovakis et al., 1988b). It has been suggested that the cMRF also has projections to the PPRF (Büttner-Ennever and Büttner, 1988). Specifically, retrograde and anterograde studies in cats indicate the MRF provides a bilateral input to the PRF (Edwards, 1975; Stanton and Greene, 1981).

In light of these findings, we undertook a more detailed investigation of this cMRF projection in a primate, *Macaca fascicularis*, in order to specifically test whether the cMRF has appropriate patterns of connectivity to serve the spatiotemporal transformation of the collicular saccade signal. Biotinylated dextran amine (BDA) or *Phaseolus vulgaris* leucoagglutinin (PhaL) was injected into the cMRF of macaque monkeys, in order to anterogradely labeled reticuloreticular axons. We expected that the crossed projection would be excitatory and the ipsilateral projection would be either inhibitory, or end on inhibitory interneurons, since cMRF stimulation produces contraversive saccades and PPRF stimulation produces ipsiversive saccades, and because cells in both regions display a burst of action potentials when saccades are made in their on direction, but are silent when saccades are made in their off direction. To test whether the ipsilateral pathway was inhibitory, we also prepared material for electron microscopic investigation. Postembedding, gamma-aminobutyric acid (GABA) immunohistochemistry was used to examine the possible GABAergic nature of cMRF reticuloreticular axon terminals and targets in the ipsilateral PPRF. Finally, we tested whether the SC has direct access to this reticuloreticular projection by the use of dual tracer studies. Portions of these results have been presented in abstract form previously (Warren and May, 2004; May et al., 2005; Zhou et al., 2006).

MATERIALS AND METHODS

All animal procedures were undertaken in accordance with the animal care and use guidelines of the NIH, including the Guide for the Care and Use of Laboratory Animals, and with the approval of the University of Mississippi Medical Center IACUC. A total of 13 adult or young adult *Macaca fascicularis* monkeys of both sexes underwent surgeries performed with sterile techniques under isoflurane anesthesia (1%–3%; some of these animals were also used in other non-conflicting studies). Animals were sedated with ketamine HCl (10 mg/kg, IM). They were also treated with atropine sulfate (0.2 mg/kg, IV) to reduce airway secretions and dexamethasone (0.4 mg/kg, IV) to minimize cerebral edema. Vital signs, including core temperature and blood O_2 levels, were monitored and maintained at physiological levels. After the tracers were injected, the aspirated area was

filled with hydrated Gelfoam, the incision was closed with suture, and the wound edges were infused with Sensorcaine. Buprenex (0.01 mg/kg, IM) was administered as a postsurgical analgesic.

Anterograde Tracer Cases

Pressure injections of BDA (Molecular Probes; $n = 6$) were made with a 1.0 μl Hamilton microsyringe attached to a micromanipulator. To avoid the SC, the needle was inserted through the pulvinar (for details, see Wang et al., 2010, 2013). The injection depth was adjusted with respect to the SC surface. Between 0.1 μl and 0.2 μl of a 10.0% solution of BDA was delivered into the left cMRF along each of 1 or 2 penetrations. The same approach was used for the injections of PhaL ($n = 2$). A 2.0% solution in 0.1 M, pH 8.0 phosphate buffered saline solution was injected by means of iontophoresis. A positive current of 7 mA was passed through the PhaL solution, which was held in a glass micropipette with a tip diameter of 20–30 μm. Current was passed for 10–20 min (50% duty cycle, 7 s/pulse).

After a 3 week survival period for BDA injections or a 2 week survival period for PhaL injections, animals were sedated with ketamine HCl (10 mg/kg, IM) and deeply anesthetized with sodium pentobarbital (50 mg/kg, IP). They were perfused via the heart with phosphate buffered saline, followed by a fixative containing 1% paraformaldehyde and 1.25%–1.5% glutaraldehyde in 0.1 M, pH 7.2 phosphate buffer (PB). The brainstem was blocked in the frontal plane, removed and stored in cold 0.1 M, pH 7.2 PB. Frontal sections were cut at 100 μm with a vibratome (Leica VT1000S) for the BDA cases or at 50 μm for the PhaL cases, and collected in PB.

For BDA injections, at least two rostrocaudal 1 in 3 series at 300 μm intervals were reacted to reveal the presence of the tracer. As previously described (Barnerssoi and May, 2016), the sections were incubated overnight at 4°C in a solution containing Avidin D conjugated to horseradish peroxidase (Vector Laboratories, 1:5000) dissolved in 0.05% triton X-100 in 0.1 M, pH 7.2 PB. They were then rinsed with 0.1 M, pH 7.2 PB and reacted in a solution containing 5.0% diaminobenzidine (DAB) dissolved in 0.1 M, pH 7.2 PB. This solution also contained 0.011% hydrogen peroxide, 0.05% nickel ammonium sulfate and 0.05% cobalt chloride. In preparation for light microscopy, the sections were mounted on gelatinized slides, air dried, counterstained with cresyl violet, dehydrated in a graded series of ethanols, cleared in toluene, and cover slipped.

For PhaL injections, at least two rostrocaudal 1 in 3 series at 150 μm intervals were reacted following previously described procedures (Gerfen and Sawchenko, 1984; Perkins et al., 2009). Specifically, the sections were incubated in a 0.3% triton X-100 in 0.1 M, pH 7.2 PB solution for 20 min, rinsed and then placed in a 10.0% solution of normal goat serum in PB, as a blocking agent. Next, they were incubated in a solution containing biotinylated anti-PhaL (0.5% in 0.01 M, pH 7.2 PB solution), first at room temperature for 1 h, and then overnight at 4°C, with agitation. The next day, the sections were incubated in the final solution of an ABC kit (Vector Laboratories) for 1–2 h. After rinsing in a 0.1 M, pH 7.2 PB solution, the HRP tagged Avidin-Biotin complex was revealed as follows. Sections were placed in a 5.0% DAB solution in 0.1 M, pH 7.2 PB for 10 min, and the reaction was then initiated with H_2O_2 (0.011%) and allowed to run for up to 30 min. Reacted sections were mounted and prepared for light microscopy in the same manner as the BDA labeled sections.

Ultrastructural Procedures

Two of these same BDA cases with the most discrete cMRF injections were used for ultrastructural examination. Under a stereomicroscope (Leica Wild M8), small tissue blocks containing labeled terminals were cut out of free-floating sections and collected in 0.1 M, pH 7.2 PB. The details of the EM preparation are provided in previous reports (Wang et al., 2010, 2013). Ultrathin sections for conventional EM analysis were collected on copper mesh grids, while those used for GABA postembedding were collected on nickel slot grids. The latter were processed using rabbit anti-GABA (Sigma) and anti-rabbit IgG conjugated to15 nm gold particles (EM Sciences; for details, see Barnerssoi and May, 2016).

Dual Tracer Experiments

In dual tracer experiments ($n = 3$), injections of BDA and of wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) were made in sequential surgical procedures. Both tracer injections were placed on the left side. For the first surgery, the injection of the SC was performed following an approach similar to that described for the cMRF injection, above. After cortical aspiration, a 1.0 μl Hamilton injection syringe containing BDA was angled at 30°, tip rostral in the sagittal plane, and visually guided into the colliculus. Injection depth ranges from 1.0 mm to 1.5 mm from the surface. Post injection procedures were the same as for the cMRF injection.

Two to three weeks following the initial surgery, animals had a second surgery to inject a retrograde tracer into the PPRF. Injections into PPRF were done using stereotaxic coordinates for the PPRF (AP = 0.6, ML = 1.5, and DV = 0; Szabo and Cowan, 1984). The initial incision from the first surgery was reopened and the Gelfoam aspirated to reveal the tentorium cerebelli. A small incision was made in the tentorium to allow the injection syringe needle to penetrate the underlying cerebellum. A 1.0 μl Hamilton injection syringe attached to a stereotaxic manipulator set at an angle of 10°, tip rostral in the sagittal plane, was passed through the dorsal surface of the cerebellum to penetrate the PPRF. Each injection consisted of a 2.0% solution of WGA-HRP combined with a 10.0% solution of HRP dissolved in dH_2O. The injection volume ranged from 0.01 μl to 0.05 μl. Two additional animals just received the PPRF injection of WGA-HRP.

Within 48 h of the PPRF injection, animals were perfused and their brains were sectioned at 100 μm, as described above. Tissue sections were reacted first to demonstrate the HRP reaction product (Olucha et al., 1985; Perkins et al., 2009). The sections were rinsed with 0.1 M, pH 6.0 PB. This was followed by incubation in a chromagen for 20 min: 5% tetramethylbenzidine (TMB; Free Base) in a solution containing 0.025% ethanol and 0.25% ammonium molybdate in the 0.1,

pH 6.0 PB. Next, the sections were reacted by the addition of 0.3% H_20_2 solution (0.011% final concentration) at room temperature for 1 h. The sections continued to react overnight at 4°C, with gentle agitation. They were then transferred to a stabilizer solution of 5.0% ammonium molybdate in 0.1 M, pH 6.0 PB, for 15 min, followed by multiple buffer rinses. Sections from animals that had only received the PPRF injection were mounted, counterstained and coverslipped at this point. Sections from the dual injection cases were then incubated in a solution of DAB in 0.1 M, pH 7.2 PB and reacted with addition of 0.3% H_2O_2 (0.011% final concentration) to stabilize the TMB reaction product. The tissue was then rinsed in buffer and reacted to reveal the BDA following the procedures outlined above.

Analysis

For light microscopy, the distribution and morphology of anterogradely labeled terminals and retrogradely labeled neurons were charted using Olympus BH-2 or Nikon Eclipse 80i microscopes equipped with drawing tubes. Selected areas containing labeled terminals and/or neurons were digitally photographed with a Nikon Eclipse E600 photomicroscope equipped with a Nikon Digital DXM1200F color camera and Nikon Elements analysis software. In some cases, images from multiple focal planes were digitally combined using the Nikon Elements *Z*-axis program. The digitized images were adjusted in Adobe Photoshop to appear as close as possible to the visualized image.

For electron microscopic (EM) analysis, ultrathin sections were examined, and labeled profiles were photographed with a transmission electron microscope (Zeiss LEO 906). EM photographs of terminals were generally taken at magnifications of 21,560×. For characterizing labeling after postembedding immunohistochemistry for GABA, the number of gold particles in a 0.25 μm^2 square sampled from 3 or more regions over axon myelin sheaths, per grid, was counted to provide a background particle density for use as a baseline. We classified the terminals into GABA-positive ($GABA^+$, $\geq$3× baseline), intermediate (>baseline and <3× baseline) and GABA-negative ($GABA^-$, $\leq$ baseline) categories. Somatic and dendritic profiles were classified into $GABA^+$ ($\geq$2 × baseline), intermediate (>baseline and <2× baseline) and $GABA^-$ ($\leq$ baseline).

RESULTS

Anterograde Studies

An example of a BDA injection in the cMRF is illustrated in **Figure 1**. This injection site lay in the left cMRF (**Figures 1A,B**) and it was largely confined to the center of the nucleus. A small amount of BDA was found within the needle track, which extended through the caudal pulvinar and part of the posterior pretectal nucleus (PPt). Within the midbrain, BDA labeled terminals (stipple) were found bilaterally, with an ipsilateral predominance, in the nucleus of posterior commissure (nPC; **Figures 1A,B**). A relatively intense terminal field was present in the lateral part of periaqueductal gray (PAG; **Figures 1B–E**) and the supraoculomotor area (SOA; **Figures 1A,B**), but few terminals were found in the oculomotor nucleus (III), as reported recently (Bohlen et al., 2016). A fairly dense terminal field was present in the contralateral cMRF (**Figures 1A–D**), which may underlie the presence of an inhibitory off direction in tonically active cMRF neurons (Waitzman et al., 1996). More caudally, an extensive terminal field was found in the SC (**Figures 1C–F**). Terminals were distributed to both sides, and were densest within intermediate gray layer (SGI), in agreement with previous reports (Zhou et al., 2008; Wang et al., 2010). Labeled fibers extended caudally from the cMRF injection site and terminated densely and bilaterally in the cuneiform nucleus (Cun; **Figure 1E**).

In the pons, labeled terminals were found throughout the PRF, including both its medially located magnocellular division and, to a lesser extent, in the laterally located parvocellular division. These terminals distributed to both sides, with an ipsilateral predominance (**Figures 1B–G**). On the ipsilateral side, numerous fibers coursed diagonally, dorsolateral to ventromedial in the rostral end of the PRF (**Figures 1B–E**), before taking up a medial location. On the contralateral side, labeled axons that had decussated beneath the rostral pole of III took up a position just off the midline, beneath the brachium conjunctivum (**Figures 1A,B**), and maintained this position, running just lateral to the raphe nuclei through the pons and into the medulla. They terminated extensively in the nucleus reticularis tegmenti pontis (nRTP; **Figure 1D**). Labeled terminals were densest in the rostral, ipsilateral PRF (**Figures 1C,D**). More caudally, the ipsilateral PRF terminals were concentrated medially, ventral and rostral to the abducens nucleus, where the horizontal gaze center is located (**Figures 1E–G**). However, scattered terminations were also present throughout the parvocellular regions found laterally in the PRF. On the contralateral side, there were fewer terminal arbors and they tended to be concentrated medially in the PPRF (**Figures 1D–G**). This trend becomes more evident as one precedes caudally. Numerous labeled terminals were present on the midline in the nucleus raphe interpositus (RIP), where the omnipause neurons lie (**Figures 1C–E**), as has been reported previously (Wang et al., 2013). Finally, BDA labeled terminals were present in the parabrachial nuclei (PB), mainly ipsilaterally (**Figure 1E**).

In the medulla, labeled cMRF terminals continued to target the reticular formation bilaterally (**Figures 1G–I**), but far more were found ipsilaterally, as previously described (Perkins et al., 2009). In the rostral medulla (**Figure 1H**), terminals were present dorsally in the medullary reticular formation (MdRF), where the IBN component of horizontal gaze center is located. The vast majority of MdRF terminals were located dorsal to the inferior olive (IO), particularly at more caudal levels (**Figures 1G–I**). Terminals were quite evident ipsilaterally, in the medial accessory nucleus of the IO (**Figure 1I**). Caudal to the area illustrated, the quantity of labeled terminals dropped off dramatically, so that only a small number of labeled terminals were present in the cervical spinal cord (for details, see Warren et al., 2008; Perkins et al., 2009).

A more detailed example of the pattern of terminal labeling at the level of the abducens nucleus is presented in **Figure 2**. In this case, the BDA injection was slightly larger than the

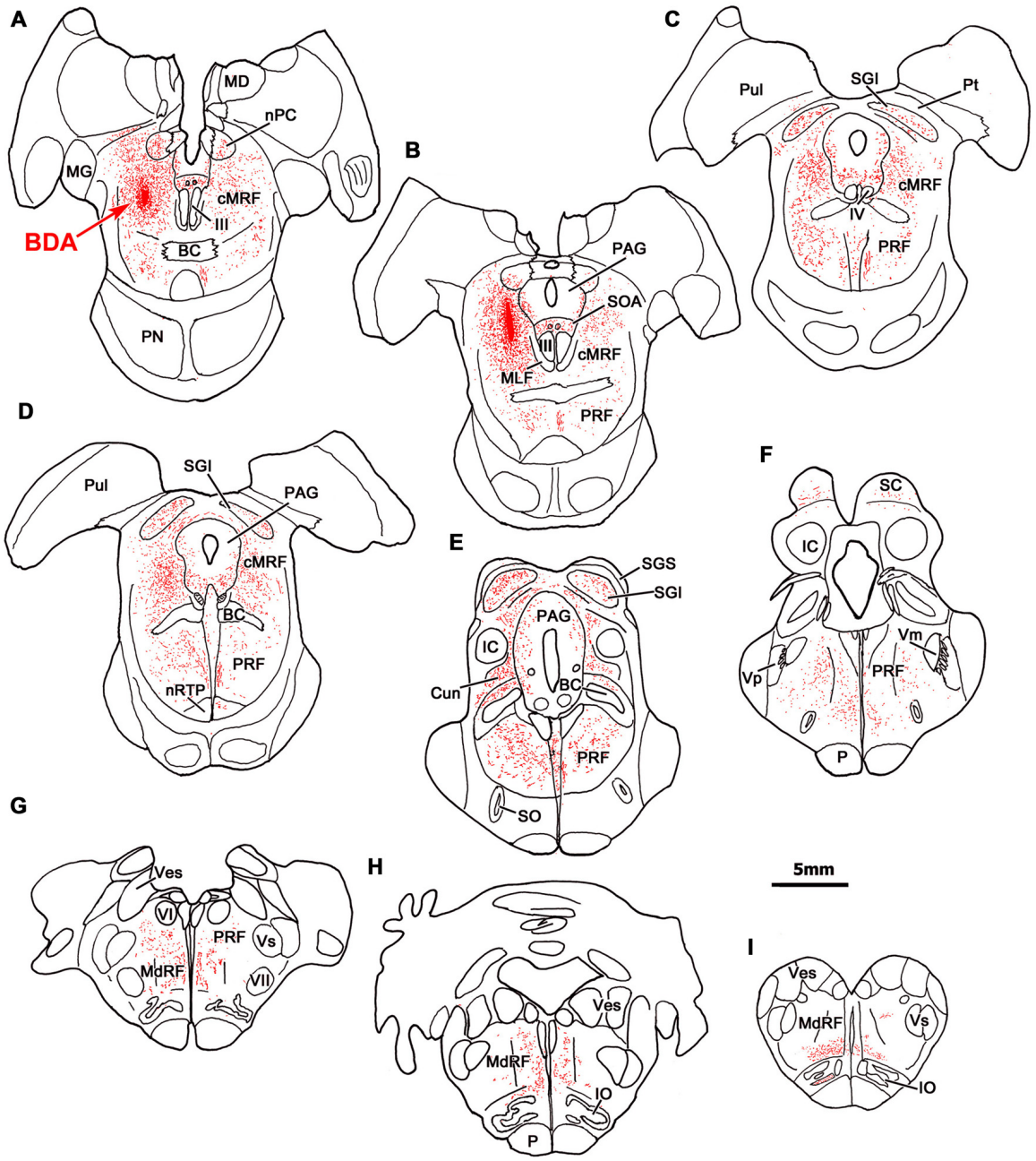

FIGURE 1 | Distribution of central mesencephalic reticular formation (cMRF) terminals labeled by biotinylated dextran amine (BDA). The distribution of labeled terminals (stipple) and axons (lines) observed following a BDA injection into the cMRF **(A,B)** is charted on a rostral to caudal series of sections through the midbrain **(A–F)**, pons **(A–G)** and medulla **(G–I)**.

case shown in **Figure 1**, but was still largely confined to the cMRF (**Figure 2B**). A few axonal arbors were located within the abducens nuclei (VI) on both sides (**Figure 2A**). RIP was filled with many small labeled boutons that were often organized in clusters. Within the PRF, the ipsilateral labeling was distinctly denser, and most of the terminal labeling was distributed medially (**Figure 2A**). On both sides, thicker, dorsoventrally oriented axons were found just lateral to the raphe nucleus. These were relatively short, indicating their rostrocaudal course through a frontal section. The presence of these axons suggests that the descending projections of cMRF travel in a position similar to that of the predorsal bundle, which contains crossed tectobulbospinal axons. Thinner axons extended mediolaterally, presumably branches of these parent axons. Numerous fine axons with small boutons filled the neuropil ventral to VI, in the area of the PPRF. Only a few labeled axons and terminals

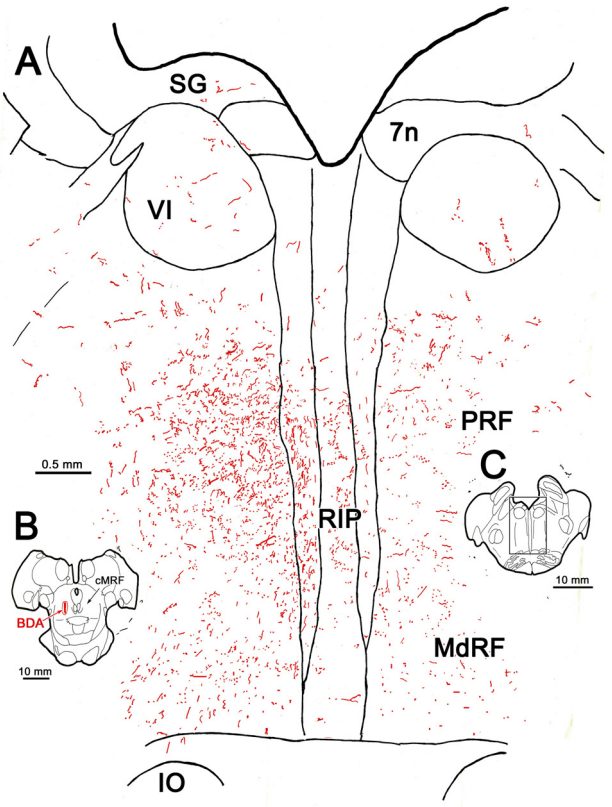

FIGURE 2 | BDA labeled reticuloreticular axons at the level of the abducens nucleus. The pattern of reticuloreticular axons (lines) and terminals (stipple) is illustrated for the region shown by a box in **(C)** at higher magnification in **(A)**. The center of the BDA injection within the cMRF that produced the labeling is shown in **(B)**. Note the ipsilateral predominance of the terminal labeling in the pontine reticular formation (PRF) and medullary reticular formation (MdRF).

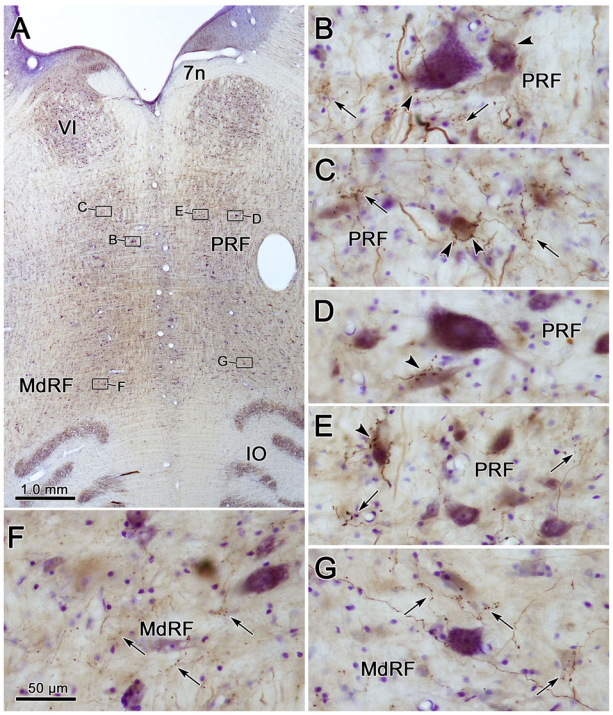

FIGURE 3 | Morphology of reticuloreticular axons labeled by BDA. **(A)** Low magnification photomicrograph of a section through the abducens nucleus showing the location of the higher magnification plates in this figure (labeled boxes). Some of the BDA labeled axons displayed close associations with counterstained somata (arrowheads), while others terminated in the neuropil (arrows). The images were taken from the ipsilateral PRF **(B,C)**, contralateral PRF **(D,E)**, ipsilateral MdRF **(F)** and contralateral MdRF **(G)**. The case shown is the same as illustrated in **Figure 2**. (Number of 1.0 µm *Z* axis planes merged: **B,C,E** = 3, **D,F** = 1, **G** = 4).

were observed more laterally, in the parvocellular reticular formation. More ventrally in the section, labeled axons were found within the MdRF. These tended to run obliquely, dorsal to the IO.

Examples of the terminal patterns produced by BDA injection of the cMRF shown in **Figure 2B** are found in **Figure 3**. A section through VI is shown for reference (**Figure 3A**). Within the ipsilateral PRF, small, BDA labeled boutons formed close associations (arrowheads) with both the larger (**Figure 3B**) and more commonly the smaller (**Figures 3B,C**) cresyl violet stained somata, but most terminated in the neuropil. Thick labeled axons coursed through the neuropil and finer axons formed terminal arbors (arrows; **Figures 3B,C**). A similar pattern was observed in the contralateral PRF. There, close associations (arrowheads) between the small, BDA labeled boutons and cresyl violet stained somata were evident (**Figures 3D,E**), with most being related to the smaller cells. The axosomatic contacts were more evident contralaterally, but the main target of the terminal arbors (arrows) was the neuropil. A fine network of labeled axons was found within the MdRF, just dorsal to the IO (**Figures 3F,G**). Almost all the terminal boutons were located in the neuropil, and were not associated with counterstained somata. Fewer terminals were present on the contralateral (**Figure 3G**) than the ipsilateral side (**Figure 3F**).

To ensure that the pattern of label seen with the BDA injections was not due to fiber-of-passage uptake, we also performed PhaL injections, as this tracer shows little fiber-of-passage uptake (Gerfen and Sawchenko, 1984). In the illustrated case, the PhaL injection into the left cMRF was centered within the region, and included much of its dorsoventral extent (**Figures 4A,B**). It extended into the pretectum. In the rostral midbrain, PhaL labeled cMRF axon terminals (stipple) were found bilaterally, with an ipsilateral predominance. Their pattern of termination (**Figures 4A–G**) was very similar to that seen with the BDA injections (**Figure 1**). In the pons, large numbers of terminals were found in the PRF (**Figures 4B–G**). Far more axons and terminal boutons were located on the ipsilateral side, and the density of termination decreased, and became more medially concentrated at more caudal levels. Notably less evident was the labeled fiber track found in a paramedian position on the contralateral side following the BDA injection. This suggests this track mainly represented predorsal bundle axons labeled via fiber-of-passage uptake of BDA. Terminals were present throughout RIP (**Figures 4F–H**). Fewer labeled

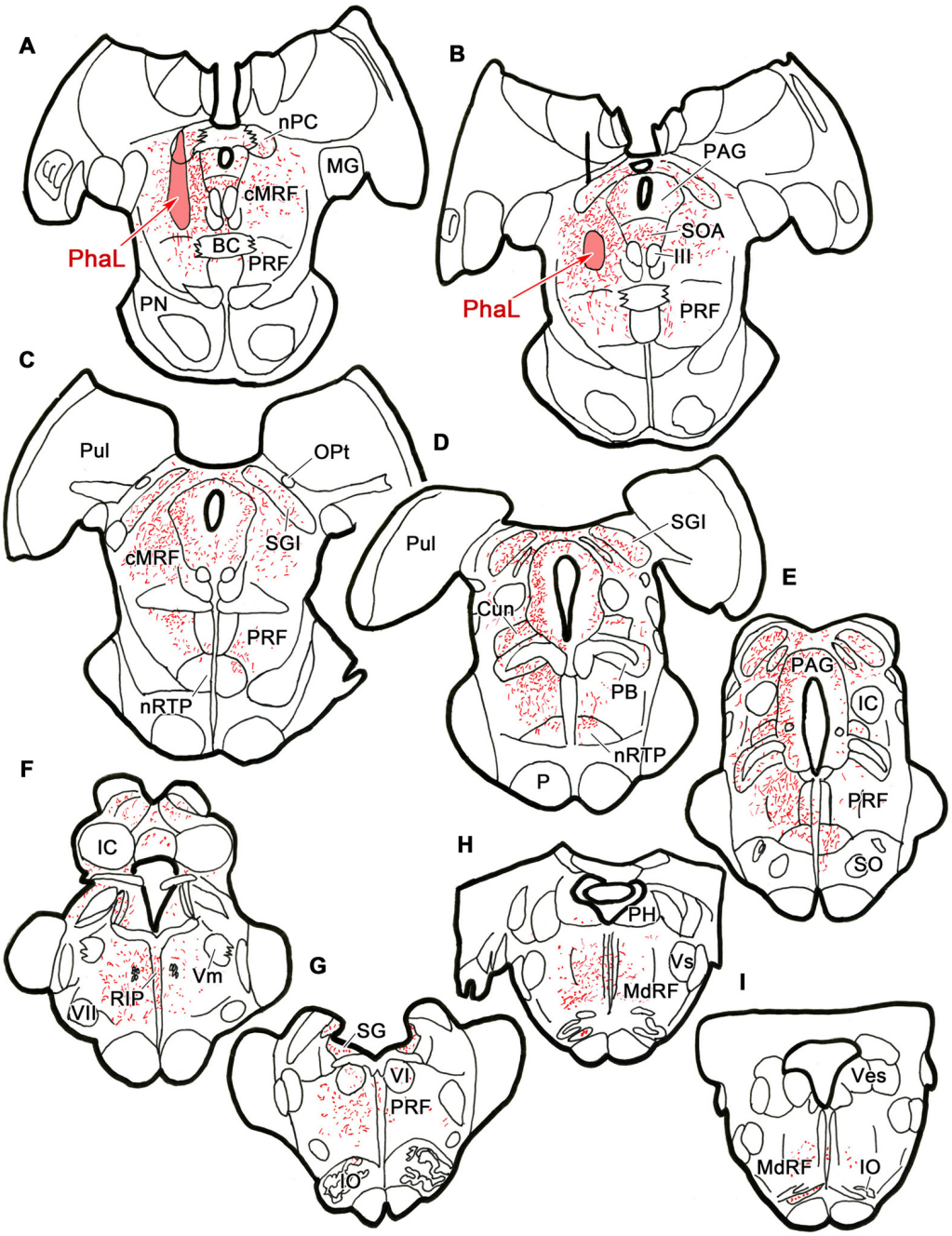

FIGURE 4 | Distribution of cMRF terminals labeled by *Phaseolus vulgaris* leucoagglutinin (PhaL). The distribution of labeled terminals (stipple) and axons (lines) observed following a PhaL injection into the cMRF **(A,B)** is charted on a rostral to caudal series of sections through the midbrain **(A–F)**, pons **(A–G)** and medulla **(H–I)**.

terminals were observed within the medulla (**Figures 4H,I**) than in the pons, but the ipsilateral predominance was still evident.

The pattern of axonal labeling following this PhaL injection is shown in greater detail in an illustration of a section at the level of the abducens nucleus (**Figure 5**). Note the relatively extensive labeling in the supragenual region (SG) above the facial nerve. There were a few terminal arbors within both abducens nuclei, but they were scattered. The main terminal field was in the ipsilateral PRF. This field was densest medially, within the PPRF. Labeled terminal arbors were also present contralaterally, but they were considerably fewer in number. The labeling within the MdRF was consistent with the BDA cases (**Figure 2**).

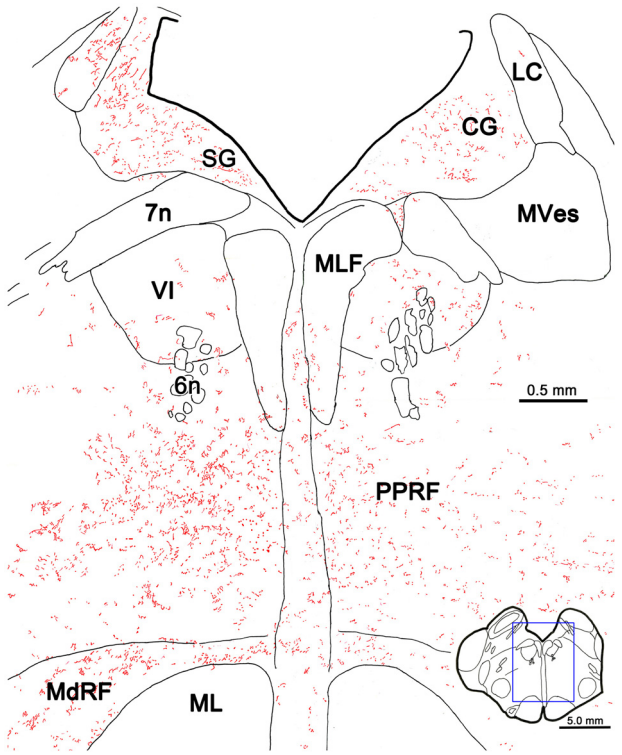

FIGURE 5 | PhaL labeled reticuloreticular axons at the level of the abducens nucleus. The pattern of reticuloreticular axons (lines) and terminals (stipple) is illustrated for the region shown by a box in the inset. The injection site is illustrated in **Figure 4**. Note the ipsilateral predominance of the terminal labeling in the PRF and MdRF.

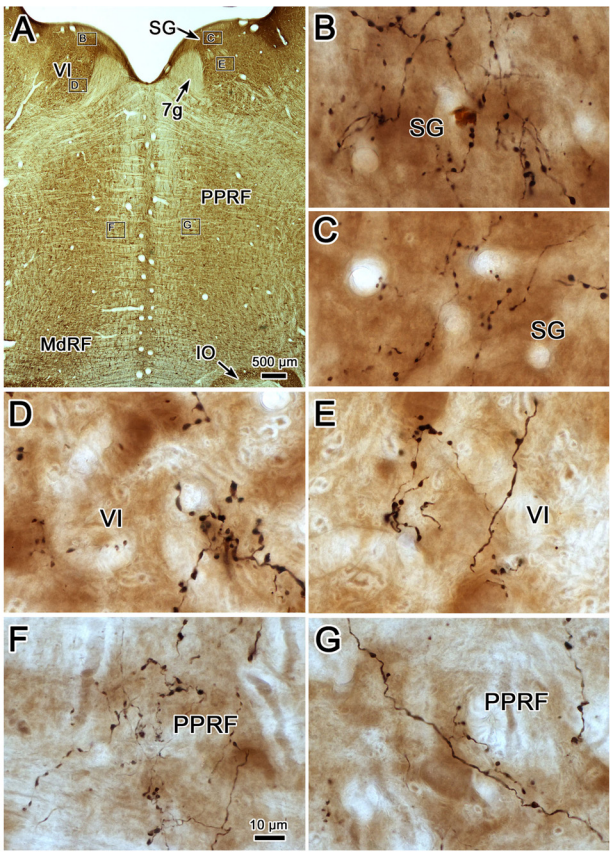

FIGURE 6 | Morphology of reticuloreticular axons labeled by PhaL. **(A)** Low magnification photomicrograph of a section through the abducens nucleus showing the location of the higher magnification plates (labeled boxes) in this uncounterstained section. The images were taken from the ipsilateral and contralateral supragenual region (SG; **B,C**, respectively), ipsilateral and contralateral abducens nucleus (VI) (**D,E**, respectively), and ipsilateral and contralateral PRF (**F,G**, respectively). Note the larger number of boutons ipsilaterally in the PRF. The case shown is the same as illustrated in **Figures 4**, **5**. (Number of 1.0 µm *Z* axis planes merged: **B,E,G** = 10, **C,F** = 13, **D** = 4).

Photomicrographs showing examples of PhaL labeled terminal arbors from the level of the caudal abducens nucleus (**Figure 6A**) are presented in **Figures 6B–G**. Thin, PhaL labeled axons could be observed with occasional branch points and terminal arbors within this region. The numerous *en passant* and terminal boutons varied in diameter. The density of the labeling was always heavier on the ipsilateral side (**Figures 6B,D,F**) than the contralateral side (**Figures 6C,E,G**). In the abducens nucleus, the boutons were clustered near individual cells, but most of the cells in the nucleus did not show adjacent terminals (**Figures 6D,E**). However, a dense network of terminals was observed dorsally, in the SG (**Figures 6B,C**). (Note that this is a complex region, that includes a number of supragenual nuclei (Büttner-Ennever et al., 1989; Biazoli et al., 2006; McCrea and Horn, 2006), but we will not examine this in detail here.) As can be seen in the PPRF samples (**Figures 6F,G**), the ipsilateral terminals seem more widespread in the neuropil, while associations with somata are more common contralaterally.

Terminal Ultrastructure

Figure 7 shows an example from a BDA case with a pair of small injections located laterally in the cMRF (**Figures 7A,B**). While the pattern of terminal label was similar to that described above, the number of labeled terminals was far smaller. The small windows in this figure indicate the areas where EM samples were taken. We concentrated our analysis on ipsilateral samples, where we expected to find solely inhibitory contacts. BDA labeled terminals (At*) were observed in material from the ipsilateral PPRF (**Figure 8**). Due to the BDA reaction product, they had greater electron density than profiles in the surrounding neuropil. Their morphological characteristics were similar to those we have described in the SC and RIP following cMRF injections (Wang et al., 2010, 2013). They were roughly round or oval in shape, and ranged in size between 0.40 µm and 3.60 µm along their long axis. These profiles were densely packed with small, clear vesicles that were either pleomorphic (**Figures 8D–F**) or spherical (**Figures 8A–C**) in shape. No dense core vesicles were seen in these terminals. The labeled terminals mostly contacted dendritic profiles. Both symmetric (**Figures 8D–F**) and slightly asymmetric (**Figure 8C**) synaptic densities were observed between the labeled terminals and dendritic profiles.

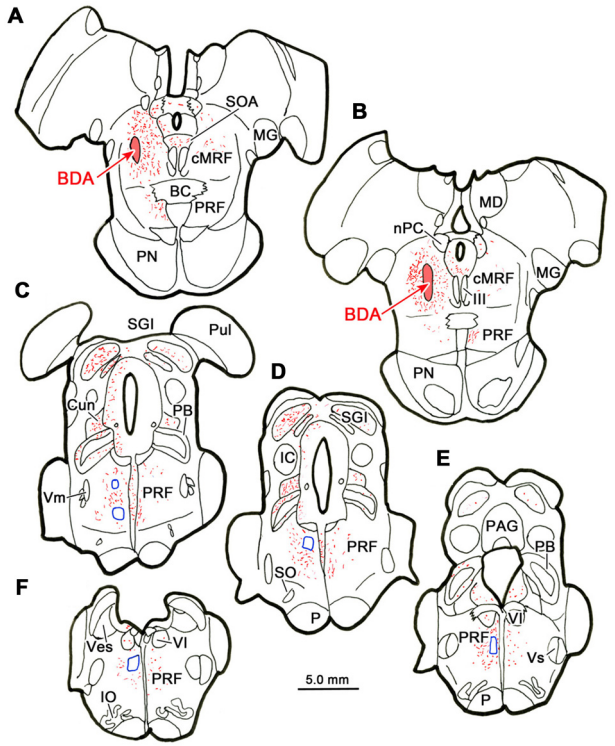

FIGURE 7 | Location of electron microscopic (EM) samples from the PRF. The distribution of labeled terminals (stipple) and axons (lines) observed following a small BDA injection into the cMRF **(A,B)** is charted on a rostral to caudal series of sections through the midbrain **(A–E)** and pons **(A–F)**. The location of areas sampled for electron microscopy is indicated by blue boxes.

For brevity, the characteristics of the BDA labeled terminals are demonstrated here from examples observed following GABA postembedding immunohistochemistry. BDA labeled profiles that were judged to be either GABA-positive ($GABA^+$) or GABA-negative ($GABA^-$) were both found in the ipsilateral PPRF. **Figures 8D–F** shows examples of these BDA labeled, $GABA^+$ terminals (At^{*+}). They were overlain by numerous gold particles, indicating their GABA-positive nature. Most of them presented with pleomorphic vesicles. Symmetric synapses were observed between these terminals and $GABA^-$ dendritic profiles (arrowheads). BDA labeled, $GABA^-$ terminals are shown in **Figures 8A–C**. These BDA labeled terminals were overlain by very few, if any, gold particles (At*), compared to unlabeled, $GABA^+$ terminals in the area (At^+). Often, these terminals were nearly filled with densely packed, slightly larger, round, clear vesicles. These vesicle characteristics of homogeneity and high density were present in more than 90% of BDA labeled, $GABA^-$ terminals throughout our EM samples. In **Figure 8C**, an asymmetric synapse (arrowhead) is shown between a BDA-labeled, $GABA^-$ terminal (At*) and a $GABA^-$ dendritic profile (Den).

We quantified the sample of terminals we observed. Among 64 terminals we observed in BDA/GABA double labeled material in the ipsilateral PPRF, 53.13% (34) were identified as $GABA^-$, 35.93% (23) proved to be $GABA^+$, while 10.94% (7) fell into the intermediate (undefined) category. All the postsynaptic elements contacted by these BDA labeled profiles were GABA-negative. Among all the BDA labeled terminals observed in both BDA labeled material and BDA/GABA labeled material, the vast majority contacted dendritic profiles.

Retrograde Study

Figure 9 shows the location of a WGA-HRP injection placed in the PRF and the distribution of the resultant retrogradely labeled neurons. The injection site involved the core of the PRF at the level of the abducens nucleus, and spread rostral to this level (**Figures 9G–I**). It included the abducens nucleus dorsally, and at its ventral end, spread slightly into the medullary reticular formation and IO. WGA-HRP labeled cells were observed within the cMRF (red dots) bilaterally (**Figures 9C–F**). However, the clear majority of the labeled neurons were located ipsilaterally. The dorsoventral spread of labeled cells extended throughout the entire MRF, so it included areas dorsal and particularly ventral to the region connected to the SC that has previously been defined as the cMRF (Chen and May, 2000). Labeled cells were also present with an ipsilateral predominance adjacent to the interstitial nucleus of Cajal (InC), in the piMRF (**Figures 9A,B**). **Figure 9** also shows retrogradely labeled cells in other midbrain structures (blue dots). They were located in the PAG, the SOA and within III. A number of labeled cells were present in the ipsilateral PPt (**Figure 9D**). In a second case (not illustrated), the injection site was located just off the midline, and it produced more labeled cells in the contralateral than ipsilateral cMRF; but in this case, the injection site extended slightly across the midline.

Dual Tracer Study

BDA labeled tectoreticular terminals together with WGA-HRP labeled reticuloreticular neurons were identified within the ipsilateral cMRF following combined injections of BDA into the left SC and WGA-HRP into the left PRF in three monkeys. In the illustrated case, the WGA-HRP injection was centered in the PRF (**Figures 10F–H**). The BDA injected in the left SC involved portions of all the collicular layers, with the largest concentration of tracer centered in SGI (**Figures 10A–E**). At the rostral end of the SC, the tracer spread to include a small portion of the dorsolateral PAG (**Figures 10A,B**). The resultant distribution patterns of the labeled elements are plotted in **Figure 11**. The BDA labeled tectoreticular terminals (stipple) were distributed throughout the rostrocaudal extent of the cMRF. In addition, reticulotectal cells, which were retrogradely labeled from the BDA injection in the SC (black dots), were distributed along a mediolateral band within this terminal field, defining the core of the cMRF (Chen and May, 2000). The injection of WGA-HRP into the PRF retrogradely labeled numerous reticuloreticular neurons (red diamonds). These neurons were observed throughout the rostrocaudal and mediolateral extent of cMRF (**Figures 11B–F**), with a heavier concentration of labeled cells at more rostral levels, including the piMRF (**Figure 11A**). The distribution

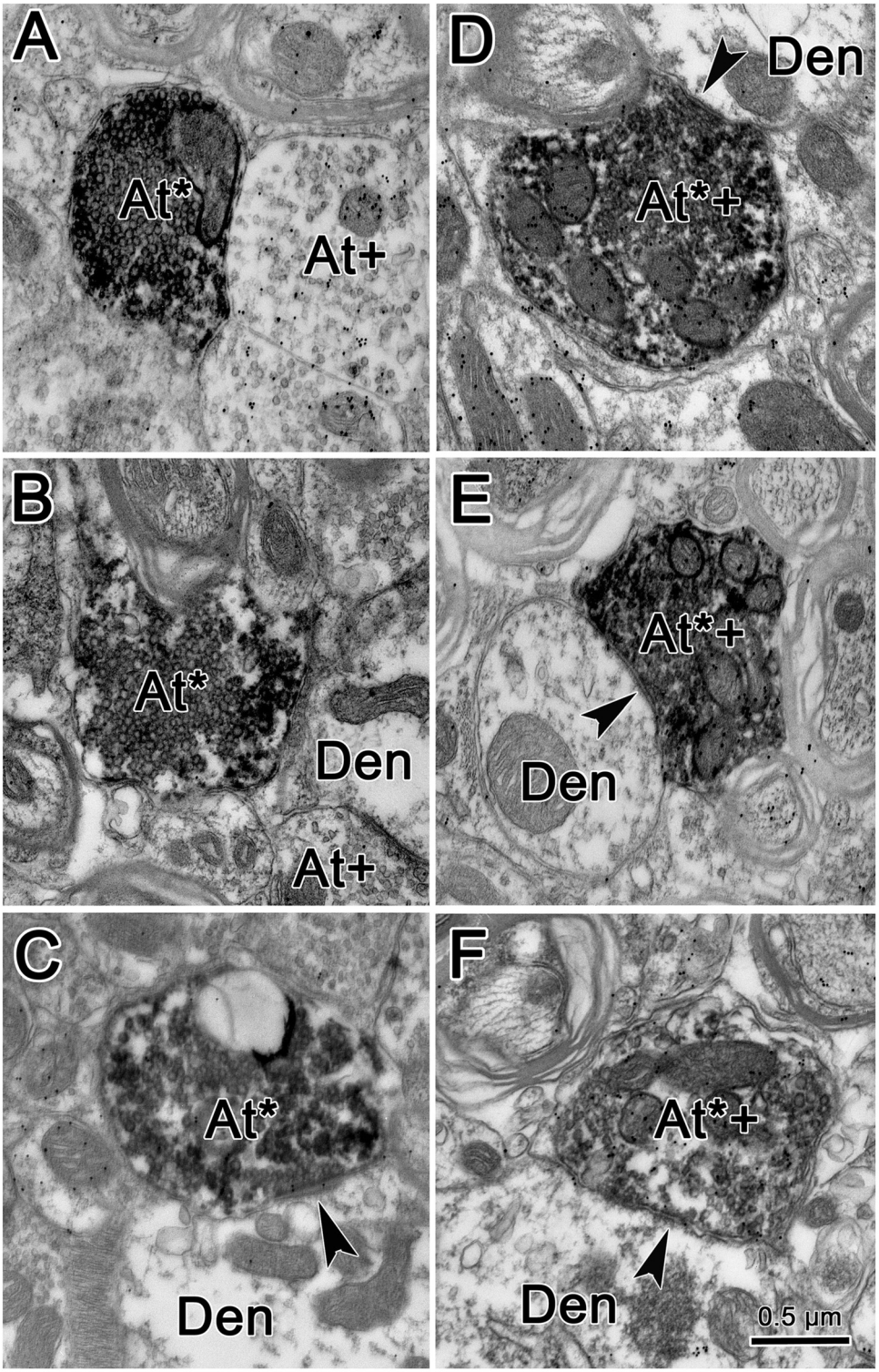

FIGURE 8 | Ultrastructure of reticuloreticular axon terminals. Axon terminals that were labeled with BDA (At*) following the injection of the cMRF shown in **Figure 7** were electron dense. Most of these terminals contacted (arrowheads) small dendrites (Den). These labeled terminals were heterogeneous: some were packed with clear spherical vesicles **(A–C)** and displayed asymmetric synaptic densities **(C)**. Others contained pleomorphic vesicles and made symmetric contacts **(D–F)**. Postembedding immunohistochemistry for gamma-aminobutyric acid (GABA) labeled a portion of the BDA labeled axon terminals (At^{*+}), as well as some terminals not labeled with BDA (At^{+}). The BDA labeled terminals that were overlain with numerous gold particles indicating they were GABAergic contained pleomorphic vesicles and made symmetric synaptic contacts **(D–F)**.

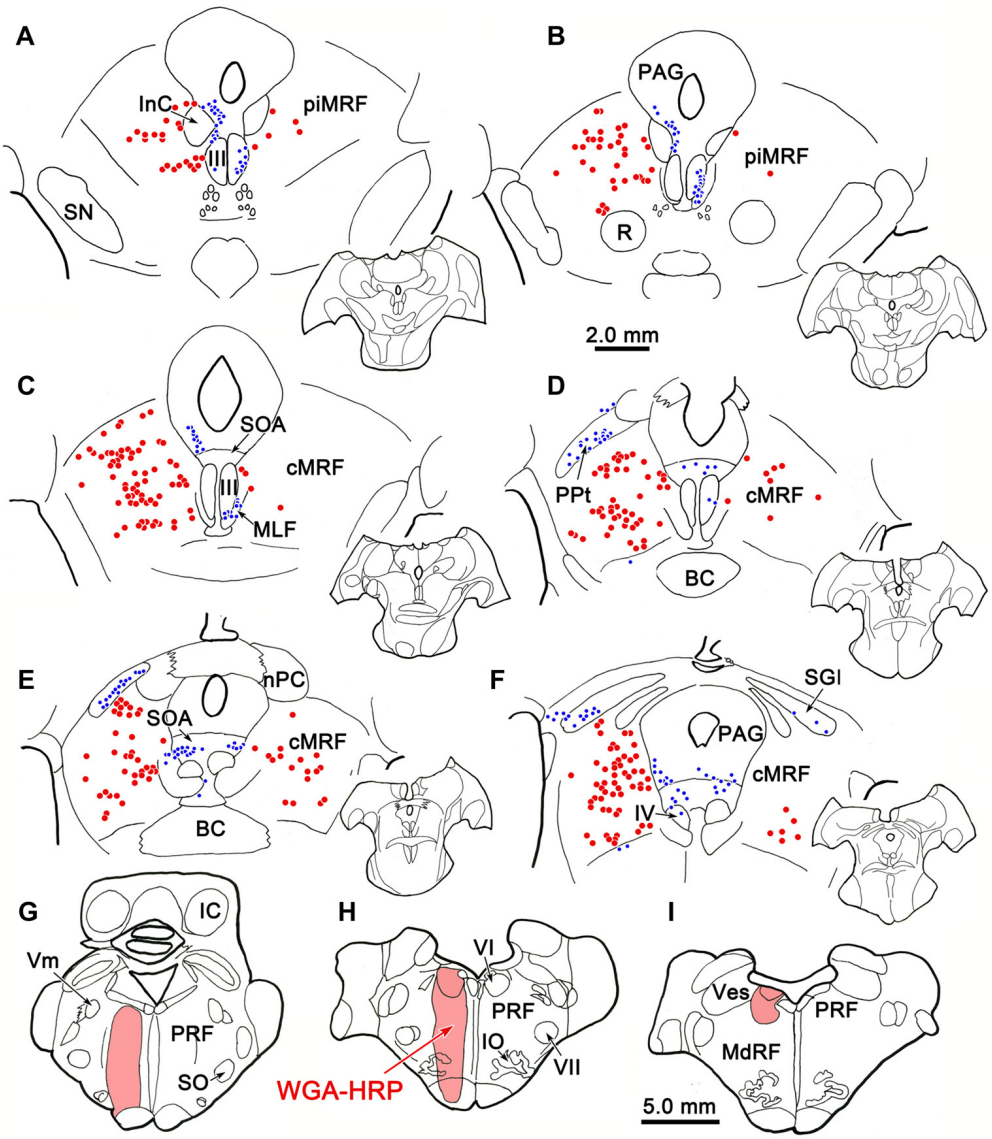

FIGURE 9 | Distribution of midbrain reticuloreticular neurons charted on a rostral to caudal series of sections. An injection of wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) centered in the PRF **(G–I)** labeled reticuloreticular neurons (red dots) in the cMRF **(C–F)** and the piMRF **(A,B)**, as well as other midbrain cells (blue dots). Note the primarily ipsilateral distribution. Adjacent insets show the level of the selected sections.

of the labeled reticuloreticular cells exhibited an obvious overlap with the distribution of BDA labeled tectoreticular terminals. Other labeled neurons from the WGA-HRP injection (small black diamonds) were also observed in the PAG, nPC, III and SOA (**Figures 11A–F**). A few WGA-HRP labeled neurons were also seen in the contralateral cMRF (not illustrated).

Examples of WGA-HRP labeled cells that were associated with BDA labeled terminals are illustrated in **Figure 12**. Their distribution in the cMRF is demonstrated in **Figure 12A**. These reticuloreticular neurons are multipolar neurons that possess three or more primary dendrites extending from their somata. Most were on the small side (10–20 μm for long axis). The BDA labeled terminals arbors consisted of thin fibers interrupted at numerous points by varicosities (boutons) of various sizes. These varicosities were seen in close association (arrowheads) with the somata (Cells B, C, E, F, H, I), as well as the proximal dendrites (Cells B–J), of the labeled reticuloreticular neurons. The close associations between the labeled boutons and neurons suggest synaptic contact, although some of the boutons sat above or below the labeled cell, and so were clearly in contact with other elements. It should be noted that some cells received only a few contacts (Cell D) and some received none at all (not illustrated).

High magnification photomicrographs (**Figure 13**) further demonstrate the relationship between the brown, WGA-HRP labeled reticuloreticular neurons and the black, BDA labeled tectoreticular axon terminals. The terminal boutons appear as beads on a string in the neuropil. These varicosities display close associations (arrowheads) with both the somata and dendrites of labeled reticuloreticular cells, although most are associated with unlabeled elements in the neuropil (**Figures 13A–E**). For comparison, **Figure 13F** shows an example of anterogradely labeled terminal boutons in close association with a reticulotectal neuron in cMRF that was retrogradely labeled from the BDA injection in the SC. The reticulotectal neurons were often larger than the reticuloreticular neurons. Furthermore, they were heavily invested with tectoreticular terminals that ran along their dendrites. Note that due to their color, the two classes of retrogradely labeled neuron could be easily discriminated.

DISCUSSION

The results of this study demonstrated that the descending reticuloreticular projections of the cMRF in *M. fascicularis* monkeys are concentrated in the PRF, where they display an ipsilateral predominance. The terminal field is densest in a paramedian position over the horizontal gaze center. By using GABA postembedding immunohistochemistry, it was revealed that cMRF sends both GABA$^+$ and GABA$^-$ ipsilateral projections to the region containing presaccadic, medium-lead burst neurons. These terminal types differ in their ultrastructure. The majority are GABA$^-$ terminals that contain densely packed, spherical vesicles and make asymmetric contacts suggestive of excitatory input, while the minority are GABA$^+$ terminals that have more dispersed, pleomorphic vesicles, and make symmetric contacts suggestive of inhibitory input. In the cMRF, the reticuloreticular cells that provide this output to the PPRF are relatively smaller, compared to reticulotectal cells. Close associations between BDA labeled tectoreticular terminals and some of these reticuloreticular cells suggest direct synaptic input. Thus, they are in a position to provide a conduit whereby tectal signals can gain access to the pontine gaze centers.

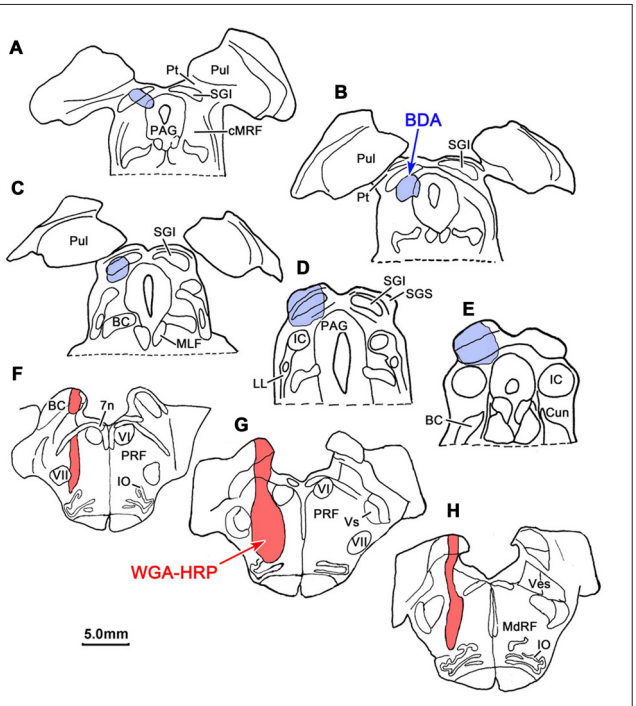

FIGURE 10 | Injection sites for a dual tracer case indicated on a rostral to caudal series of sections. BDA was injected into the superior colliculus (SC; Blue area in **A–E**) and WGA-HRP was injected into the lateral PRF (Red area in **F–H**).

The circuits that have been demonstrated by the present results and our previous work (Chen and May, 2000; Zhou et al., 2008; Wang et al., 2010) are illustrated in **Figure 14**. The SC projects to burst neurons in the contralateral horizontal gaze center to direct contraversive saccades. It also provides collaterals to the ipsilateral cMRF where neurons increase their firing for contraversive saccades and decrease their firing for ipsiversive saccades. The cMRF provides bilateral feedback to the SC. In addition, we have shown that it provides a crossed projection to the horizontal gaze center that is presumably excitatory and initiates a contraversive saccade. Within the cMRF, the tectal inputs targets two populations of ipsilaterally projecting reticuloreticular neurons. The inhibitory ipsilateral projection presumably silences the ipsilateral burst neurons during a contraversive saccade. The possible roles of the excitatory ipsilateral projection will be discussed below.

Technical Considerations

One advantage of this study is that it utilized two different tracers to reveal the cMRF's efferents, and showed labeled terminals in the same target structures. While false positive labeling due to tracer spread into areas outside the cMRF, particularly the pretectum, is possible, cross-case analysis showed that injections that did not involve these regions still produced the same basic pattern of terminal labeling. Labeling of axons of passage could also produce false positive labeling, as crossed tectobulbar projections (predorsal bundle axons) travel through the medial aspect of the cMRF. This does appear to have occurred with the BDA injections, as iontophoretic injections of PhaL, which produce little fiber of passage uptake, did not label the contralateral, paramedian axons (predorsal bundle) in the pons. Nevertheless, contralateral terminal fields were still present with PhaL injections.

In a postembedding immunohistochemical study, it is always possible that false positive and false negative labeling may be present. However, the fact that the antibody label was generally associated with a specific ultrastructural pattern that is characteristic of inhibitory synapses, and did not overlay terminals whose ultrastructural characteristics are generally associated with excitatory synapses, strongly suggests that our conclusion that the ipsilateral reticuloreticular projection to the pons is not exclusively inhibitory is correct. We found that all the targets of these cMRF terminals were GABA$^-$, which

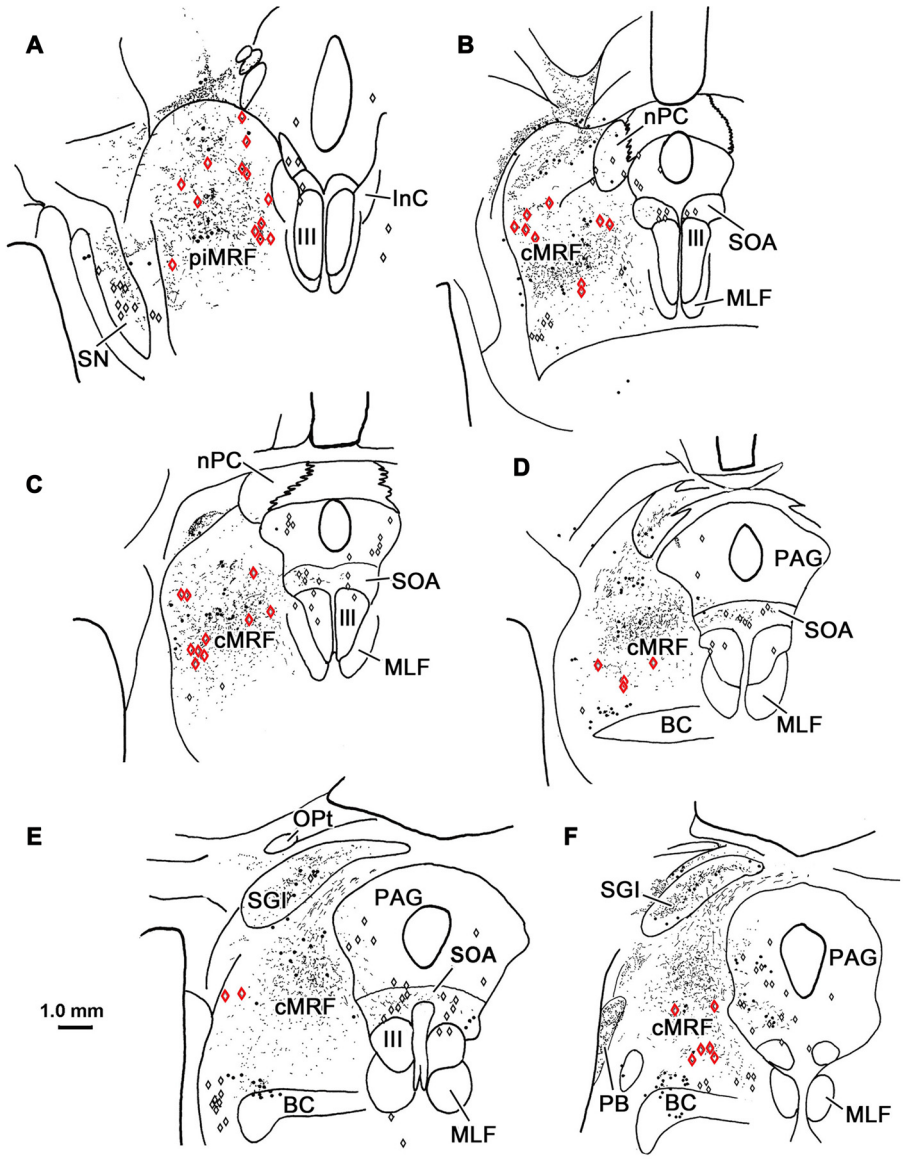

FIGURE 11 | Overlapping distribution of tectoreticular terminals and reticuloreticular neurons. The distribution of tectal terminals (stipple) labeled by BDA and reticuloreticular neurons (red diamonds) labeled by WGA-HRP that resulted from the injections illustrated in **Figure 10** are shown for the ipsilateral midbrain reticular formation (MRF). Note the overlap in their distributions within the cMRF **(B–F)** and the piMRF **(A)**. The reticuloreticular cells were scattered amongst BDA labeled reticulotectal neurons (black dots) in the cMRF. Other WGA-HRP labeled cells are indicated by black diamonds.

suggests that cMRF axons contact cells other than GABAergic interneurons. This is not surprising, as IBNs use glycine as a transmitter (Spencer et al., 1989). Therefore, the targets of cMRF terminals in PPRF are very likely to be presaccadic burst neurons. We cannot tell from our results whether these target cells are LLBNs, EBNs or IBNs.

In the dual tracer experiments, the WGA-HRP injections of the pons spread into the MdRF. However, the reticuloreticular cells that supply the MdRF are restricted to the medial cMRF (Perkins et al., 2009), and we did not see this cell distribution pattern in the present study. These retrograde results support the presence and the degree of laterality of the reticuloreticular projection from the cMRF observed anterogradely. The injections of the SC, were relatively discrete, and so were unlikely to have produced false positive terminal labeling. In these experiments, we observed close associations between anterogradely labeled tectoreticular terminals and retrogradely labeled reticuloreticular neurons in the cMRF at the light microscopic level. While these close associations suggest synaptic contact, this assertion remains to be confirmed by ultrastructural analysis. Unfortunately, the collicular injections were too large to indicate any topography with respect to cMRF

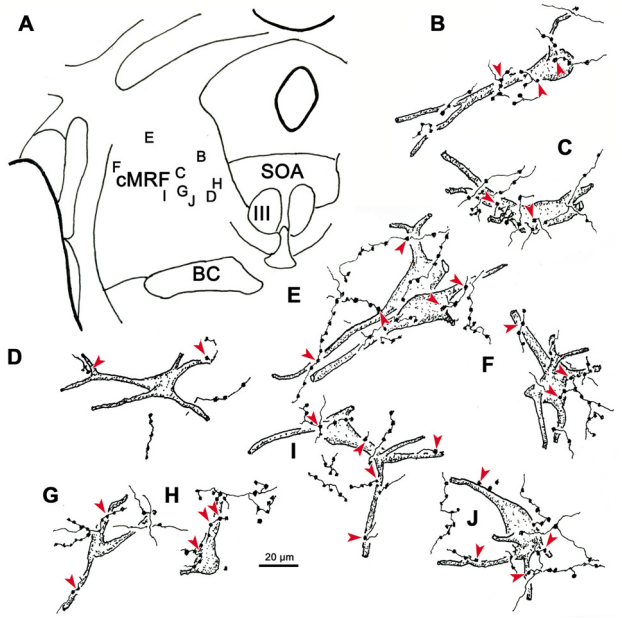

FIGURE 12 | The relationship between tectal inputs and reticuloreticular neurons in the cMRF. Drawings reveal the presence of close associations (arrowheads) between BDA labeled tectoreticular terminals and reticuloreticular cells **(B–J)** labeled from a PRF injection of WGA-HRP. The location of the example cells within the cMRF is shown in **(A)**. While a number of close associations are present, the labeled axons rarely **(E,H)** follow the contours of the cell, and the number of contacts varied.

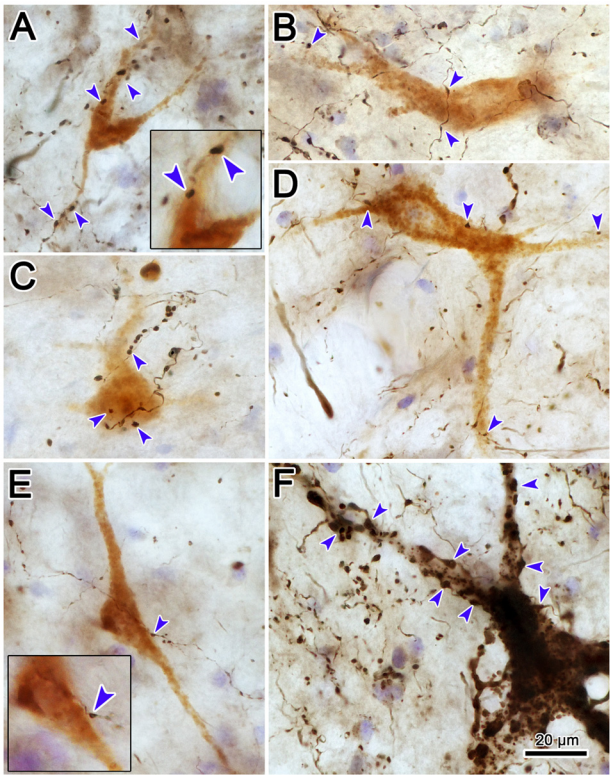

FIGURE 13 | Pattern of contacts between tectoreticular terminals and cMRF cells. Photomicrographs show examples of close associations (arrowheads) between brown, retrogradely labeled reticuloreticular neurons and black, anterogradely labeled tectoreticular terminals **(A–E)**. Individual axons display one or two boutons associated with the cell in most **(B,D,E)**, but not all **(A,C)**, cases. In contrast, black, BDA labeled tectoreticular terminals line up along the dendrites of the black, BDA labeled reticulotectal neurons in the same case **(F)**.

projections, such as has been reported by other means (Cohen et al., 1985).

Comparison to Previous Findings

Similar to the pattern of anterograde label in the present study, Edwards (1975) found ^{3}H-leucine labeled terminals in nRTP and in the rostral and caudal portions of the PRF after injecting tritiated amino acids into the Cun in cats. His Cun included the region termed the cMRF here. However, the projection observed in cats was more evenly bilateral than observed in the present findings. A fluorescent retrograde tracer study of inputs to feline PRF has also shown a stronger contralateral projection than we have seen in the present study (Perkins et al., 2009). On the other hand, Cowie and Robinson (1994) reported a primarily ipsilateral cMRF distribution in monkeys after injecting the rostral medullary reticular formation, similar to the present findings. Thus, there appears to be a species difference in the degree of laterality of the cMRF projection. Although there are differences in the orienting movements of the cat and macaque (Guitton et al., 1990; Fuller, 1992), a specific explanation for this difference in laterality must await a better understanding of the role of the ipsilateral reticuloreticular projection of the cMRF. We also illustrated a sparse, bilateral projection to the abducens nucleus, which we previously described (Bohlen et al., 2016). This projection was not seen in the cat (Edwards, 1975), most likely for technical reasons. When Ugolini et al. (2006) injected the retrograde transneuronal tracer, rabies virus, into the lateral rectus muscle of monkeys, they also found transneuronally labeled neurons in the medial portion of caudal cMRF, supporting our finding that the cMRF projects directly to abducens motoneurons.

The results of these experiments may also speak to the question of the relative importance of the feed forward and feedback circuits in cMRF function. Comparison of the numbers of labeled reticulotectal, reticulopontine and reticulomedullary neurons in the cMRF show consistent differences (Chen and May, 2000; Warren et al., 2008; Perkins et al., 2009, 2014; Wang et al., 2010, 2013; Present Results). In cats, reticulotectal neurons outnumbered the cells with descending axons (Perkins et al., 2014) and the reticuloreticular projection seen here in monkeys is less intense than the cMRF's projections to the ipsilateral SC (**Figures 1**, **4**, **9**; Zhou et al., 2008; Wang et al., 2010). In addition, cMRF terminals tended to have a distal location on the dendritic tree of PRF neurons. Thus, the feedback signal may be more robust than the feed forward signal, and perhaps more central to cMRF function. An additional difference was noted here. The number of tectal terminals contacting individual cMRF neurons varied with respect to the target of the neuron. Specifically, the reticulotectal neurons

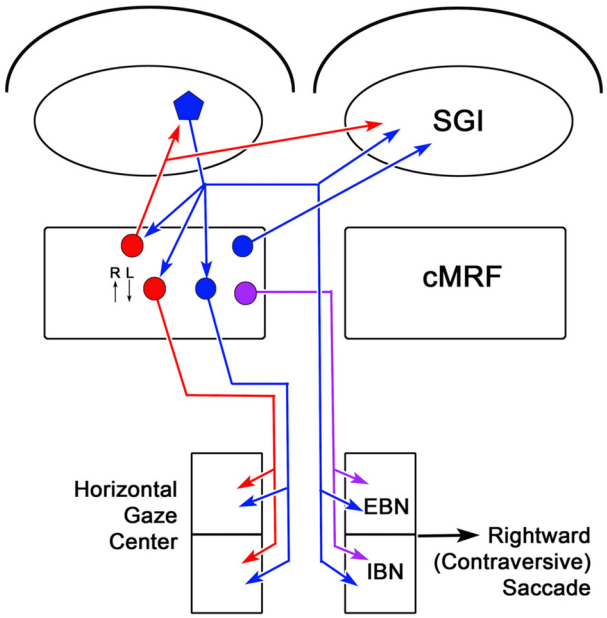

FIGURE 14 | Circuit diagram showing the cMRF as a conduit for collicular influence on lower brainstem centers. Connections from the left side of the midbrain that would produce rightward (contraversive) saccades are shown. Neurons located in the cMRF receive direct input from the SGI of the SC and forward a signal to the horizontal gaze center, the brainstem center that controls horizontal saccadic eye movements. The horizontal gaze center contains both excitatory burst neurons (EBNs) and inhibitory burst neurons (IBNs) that are premotor cells projecting to the ipsilateral and contralateral abducens nucleus, respectively. Axons of collicular neurons (blue pentagon) that travel via the predorsal bundle, provide collaterals to the ipsilateral cMRF before crossing and terminating in the horizontal gaze center. The cMRF contains both inhibitory (red circle) and excitatory (blue circle) cells that project to the ipsilateral horizontal gaze center. In addition, it contains a population of cells (purple circle) that project to the contralateral horizontal gaze center. The cMRF also provides feedback to the SC, which is purely inhibitory (red circle), ipsilaterally, and both inhibitory and excitatory (red and blue circles, respectively), contralaterally. The activity of cMRF neurons for rightward (R) and leftward (L) saccades is indicated by small arrows.

had a far greater number of tectoreticular terminal associations (**Figure 13E**; Chen and May, 2000), when compared to the reticuloreticular neurons (**Figures 12**, **13A–D**). The extensive tectal input onto cMRF reticulotectal neurons suggests that their physiologic characteristics are largely controlled by inputs from predorsal bundle axons. This anatomical finding correlates with the physiological descriptions of these neurons given by Moschovakis et al. (1988a), who suggested there was little difference between the firing characteristics of these cMRF cells and tectal LLBNs. In contrast, the more limited input observed on cMRF reticuloreticular neuron suggests that while the collicular input influences these cells, it is not nearly so dominant an input as that targeting the feedback circuit.

The Contralateral cMRF-PPRF Projection

The PPRF is a primary target of the descending predorsal bundle axons from the SC (cat: Cowie and Holstege, 1992; monkey: Harting, 1977; Basso and May, 2017). In the present study, we have demonstrated that the horizontal gaze center is also targeted by the cMRF in monkeys. The terminations are more prevalent rostrally, which is where EBNs are more common, compared to caudally, where IBNs are more common. Since the SC provides the major input to the cMRF via collaterals of the predorsal bundle axons (Harting, 1977; Grantyn and Grantyn, 1982; Moschovakis et al., 1988a), then it is likely that it targets reticuloreticular neurons in the cMRF. Thus, the presence of a crossed cMRF projection to the PPRF, as shown here, supports the existence of a crossed tectoreticuloreticular pathway that traverses the cMRF, as proposed by Waitzman et al. (1996). They suggested that this pathway might help produce the spatiotemporal transformation needed to change the topographic code of the SC into the firing rate code of medium lead burst neurons. Further studies demonstrated that the firing of some cMRF cells do appear to represent a partial transformation of the collicular signal (Cromer and Waitzman, 2006, 2007). On the other hand, the cMRF terminals we observed often had a relatively distal distribution on pontine reticular neurons, suggesting a modulatory, as opposed to a driving influence central to the spatiotemporal transformation. Indeed, the function of this trans-cMRF pathway from the SC to the PPRF is not entirely clear. Muscimol inactivation of the cMRF, which leaves the collicular projections to the PPRF intact, but eliminates the trans-cMRF pathways, leads to hypermetric contralateral horizontal saccades (Waitzman et al., 2000b). This is not necessarily what one would expect if the cMRF is a key factor in the spatiotemporal transformation and if this effect is due to loss of downstream projections of the cMRF. Of course, the hypermetric saccades might be due to loss of feedback projections to the SC, although stimulation of the cMRF in animals whose ipsilateral SC has been ablated still produces horizontal eye movements, indicating that pathways from the cMRF are capable of inducing eye movements without any contribution of cMRF-SC circuits (Cohen et al., 1986; Luque et al., 2006).

The Ipsilateral cMRF-PPRF Projection

It is interesting that the cMRF projection in the monkey mirrors the tectal projection, with the cMRF terminal field being predominantly ipsilateral and the collicular terminal field being predominantly contralateral (**Figure 14**). There is evidence that pathways from the ipsilateral SC influence activity observed in horizontal gaze center neurons, specifically IBNs (Strassman et al., 1986b; Sugiuchi et al., 2005; Takahashi et al., 2005). However, the authors of these articles ascribe most of these effects to tectotectal interactions, not tectoreticular pathways. If the collicular and cMRF pathways work together in a push-pull manner while producing a contraversive saccade, then the ipsilateral cMRF projection should be inhibitory, as the left PPRF is generally silent for a rightward movement, while the right PPRF is active (Hepp and Henn, 1983; Strassman et al., 1986a). This hypothesis is based on the fact that electrical stimulation of the cMRF in the monkey produces contraversive horizontal saccades (Cohen et al., 1985). Furthermore, when Cromer and Waitzman (2007) recorded the presaccadic responses of cMRF neurons, they found neurons that fired in association

with contraversive horizontal saccades. Our observation of a $GABA^+$ component among the cMRF axons that terminate in the ipsilateral PPRF is consistent with this directional preference. Many of the neurons recorded by Waitzman et al. (1996) showed a fairly high tonic firing rate, which was silenced by ipsiversive eye movements. If these cells represent the ipsilaterally projecting GABAergic population observed here, they could inhibit the activity of burst neurons in the ipsilateral PPRF during contraversive eye movements and so help to eliminate activity in antagonist eye muscles. This GABAergic input would be turned off for ipsiversive eye movements when this side of the PPRF would be activated.

Not all the findings are consistent with this push-pull hypothesis. Specifically, the ipsilateral projection is heterogeneous ($GABA^+$ and $GABA^-$), instead of purely inhibitory, as would be expected if the sole function of this projection was to drive contraversive eye movements. While the presence of an excitatory ipsilateral cMRF-PPRF projection could help to explain why inactivation of the cMRF also affected ipsiversive saccades, making them hypometric (Waitzman et al., 2000a), it is not clear what the role of this excitatory ipsilateral pathway is.

One possibility is that cMRF saccadic burst neurons are not the actual target of this excitatory ipsilateral projection. Pathmanathan et al. (2006a,b) described "postsaccadic" cMRF neurons, characterized by firing after gaze shift onset or at the end of the gaze shift. In head-free animals, the firing of these postsaccadic cMRF neurons was found to be most closely associated with head movements. In fact, the cMRF has mainly ipsilateral projections to the medullary reticular formation and spinal cord that presumably affect head movements (Warren et al., 2008; Perkins et al., 2009). Perhaps the $GABA^-$ cMRF terminals found in the PPRF in the present study actually contact the dendrites of reticulospinal neurons. If this is true, the excitatory ipsilateral reticuloreticular projections could be there to adjust the activity of reticulospinal neurons that act to brake the movement of the head or maintain head position (Corneil et al., 2001; Perkins et al., 2009).

Another possibility comes from consideration of the fact that the heterogeneous pattern of cMRF termination in the ipsilateral PPRF resembles the pattern of cMRF termination in the contralateral SC (**Figure 14**; Wang et al., 2010). Both structures have been presumed to be silent during the production of saccades in the off direction. Perhaps, the excitatory ipsilateral cMRF projection to the PPRF, like the crossed cMRF reticulotectal projection, may function in organizing complex saccadic behaviors. Mays and Sparks (1980) utilized two targets to trigger sequential saccades. When they arranged the target sequence to produce a leftward and then a rightward saccade, the activity corresponding to the rightward saccade was in the left SGI, even though the visual activity all occurred in the right superficial gray layer. If the movement of the activity into the other side of the SC is transmitted by the crossed excitatory reticulotectal projection of the cMRF, direct excitation to the ipsilateral PPRF by the cMRF may be assisting the downstream effects of the crossed tectoreticular projection during such saccade sequences.

A third possibility that must be considered in light of the dominant, excitatory projection to the ipsilateral PRF is the idea that the cMRF supports behaviors other than orienting saccades. The SC is known to also help direct avoidance movements through its ipsilateral descending projections (Ingle, 1983; Dean et al., 1986; Ellard and Goodale, 1986; Furigo et al., 2010; Comoli et al., 2012). Perhaps the cMRF also takes part in directing the eyes and head away from threats. In this case, the ipsilateral cMRF projections would parallel the ipsilateral descending collicular projections. In effect, they would provide a pathway for supporting the spatiotemporal transformation in an avoidance movement, instead of an orienting movement. Arguing against this idea is the fact that Comoli et al. (2012) saw relatively few terminals in the MRF following collicular injections into the region associated with avoidance in rats.

A final possibility is suggested by recent findings that indicate that abducens motoneurons do not always act in a manner that can be predicted by a purely antagonistic and purely conjugate model. Indeed, there is evidence that some extraocular motoneurons appear to display signals that are better related to the contralateral eye (Zhou and King, 1998; Van Horn and Cullen, 2009). The mixed ipsilateral projections found in this study might help to explain these signals. These investigators have also provided evidence that medium lead burst neuron activity is also not purely predicted by conjugate movement models. During disjunctive saccades between targets that lie at different distances from the observer, the activity of an individual burst neuron in the left PPRF can be best correlated to the action of either the left or right eye (Zhou and King, 1998; Van Horn et al., 2008). Similar eye-specific signals have been observed on saccade-related cells in the cMRF (Waitzman et al., 2010). We have recently reported projections to the SOA that terminate on neurons in the Edinger-Westphal nucleus and medial rectus motoneurons in the C-group in a pattern that is consistent with the idea that the cMRF plays a role in disjunctive saccades, where each eye is independently directed to compensate for target distance (Bohlen et al., 2016, 2017; May et al., 2016). Thus, the heterogeneous cMRF projection to the ipsilateral PPRF may be part of this same system that modulates burst neuron activity during disjunctive saccades. In this light, it would be worth investigating whether the crossed cMRF projection also displays both GABAergic and non-GABAergic components.

In summary, the cMRF provides both excitatory and inhibitory inputs to burst neurons in the pontine gaze centers. Compared to the SC, its descending projections to the contralateral PPRF are less robust, but its ipsilateral projections are much stronger. The heterogeneity of the ipsilateral projection and the distribution of tectoreticular inputs on reticuloreticular neurons suggest that saccadic signals are not just relayed through the SC-cMRF-PPRF pathway. Instead, cMRF may modify the activity of presaccadic burst neurons on both the ipsilateral and contralateral side in a complex pattern. Considering the fact that the cMRF exerts its influence via both $GABA^+$ and $GABA^-$ terminals and has both ipsilateral and contralateral components, it is likely that it springs from several different cMRF cell populations that display various target-specific

patterns of gaze-related activity. These should be a target of further investigation if we are to have a better idea of the role of the cMRF in gaze.

AUTHOR CONTRIBUTIONS

PJM: study concept and design; critical revision of the manuscript. NW, LZ and EP: acquisition of data; drafting the manuscript. NW, LZ, EP, SW and PJM: analysis and interpretation of the data. PJM and SW: obtaining funding; study supervision. All authors had full and open access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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frontiers in Neuroanatomy

ORIGINAL RESEARCH
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Short-Term Effects of Chewing on Task Performance and Task-Induced Mydriasis: Trigeminal Influence on the Arousal Systems

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Trigeminal input to the ascending activating system is important for the maintenance of arousal and may affect the discharge of the noradrenergic neurons of the *locus coeruleus* (LC), whose activity influences both vigilance state and pupil size, inducing mydriasis. For this reason, pupil size evaluation is now considered an indicator of LC activity. Since mastication activates trigeminal afferent neurons, the aims of the present study, conducted on healthy adult participants, were to investigate whether chewing a bolus of different hardness may: (1) differentially affect the performance on a cognitive task (consisting in the retrieval of specific target numbers within numerical matrices) and (2) increase the dilatation of the pupil (mydriasis) induced by a haptic task, suggesting a change in LC activation. Results show that chewing significantly increased both the velocity of number retrieval (without affecting the number of errors) and the mydriasis associated with the haptic task, whereas simple task repetition did not modify either retrieval or mydriasis. Handgrip exercise, instead, significantly decreased both parameters. Effects were significantly stronger and longer lasting when subjects chewed hard pellets. Finally, chewing-induced improvements in performance and changes in mydriasis were positively correlated, which suggests that trigeminal signals enhanced by chewing may boost the cognitive performance by increasing LC activity.

Keywords: chewing, trigeminal input, locus coeruleus, mydriasis, cognitive performance

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INTRODUCTION

Earlier experiments have shown that trigeminal signals play a particularly important role in the control of cortical desynchronization and arousal (Roger et al., 1956). This can be accounted for by the influence exerted by trigeminal afferents on the Ascending Reticular Activating System (ARAS). Trigeminal primary and/or secondary afferents reach the pontomedullary reticular formation (McKinley and Magoun, 1942), the noradrenergic neurons of the locus coeruleus (LC) (Cedarbaum and Aghajanian, 1978; Luo et al., 1991; Craig, 1992; Couto et al., 2006), the cholinergic neurons located within the pedunculopontine and the laterodorsal tegmental nucleus (Semba and Fibiger, 1992), the tuberomammillary hypothalamic histaminergic neurons

(Fujise et al., 1998; Sakata et al., 2003) and the thalamic non-specific intralaminar and midline nuclei (Krout et al., 2002; Clascá et al., 2012). Although it has been claimed that mesencephalic trigeminal neurons (see Wang and May, 2008) are electrotonically coupled to LC neurons (Matsuo et al., 2015), there is at present no evidence of this statement, beyond a Fluorogold labeling passage from mesencephalic trigeminal neurons to the LC area (Fujita et al., 2012), which only implies the possibility of such a speculation. LC neurons modulate arousal (Samuels and Szabadi, 2008; Carter et al., 2010), sensorimotor excitability (Matsutani et al., 2000) and provide an essential contribution to arousal-associated (Bradshaw, 1967) pupil mydriasis (Gabay et al., 2011), which reflects "mental efforts" (Hess and Polt, 1964) and task performance (Rajkowski et al., 1993). In fact, LC controls the preganglionic parasympathetic neurons of the Edinger–Westphal nucleus (Breen et al., 1983), which innervate the iris constrictor, inhibiting their discharge through an α2-mediated mechanism (Szabadi and Bradshaw, 1996; Samuels and Szabadi, 2008). Such inhibition is necessary to increase the pupil size, since the tonic activity of the iris constrictor would prevent pupil enlargement by dilatator iris (Wilhelm et al., 2011). As a consequence, the LC neurons activity is strongly and positively correlated with the pupil size, both in animals (Rajkowski et al., 1993, 1994; Joshi et al., 2016) and humans (Alnaes et al., 2014; Murphy et al., 2014). For this reason, several studies have utilized the pupil size as an index of LC activity (Silvetti et al., 2013; Hoffing and Seitz, 2015; Kihara et al., 2015; Hayes and Petrov, 2016; see also Laeng et al., 2013). The connection of the trigeminal system with the ARAS and LC suggests that the modifications of trigeminal input occurring during chewing may induce relevant changes in the whole brain, leading to an enhancement in the arousal/alertness level and, as a consequence, in the cognitive performance (Sakamoto et al., 2009).

Mastication is driven by a central pattern generator (Dellow and Lund, 1971), controlled by oral sensory feedback (Appenteng et al., 1982; Lund, 1991), according to the changes in the consistency and texture of the food bolus (Horio and Kawamura, 1989). Masticatory muscle activation (Peyron et al., 2002), together with feedback signals from periodontal receptors and muscle spindles (Lavigne et al., 1987), increases with food hardness and influences brain function. In fact, animals submitted to long-term soft-diet feeding undergo a decrease in learning and memory (Tsutsui et al., 2007; Weijenberg et al., 2013), whereas mastication prevents the degradation of brain functions (Gatz et al., 2006; Okamoto et al., 2010; Ohkubo et al., 2013). Moreover, animal models showed that bilateral molar extractions, leading to long-term masticatory dysfunction, decrease the number of pyramidal cells in the hippocampal CA1 and gyrus dentatus (Oue et al., 2013), with impairment of spatial learning and memory in water maze tests (Kato et al., 1997). These deficits increase with aging and time after teeth loss (Onozuka et al., 2000). On top, teeth loss increases the proliferation and the hypertrophy of the astrocytes within the hippocampus, as it occurs following neuronal degeneration and senescence processes (Onozuka et al., 2000), while decreasing c-Fos expression during spatial task (Watanabe et al., 2002), dendritic spines density (Kubo et al., 2005) and neurogenesis (Aoki et al., 2010).

In humans, beyond the trophic, long-term effects that chewing exerts on the brain (Gatz et al., 2006; Okamoto et al., 2010), gum chewing improves cognitive processing speed (Hirano et al., 2013), alertness (Allen and Smith, 2012; Johnson et al., 2012), attention (Tucha et al., 2004), memory and learning (Allen et al., 2008; Smith, 2009); it also reduces reaction times (Hirano and Onozuka, 2014) and event-related potentials latencies in an auditory oddball paradigm (Sakamoto et al., 2009). Moreover, shortening of the visual reaction time in a button press task following chewing (Hirano and Onozuka, 2014) was found associated with an increase of the blood-oxygen-level dependent signal in the anterior cingulate, left frontal gyrus and motor related regions. On the other hand, chewing-induced improvement of short-term memory processing was coupled to an increased blood flow in the middle frontal gyrus of the dorsolateral prefrontal cortex, the right premotor cortex, precuneus, thalamus, hippocampus and inferior parietal lobe (Hirano et al., 2008).

Chewing-induced advantages in cognitive performance were observed following, but not during chewing bouts for a time period of 15–20 min (Onyper et al., 2011). The consistency of the chewed gum pellet influences chewing-induced performance changes. In fact, at variance with an ordinary chewing gum, a soft chewing gum like bubble gum did not improve memory (Davidson, 2011). On the other hand, the prolonged chewing of a hard gum significantly increased the fatigue of the masticatory muscles, blunting cognitive performance (Farella et al., 2001).

Chewing effects on cognitive performance and arousal could be related to trigeminal action on different ARAS components. More specifically, the involvement of the LC should result in an increased task-related mydriasis, due to the coupling between LC activity and pupil dilatation, well documented by several investigations (Rajkowski et al., 1993, 1994; Alnaes et al., 2014; Murphy et al., 2014; Joshi et al., 2016). Therefore, based on the assumption of the proposed LC contribution to chewing effects on the brain, a correlation can be expected between chewing-induced changes in performance and in task-related mydriasis. Thus, the aim of the present study was to investigate whether short bouts of masticatory chewing influence (1) the velocity of retrieval of specific target numbers within numerical matrices and relative errors as well as (2) the mydriasis induced by a haptic task. Finally, we tested whether chewing induced changes in cognitive performance and mydriasis are correlated with each other.

MATERIALS AND METHODS

Subjects

This study was carried out in accordance with the recommendations of the Ethical Committee of the Pisa University. According to the Declaration of Helsinki, each subject signed an informed consent, approved by the local Ethical Committee. Experiments were performed in 30 right-handed subjects (15 females) aged between 18 and 55 years (36.3 ± 12.5),

not affected by pain in the masticatory/neck muscles and by neurological, psychiatric, metabolic or endocrine diseases.

Experimental Procedure

Subjects were asked to avoid caffeine and smoking for at least 2 h before the experimental session. Each subject underwent 4 experimental sessions (no activity, handgrip, soft pellet, hard pellet), separated by at least 24 h within 4–6 days. In each session subjects were engaged in activities lasting 2 min (soft pellet, hard pellet, handgrip) or invited to relax for 2 min, without specific instructions (no activity). Activities consisted of chewing a custom-made hard pellet, chewing a commercially available soft gum pellet, and rhythmically squeezing an anti-stress ball of 6 cm in diameter, respectively. Each subject performed all motor activities according to his/her own preferred rate, on the preferred side during the first minute and on the other side for the remaining time. When changing the side, the soft (but not the hard) pellet was discarded and a new pellet was delivered to the subject. The session order was pseudo-randomly varied among participants. In each session subjects were studied at T0 (control), T7 and T37, i.e., 5 min before the beginning, soon after and 30 min following the end of the activity/no activity period, respectively. Thus, the activity/no activity period began, in each session, 5 min after T0 and ended just before T7. Between T7 and T37 measurements, subjects were invited to relax.

Variables

All the listed variables were studied at T0, T7 and T37.

(1) Performance Index, Scanning Velocity and Error Rate in a cognitive task based on a modified version of the Spinnler-Tognoni numeric matrices test (Spinnler and Tognoni, 1987) (**Figure 1A**).

In this task, the subject seated in front of a table, where the experimenter displayed a paper sheet containing three numerical matrices of 10 lines and 10 columns. The subjects had been previously instructed to underline with a pencil the number 5 in the first matrix from the left, the numbers 6 and 8 in the second and the numbers 1, 4 and 9 in the third (**Figure 1A**). They were invited to retrieve as many target numbers as possible, scanning sequentially each matrix line. The target numbers were 60 out of 300 included within the 3 matrices. The numbers retrieved in 15 s were counted and divided by 15, thus obtaining the velocity of number retrieval, indicated as Performance Index. Moreover, we evaluated the sum of missed target numbers and non-target numbers wrongly underlined, which was divided by 15 for obtaining the Error Rate. Finally, we counted all the numbers (target and non-target) scanned and obtained the Scanning Velocity, by dividing it by 15.

The matrices presented at T0, T7 and T37 differed for the position of the target numbers, thus subjects could not benefit of previous spatial information for speeding up their performance.

(2) Pupil size at rest and during execution of a haptic task (**Figure 1B**) based on Tangram, a puzzle of triangular, square and parallelogram-shaped forms. During both rest and task measurements the subjects were seated with the head within a pupillometer. In the first case, the subjects were invited to relax. Measurements on both sides were taken. During task measurements, a piece of the puzzle (the parallelogram) was removed by the experimenter and placed in the right hand of the subjects, who had to reposition it in its original place without looking at their hand and the pupil size was monitored as soon as they began to fit the piece in its proper place. Left and right pupil measurements were collected in separate task repetitions. All the subjects performed this task for training, before the beginning of the first experimental session.

Pupil size measurements (mm) were performed in standard conditions of artificial lighting by using a corneal topographer-pupillographer (MOD i02, with chin support, CSO, Florence, Italy) made up of a standard illuminator (halogen lamp, white light), a camera sensor CCD1/3", with a 56 mm working distance. The operator monitored the iris image (**Figure 1B**) by the camera (acquisition time 33 ms). Measurements were performed in photopic conditions (40 lux) and values were displayed online on the computer screen. During pupil size evaluation (both at rest and during task) the dental arches were not in contact.

Pupillographic measurements have been previously used to indirectly track the activity of LC neurons (Silvetti et al., 2013; Hoffing and Seitz, 2015; Kihara et al., 2015; Hayes and Petrov, 2016; see also Laeng et al., 2013; De Cicco et al., 2014, 2016).

Pellets and Anti-stress Ball

The anti-stress ball squeezed by the subjects during the handgrip task (TB600 Artengo, Italy) was made by a polyurethane foam, characterized by a constant hardness (defined as the material's resistance to indentation when a static load is applied) of 30 Shore OO, where 0 and 100 Shore OO correspond to maximal and no indentation, respectively. The soft pellet consisted of a commercially available chewing gum (Vigorsol, Perfetti, Italy), with an initial hardness of about 20 Shore OO. It was white in color, made of base gum with the main taste of liquorice (2.0 cm × 1.0 cm × 0.5 cm in size). The hard pellet was instead manufactured (OCM Projects, Italy) by using a silicon rubber (gls50, Prochima, Italy) and was characterized by a reticular structure. The material had a constant hardness (60 Shore OO), unmodified during chewing. The induced deformation was approximately proportional to the muscular force applied (spring constant = 15.7 N/m) and the material quickly recovers its original shape when the pressure ended. The pellet was cylindrical in shape (1.0 cm × 1.0 cm × 1.5 cm). It was gray in color, sugar free, odor and tasteless. The pellets were self-administered and the subject had visual access to the pellet right before the administration.

Statistical Analysis (SPSS.13)

The average pupil size (left/right) at rest and during the haptic task, their difference (i.e., the task-induced mydriasis), the Performance Index, the Error Rate and the Scanning Velocity were analyzed. Correlations between variables were assessed by linear regression analysis. The effects of the different motor activities on the variables listed above at the different times were analyzed by a 4 Conditions (no activity, handgrip, soft

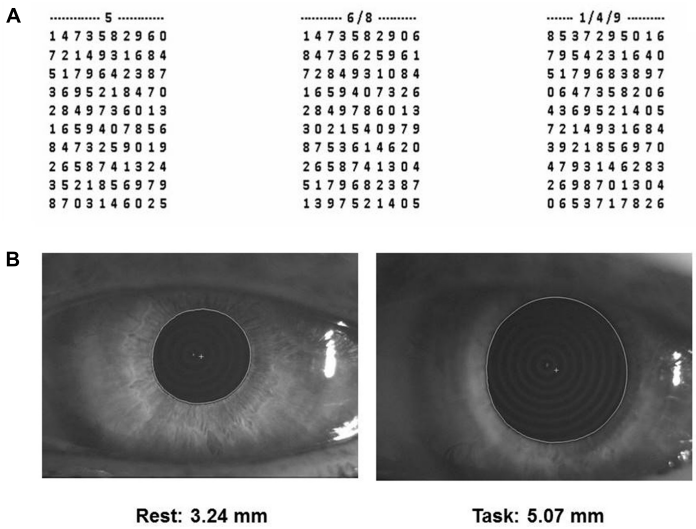

FIGURE 1 | Spinnler-Tognoni matrices and pupil size recordings. **(A)** Spinnler-Tognoni matrices. The test consists in identifying the target numbers indicated above each matrix. **(B)** Example of pupillometric recording performed at rest (left side) and during Tangram execution (right side).

pellet, hard pellet) × 3 Times (T0, T7, T37) repeated measures ANOVA, with Gender as a between-subjects factor. Since several variables showed a correlation with age, the latter was used as a covariate. The Greenhouse-Geisser ε correction was used when requested. Significance was set at $P < 0.05$. *Post hoc* comparisons were performed by paired *t*-test. In addition, the differences between T7 and T37 values with respect to T0 (ΔT7 and ΔT37, respectively) were computed for each condition and compared by paired *t*-test.

RESULTS

Correlation of Performance and Pupil Size with Age

The average values of Performance Index and Scanning Velocity recorded in control condition (T0) were negatively correlated with age (Performance Index: $r = 0.491$, $P < 0.006$, $Y = -0.02X+2.48$; Scanning Velocity: $r = 0.588$, $P < 0.001$, $Y = -0.096X+16.435$). Similar trends with comparable slope values were observed for both males and females, although, in the latter population, the correlation between Performance Index and age did not reach the significance level. Average Error Rate at T0 was not significantly correlated with age.

The T0 values of pupil size at rest and during the haptic task were highly correlated with each other ($r = 0.932$, $P < 0.0005$, $Y = 1.131X+0.901$), without differences between males and females and both of them exhibited significant negative correlation with age. Similar regression lines were observed for values obtained at rest ($r = 0.648$, $P < 0.0005$, $Y = -0.039X+5.268$) and during the haptic task ($r = 0.597$, $P < 0.001$, $Y = -0.044X+6.875$), independently of gender. The mydriasis observed during Tangram performance did not correlate with age neither in males nor in females.

Influence of Motor Activity on Spinnler-Tognoni Matrices Processing

Relevant effects and interactions of Condition, Time, Gender on Spinnler-Tognoni matrices processing are detailed in the Supplementary Table 1. In particular, significant Condition × Time interactions were observed for Scanning Velocity [$F(6,162) = 3.76$, $P < 0.002$] and Performance Index [$F(6,162) = 9.48$, $P < 0.0005$], whose decomposition can be found in the Supplementary Table 2, together with data relative to the actual number of retrieved and missed target items (Supplementary Table 3). Both variables were not significantly modified by simple test repetition neither at T7 nor at T37 with respect to T0 (**Figure 2**). They decreased significantly soon after the handgrip (T7) but not at T37 with respect to T0 (**Figure 2**). Chewing hard and soft pellet significantly increased the Scanning Velocity and Performance Index at T7. When chewing hard pellet both variables were still significantly enhanced at T37, whereas, at this time, only the Performance Index was still larger than in control condition (**Figure 2**). Analysis of ΔT7 and ΔT37 showed that the differences in Performance Index and Scanning Velocity at T7 and T37 with respect to T0 induced by chewing hard pellet were always significantly larger than those obtained by chewing soft pellet (ΔT7, $P < 0.0005$; ΔT37, $P < 0.0005$).

No Gender effects were observed. However, due to the low values of the η^2 coefficients for the Condition × Time × Gender interaction (Performance Index = 0.038, Scanning Velocity = 0.029) significant gender differences could have been present. In fact, in the handgrip condition the drop in Scanning Velocity and Performance Index observed at T7 with respect to control was observed in females (Performance Index: from 1.90 ± 0.66, SD, numb/s to 1.74 ± 0.58, $P < 0.001$; Scanning Velocity: from 13.56 ± 2.56 to 12.84 ± 2.32, SD, numb/s, $P < 0.002$), but not in males (Performance Index: from 1.56 ± 0.32 to 1.58 ± 0.30, NS; Scanning Velocity:

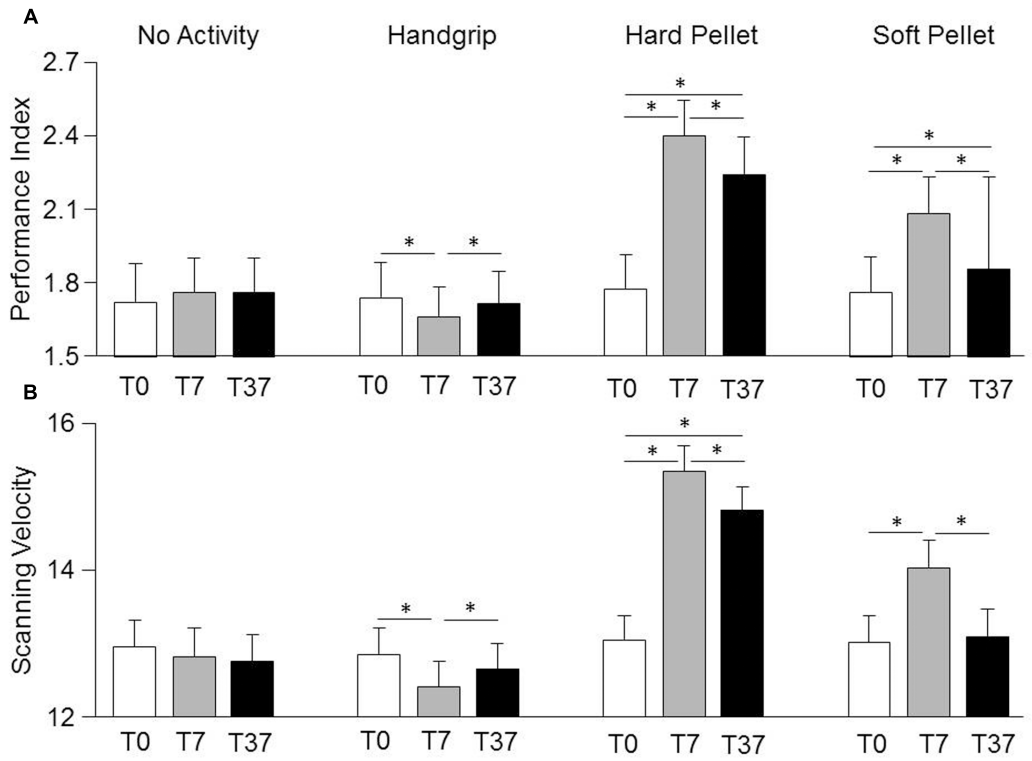

FIGURE 2 | Changes in Performance Index and Scanning Velocity induced by various sensorimotor activities. Columns represent the mean values of the Performance Index **(A)** and of the Scanning Velocity **(B)** observed in the 4 conditions (no activity, handgrip, hard pellet, soft pellet) and at the different times tested (T0, T7, T37) are represented by the height of the corresponding columns. In **(A,B)**, the error bars represent Standard Errors. Asterisks refer to significant differences between T0, T7 and T37, as indicated by the horizontal lines. See Supplementary Table 2 for details.

from 12.12 ± 1.46 to 12.00 ± 1.34, NS). At T37 no significant differences with respect to control were found in both females (Performance Index: 1.84 ± 0.62; Scanning Velocity: 13.30 ± 2.34) and males (Performance Index: 1.60 ± 0.28; Scanning Velocity: 12.00 ± 1.22). In the no activity, hard pellet and soft pellet conditions, males and females showed a similar behavior.

Only a significant Time effect was observed for the Error Rate, which decreased significantly from 0.28 ± 0.20 (T0) to 0.22 ± 0.14 (T7, $P < 0.0005$) and to 0.22 ± 0.18 (T37, $P < 0.0005$) independently from the performed activity. As shown in **Figure 3A** when data obtained at all times and conditions were pooled together, a significant correlation was found between the Performance Index and the Scanning Velocity ($r = 0.94$, $P < 0.0005$, $Y = 0.242X-1.348$). This relation was confirmed in each condition separately. On the other hand, increasing the Scanning Velocity did not significantly modify the Error Rate. Moreover, as shown in **Figure 3B**, also the pooled changes in Performance Index and Scanning Velocity obtained at T7 and T37 with respect to T0 correlated between each other ($r = 0.902$, $P < 0.005$, $Y = 0.202X+0.082$).

Independently from the Scanning Velocity, the performance accuracy indicated by the Performance Index was near maximal for all activities and times, as revealed by the saturation of percentage of retrieved targets, ranging from 99.2 ± 2.03 to 98.0 ± 2.66, for the different combinations of times and conditions.

Influence of Motor Activity on Pupil Size at Rest and during Task Execution

Condition, Time, Gender effects and their interactions on pupil size are detailed in the Supplementary Table 4. No significant effects were observed for pupil size at rest, while a significant Condition × Time interaction was observed for pupil size during the haptic task [$F(6,162) = 8.63$, $P < 0.0005$], as well as for task related mydriasis [$F(6,162) = 7.14$, $P < 0.0005$], whose decomposition can be found in the Supplementary Table 5. As shown in **Figure 4** (lower row), with respect to control (T0), pupil size during haptic task significantly increased at both T7 and T37 after chewing hard pellet, while only at T7 after chewing soft pellet (**Figure 4**, lower row). On the other hand, it decreased at both T7 and T37 after handgrip performance (**Figure 4**, upper row). No change was induced at any time by simple task repetitions (**Figure 4**, upper row). Similar results were obtained for task related mydriasis, which can be appreciated in **Figure 4** by comparing data obtained at rest and during the task (Supplementary Table 5). Task-related mydriasis exhibited also a significant Gender effect sustained by higher values in males (1.59 ± 0.40, SD, mm) with respect to

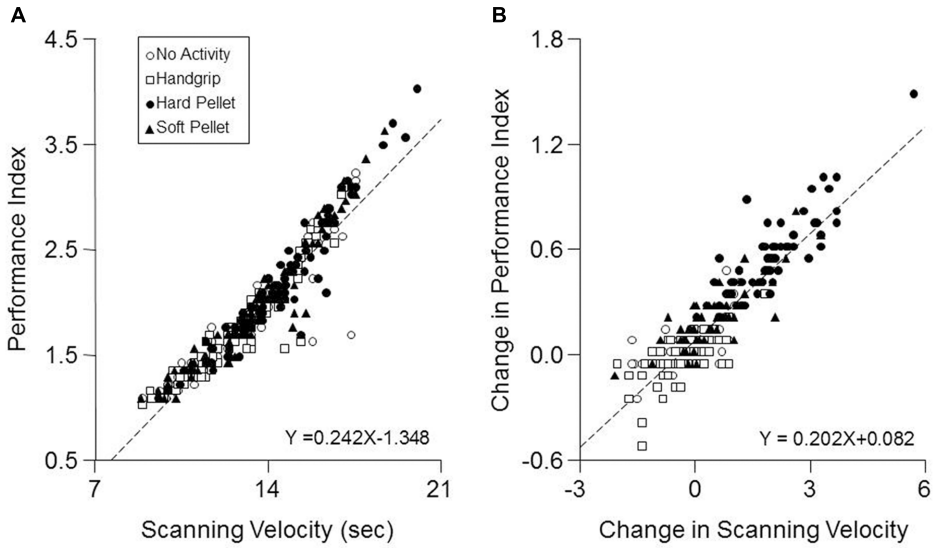

FIGURE 3 | Correlation between Performance Index and Scanning Velocity. **(A)** The values of Performance Index obtained in the different conditions and times investigated have been plotted as a function of the corresponding Scanning Velocity values. **(B)** Changes in Performance Index obtained at T7 and T37 with respect to control values (T0) have been plotted as a function of the corresponding changes in Scanning Velocity. In **(A,B)** the dashed lines are regression lines of all the data points. Open circles: no activity condition. Open squares: handgrip condition. Dots: hard pellet condition. Filled triangles: soft pellet condition.

females (1.34 ± 0.40, SD, mm), independently from times and conditions.

The changes in pupil size (during haptic task) and in task-related mydriasis observed after chewing hard pellets were significantly larger than those observed after chewing soft pellets, both at T7 ($P < 0.0005$ for both parameters) and at T37 ($P < 0.0005$ for both parameters).

Finally, as shown in **Figure 5**, a strong correlation was found between the changes in Performance Index and in Scanning Velocity induced by the various activities (at both T7 and T37) and the corresponding changes in mydriasis (Performance Index: $r = 0.688$, $P < 0.0005$, $Y = 0.638X+0.148$; Scanning Velocity: $r = 0.615$, $P < 0.0005$, $Y = 2.544X+0.361$). These correlations were observed in the handgrip, hard pellet and soft pellet conditions. In the no activity condition only changes in Performance Index were correlated with those in mydriasis.

DISCUSSION

The present findings indicate that short bouts of masticatory activity improve the velocity of number retrieval and matrix scanning and increase the task-induced mydriasis, which is considered as an indicator of arousal (Bradshaw, 1967; Bradley et al., 2008). It has to be noticed that, when a hard pellet had been chewed, the improvement in performance and the increase in mydriasis, observed immediately after mastication, persisted for at least 30 min. These findings could not be attributed to a learning effect, since simple test repetition did not significantly change sensorimotor performance and mydriasis, neither to other sensory properties as the hard pellet was tasteless, odorless and colorless. Moreover, it was specifically attributable to chewing and not to the motor activation in itself, since handgrip exercise induced slight but significant decreases in performance and mydriasis. Such decreases were significant only among females, likely owing to less developed musculature which may have been fatigued by the handgrip exercise, thus reducing their performance (White et al., 2013). To weigh the possible translational impact, the chewing effects have been replicated by administering a commercially available gum. The results show how the effects can be still observed, but they are short-lasting and reduced in amplitude. We argue that this might be related to the soft consistency of the commercially available pellets, as compared to the custom-made hard ones.

The improvement in cognitive performance induced by chewing could be attributed to an enhancement of the arousal level, as indicated by the task-related enhancement in mydriasis observed following the chewing period, and by the significant correlation between the changes in performance and mydriasis. Thus, these findings suggest that the sensorimotor orofacial activity has a strong impact on the modulatory system regulating arousal, likely due to the influence exerted on the ARAS and related structures by the central pattern generator for chewing and/or by the sensory signals elicited by masticatory movements. In fact, trigeminal input exerts a tonic action on the cortical activity, as indicated by the cortical synchronization induced by bilateral trigeminal neurectomy in encephale-isolé preparation (Roger et al., 1956) and by the cortical desynchronization associated with the stimulation of the trigeminal nerve in awake epileptic rats (Fanselow et al., 2000). Although the fibers arising from the trigeminal nuclei may affect several structures belonging to the ARAS (McKinley and Magoun, 1942; Semba and Fibiger, 1992; Fujise et al., 1998; Krout et al., 2002; Sakata et al., 2003), the present data suggest that, at least in

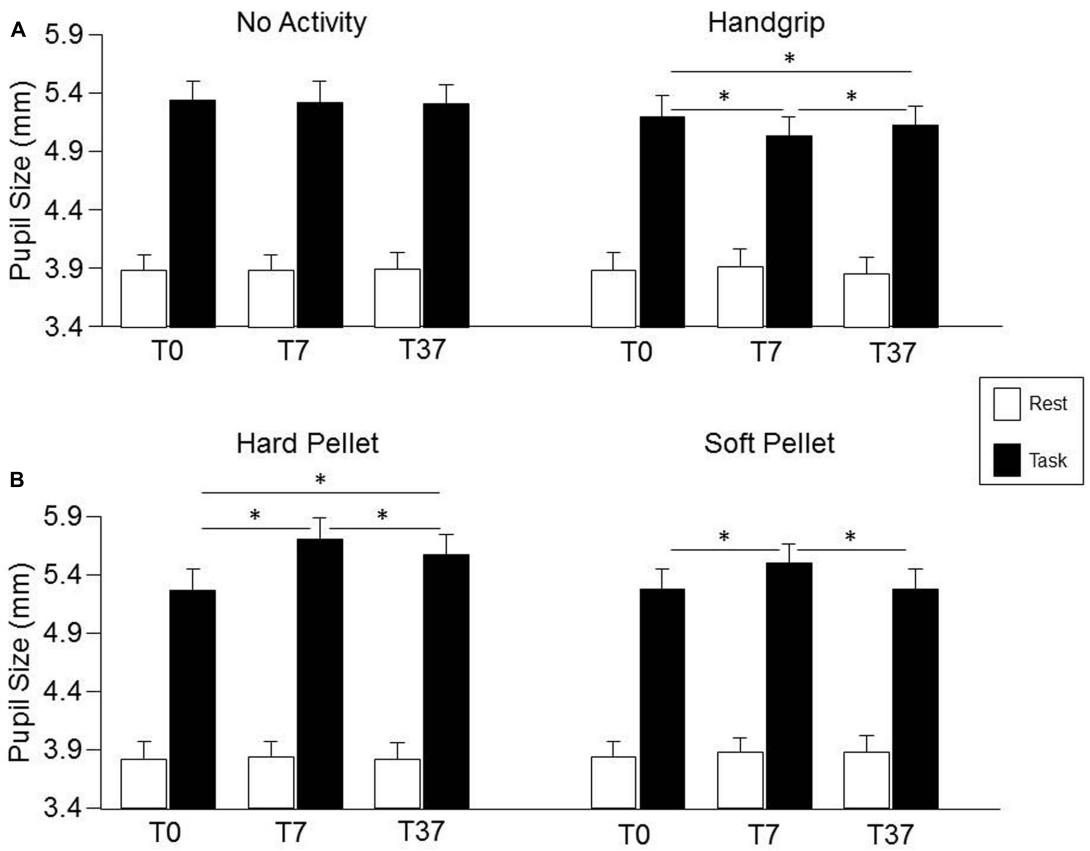

FIGURE 4 | Changes in pupil size induced by the different sensorimotor activities. Columns represent the mean values of pupil size at rest (open columns) and during task (black columns) in the "no activity" and "handgrip" conditions **(A)** and in the "hard pellet" and "soft pellet" conditions **(B)** at T0, T7, T37. In **(A,B)**, the error bars represent Standard Errors. Asterisks refer to significant differences between T0, T7 and T37, as indicated by the horizontal lines. See Supplementary Table 5 for details.

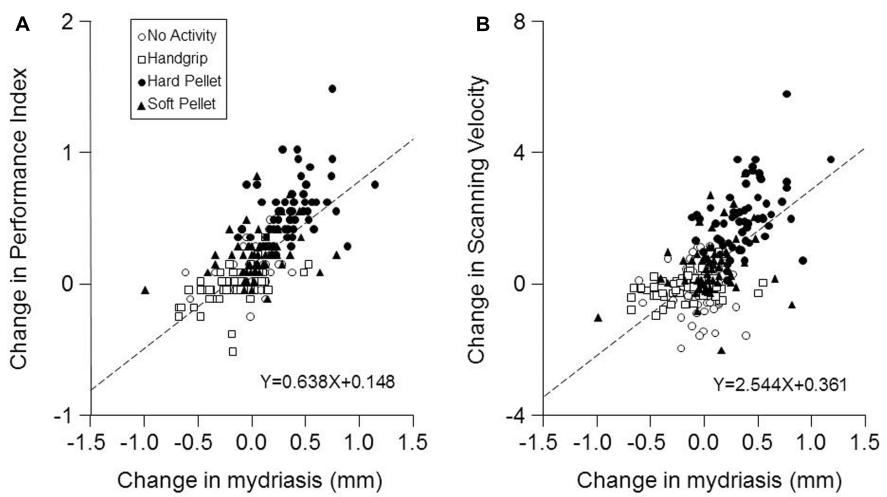

FIGURE 5 | Relation between the changes in Performance Index and Scanning Velocity with respect to control (T0) induced by the different conditions and the corresponding changes in mydriasis. The changes in Performance Index **(A)** and in the Scanning Velocity **(B)** at T7 and T37 with respect to T0 (control) have been plotted as a function of the corresponding changes in task-induced mydriasis. In **(A,B)** the dashed lines are regression lines of all the data points. Open circles: no activity condition. Open squares: handgrip condition. Dots: hard pellet condition. Filled triangles: soft pellet condition.

part, the stimulating effects of trigeminal information can be mediated by the LC. In fact, pupil size is widely considered as a proxy of LC activity (Silvetti et al., 2013; Hoffing and Seitz, 2015; Kihara et al., 2015; Hayes and Petrov, 2016; see also Laeng et al., 2013). In the present experiments, the larger changes in task-induced mydriasis corresponded to 30% of the control value (11% of the pupil size at rest) and corresponded to 0.43 mm. In humans, changes in pupil size of 0.25–0.5 mm have been shown to correlate with the corresponding changes in the LC region activity (Alnaes et al., 2014). Within this framework, our experiments document the novel finding of a significant correlation between changes in task-induced mydriasis and performance. Trigeminal inputs to the LC have been described from the spinal and principal sensory trigeminal nuclei (Craig, 1992; Couto et al., 2006) as well as from within or near the trigeminal mesencephalic nucleus (Cedarbaum and Aghajanian, 1978; Luo et al., 1991). It has been claimed that the latter could be electrically coupled to the LC (Matsuo et al., 2015). The modulatory action of the LC on a wide range of trophic brain functions (Mather and Harley, 2016) and the degenerative effects on the brain induced by teeth loss (Kato et al., 1997; Onozuka et al., 2000; Watanabe et al., 2002; Kubo et al., 2005; Aoki et al., 2010; Oue et al., 2013) could be consistent with the speculated coupling. However, there is still no direct evidence of electrocoupling between LC neurons and trigeminal mesencephalic afferents, beyond a Fluorogold labeling passage from mesencephalic trigeminal neurons to the LC area (Fujita et al., 2012). Fibers from trigeminal nuclei could affect the distal dendrites of the pericoerulear region (Aston-Jones et al., 1986, 1991), as well as, via the paragigantocellularis and the prepositus hypoglossi nuclei (Lovick, 1986; Buisseret-Delmas et al., 1999), the LC cell bodies (Aston-Jones et al., 1986). So, an increase in LC activity could occur during the trigeminal-induced enhancements in task-related mydriasis and in cognitive performance (see De Cicco et al., 2014, 2016), which would benefit of the release of noradrenaline at cerebral cortical level (Gabay et al., 2011).

It is unknown whether trigemino-coeruleus pathways modulate the activity of noradrenergic neurons projecting to the thalamus and the cortex, which are likely implicated in the arousal. Recent evidence indicates, in fact, that the LC is heterogeneous in terms of its neurochemical composition, neuronal firing patterns, intranuclear topography of target-specific projection neurons (Uematsu et al., 2015), and sub-regional extension of peri-coeruleus dendrites (Aston-Jones et al., 1991). Further investigations are necessary in order to clarify the issue. However, cells projecting to different brain region are largely overlapping (Uematsu et al., 2015), so that trigeminal afferent input to a given LC region could potentially affect different output channels.

So, it is likely that sensorimotor trigeminal signals elicit changes in the excitability of ARAS/LC which outlast mastication for at least a few minutes and may extend up to half an hour when hard pellets are chewed.

Another relevant point emerges from the improvement in performance. In fact, this resulted from an increase in the scanning velocity of the matrices without loss in accuracy, since the increase in retrieved numbers was not associated with an increase in errors. Such an increase would have been expected on the ground of the well-known trade-off between speed and precision (Fitts, 1954; Wright and Meyer, 1983; Harris and Wolpert, 2006). These data are consistent with the widespread effect of NE release (McCormick et al., 1991), which increases the signal to noise ratio of cortical and thalamic neurons (Foote et al., 1975; Moxon et al., 2007), thus enhancing sensory coding efficiency (Fazlali et al., 2016) and, possibly, network performance in highly integrated functions (Shea-Brown et al., 2008). Finally, the observation that a higher masticatory load and, as a consequence, a stronger trigeminal input induce more significant effects on task-related mydriasis and performance with respect to a less demanding chewing task with a soft, commercially available pellet is consistent with clinical and experimental animal evidence showing how chewing soft food may lead to cognitive impairments and neurodegenerative processes (Tsutsui et al., 2007; Weijenberg et al., 2013). These data have important clinical implications, paving the way to simple behavioral strategies for preventing and/or slowing the progression of neurodegenerative disorders.

CONCLUSION

Despite a few limitations (small sample size, lack of any cognitive evaluation of the participants), the present findings support the hypothesis that trigeminal activity associated with chewing activates brain arousal systems, possibly the LC, leading to persistent changes in their excitability likely improving the performance of cognitive tasks.

AUTHOR CONTRIBUTIONS

MPTF performed most of data analysis and gave a very important contribution to data understanding, manuscript preparation and revision. VDC contributed to the experimental design and executed the experiments. The contributions of MPTF and VDC were of the same importance for the work. MB gave an important contribution to data analysis and manuscript preparation and revision. EC, UF, and LB contributed to manuscript preparation and revision. DM contributed to the experimental design, supervised the whole activity and prepared most of the manuscript. All authors read, edited and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnana.2017.00068/full#supplementary-material

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REVIEW
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Trigeminal, Visceral and Vestibular Inputs May Improve Cognitive Functions by Acting through the Locus Coeruleus and the Ascending Reticular Activating System: A New Hypothesis

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It is known that sensory signals sustain the background discharge of the ascending reticular activating system (ARAS) which includes the noradrenergic locus coeruleus (LC) neurons and controls the level of attention and alertness. Moreover, LC neurons influence brain metabolic activity, gene expression and brain inflammatory processes. As a consequence of the sensory control of ARAS/LC, stimulation of a sensory channel may potential influence neuronal activity and trophic state all over the brain, supporting cognitive functions and exerting a neuroprotective action. On the other hand, an imbalance of the same input on the two sides may lead to an asymmetric hemispheric excitability, leading to an impairment in cognitive functions. Among the inputs that may drive LC neurons and ARAS, those arising from the trigeminal region, from visceral organs and, possibly, from the vestibular system seem to be particularly relevant in regulating their activity. The trigeminal, visceral and vestibular control of ARAS/LC activity may explain why these input signals: (1) affect sensorimotor and cognitive functions which are not directly related to their specific informational content; and (2) are effective in relieving the symptoms of some brain pathologies, thus prompting peripheral activation of these input systems as a complementary approach for the treatment of cognitive impairments and neurodegenerative disorders.

Keywords: ascending reticular activating system, locus coeruleus, pupil size, trigeminal nerve, visceral input, vestibular input, hemispheric imbalance, performance

INTRODUCTION: FUNCTIONAL ASPECTS OF BRAINSTEM RETICULAR FORMATION AND ASCENDING RETICULAR ACTIVATING SYSTEM (ARAS)

Reticular Formation and ARAS

The reticular formation (RF), a network of scattered cells that extends from the medulla to the hypothalamus and connects to most of brain structures, sustains nervous functions which are of crucial importance for body homeostasis and proper interaction with the environment, such as breathing, blood circulation (Guyenet, 2006, 2014), body (Luccarini et al., 1990; Pompeiano et al., 1991; Schepens and Drew, 2006; Takakusaki et al., 2016), head (Quessy and Freedman, 2004) and eye (Sparks et al., 2002) voluntary and reflex movements. Moreover, as discovered by the seminal work of Moruzzi and Magoun (1949) the RF controls arousal and sleep-waking cycle, shifting the pattern of thalamo-cortical activity between alternations of short periods of discharge and quiescence, which synchronizes within large populations (Steriade et al., 1993) and prolonged periods of continuous neuronal discharge, where only restricted populations of neurons nearby located (Steriade, 1995) or involved in the same task (Uhlhaas et al., 2009) show synchronized activity. The first pattern leads to an electroencephalographic (EEG) activity characterized by high amplitude and predominant low frequency components (synchronized or deactivated), typical of drowsiness and slow wave sleep, the second to a low amplitude, high frequency EEG (desynchronized or activated) pattern characteristic of wakefulness, but also present in the desynchronized sleep (Steriade et al., 1993; Steriade, 1995). Although there is evidence that the caudal region of the RF may include sleep-inducing, synchronizing structures (Magni et al., 1959), the studies of Moruzzi and Magoun showed the existence of an ascending reticular activating system (ARAS), which promotes the transition from a synchronized to a desynchronized EEG pattern, i.e., from sleep to wakefulness or from slow waves (synchronized) to rapid eye movement (REM, desynchronized) sleep (Steriade et al., 1993; Steriade, 1995). The ARAS extended up to the rostral region described by von Economo (1930) at the level of the junction between midbrain and thalamus, whose lesion leads to a persistent sleep in lethargic encephalitis. Later studies have shown that the ARAS includes not only glutamatergic RF neurons, as originally described, but also cholinergic and noradrenergic neurons within the dorsolateral pontine tegmentum (located in the pedunculopontine/laterodorsal tegmental (PPT/LTD) nuclei and the *Locus Coeruleus* (LC), respectively). Finally, also the histaminergic neurons in the tuberomammillary nucleus (TMN) of the posterior hypothalamic region, the peptidergic neurons in the lateral hypothalamus (LH) and the dopaminergic neurons in the mesencephalon and serotoninergic neurons in raphe nuclei (Jones, 2003; Saper et al., 2005) can be considered as ARAS components, although the precise action of the raphe neurons on cortical arousal has been questioned (Monti, 2011). These specific ARAS components regulate arousal through their connections with the specific thalamic relay nuclei and the diffuse thalamic system (Saper et al., 2005), the cerebral cortex (Saper et al., 2005) and the cholinergic basal forebrain neurons (Peyron et al., 1998; Jones, 2004; Fuller et al., 2011; Monti, 2011), which are the source of cholinergic input to the cortex, and play an essential role in the control of arousal (Fuller et al., 2011). The main connections of the ARAS with thalamo-cortical structures and basal forebrain are illustrated in **Figure 1**.

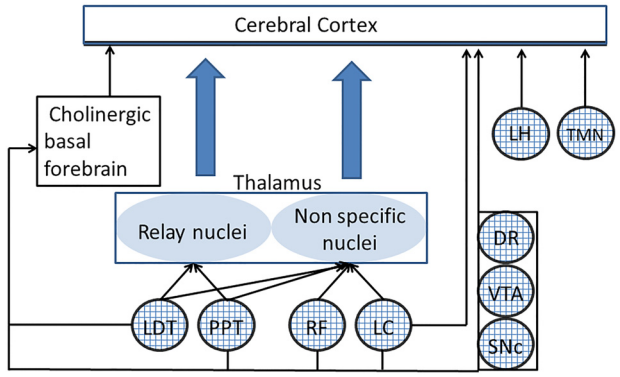

FIGURE 1 | Connections of different ascending reticular activating system (ARAS) structures to the thalamo-cortical system. Only the principal connections of ARAS structures (indicated by textured circles) have been shown. PPT and LTD act on the specific thalamic relay nuclei and, together with the RF and the LC, on the diffuse thalamic system. DR, LC, VTA, lateral hypothalamus (LH) and tuberomammillary nucleus (TMN) have a direct access to the cerebral cortex. All ARAS structures connect to basal forebrain neurons, which are the source of cholinergic projections to the cerebral cortex. DR, dorsal raphe; LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; LH, lateral hypothalamus; PPT, pedunculopontine nucleus; RF, reticular formation; SNc, substantia nigra pars compacta; TMN, tuberomammillary nucleus; VTA, ventral tegmental area.

Among the different ARAS components, the LC has attracted a large amount of investigations, which, on top of elucidating the LC effects on neuronal activity and brain networks signaling, have also disclosed neurobiological LC actions that may have a deep impact on the brain dynamics, both in health and disease.

The Noradrenergic LC System

It has been recently pointed out that the LC exerts a wide spectrum of influences on the brain, which span from tuning of neurophysiological activities related to sensorimotor/cognitive processes and setting of general brain excitability, to neurobiological functions such as the control of brain-blood barrier integrity, metabolic activity and neuroinflammatory processes (Mather and Harley, 2016). Some of these functions could place the LC at the core of the pathogenesis of neurodegenerative diseases (O'Mahony et al., 1994; Braak and Del Tredici, 2011; Del Tredici and Braak, 2013; Femminella et al., 2016).

The LC is located in the pontine brainstem, close to the forth ventricle. In humans, the LC consists of about 16,000 neurons for each side, which project virtually to the whole brain, with the exception of the basal ganglia, where LC fibers are limited to the nucleus accumbens (NAcb; see Berridge and Waterhouse, 2003 for reference) and to the substantia nigra

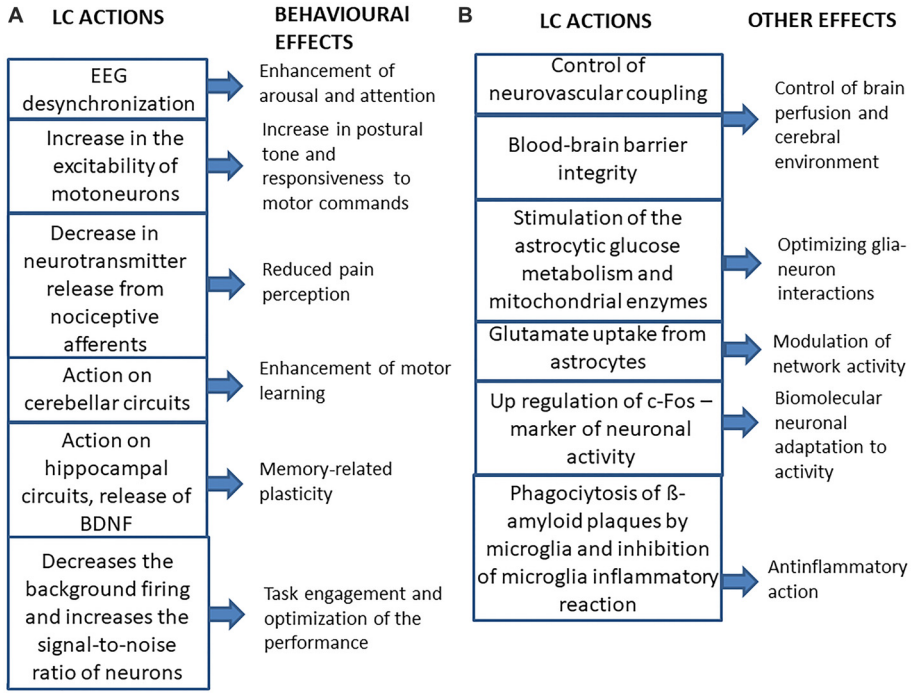

FIGURE 2 | Summary of the main effects of LC activation on the brain and of their consequences. **(A)** Behavioral effects. **(B)** Other effects.

pars reticulata (SNr; Vermeiren and De Deyn, 2017). However, due to these connections, the LC could play a role in the disorders of basal ganglia (Vermeiren and De Deyn, 2017). Interestingly, adult LC neurons tend to fire in synchrony, when their discharge rate is low, due to the presence of a weak electrotonic coupling between LC neurons (Heister et al., 2007). Such a synchronous firing could be reinforced by the existing electrical coupling of LC neurons to glial cells (Alvarez-Maubecin et al., 2000), which could in turn enhance the synchronous glutamate release from astrocytes (Pirttimaki et al., 2017).

Neurophysiological LC Influences on the Brain

The main neurophysiological effects of LC on the brain and their behavioral consequences are summarized in **Figure 2A**. As expected for a structure which is a part of the ARAS (Jones, 2003; Lee and Dan, 2012), the LC desynchronizes EEG activity, enhancing arousal and attention (Berridge, 2008; Berridge et al., 2012), thus playing an important role in behavioral control (Aston-Jones et al., 1999; Aston-Jones and Cohen, 2005; Yu and Dayan, 2005; Berridge et al., 2012; Payzan-LeNestour et al., 2013). Moreover, its activity can also affect mood (Hirschfeld, 2000; see, however Liu et al., 2017).

The LC gives some contribution to the noradrenergic control of spinal motor activity, since it has been shown that activation of the LC, similarly to local norepinephrine (NE) application, increases the excitability of motoneurons (Fung et al., 1994), leading to a strengthening of postural tone and, possibly of the general responsiveness to motor commands. Moreover, LC stimulation has been shown to exert an inhibitory role on acute pain perception, by decreasing the release of neurotransmitters from nociceptive afferents (Llorca-Torralba et al., 2016). The LC noradrenergic neurons also control the gain of vestibulospinal reflexes, probably adapting them to the level of arousal (Pompeiano et al., 1991) and their projections to the cerebellar cortex seem to be necessary for the induction of those motor learning processes that modify vestibulo-ocular and vestibulospinal reflexes (van Neerven et al., 1991; Pompeiano et al., 1994). In this respect, noradrenergic projections to hippocampal structures play a fundamental role in memory-related plasticity phenomena (Sara, 2009, 2015; Sara and Bouret, 2012). Accordingly, NE stimulates the cerebral synthesis and release from astrocytes (Juric et al., 2006) of the brain-derived neurotrophic factor (BDNF), known to facilitate memory processes (Bekinschtein et al., 2014).

In the light of the multiple effects of LC on brain activity and plasticity, it is not surprising that there is a large body of experimental evidence indicating that the cognitive improvement elicited by physical exercise can be in part attributed to the activation of LC neurons by multiple reflex pathways of somatosensory and visceral origin (McMorris, 2016).

Microiontophoretic NE application on LC target neurons, and/or electrical or chemical (by local injection of neurotransmitters) LC activation in anesthetized animals, may enhance the responsiveness of cortical neurons to subthreshold stimuli, but also decrease the background firing of the cells more than the sensory-evoked response, thus leading to an increase

in the signal-to-noise ratio of the neurons, which become responsive only to strongest inputs (Snow et al., 1999; Berridge and Waterhouse, 2003). This effect can be coupled to an increase in the responsiveness to both glutamate and GABA (Berridge and Waterhouse, 2003). The increase in signal-to-noise ratio of neuronal responses, is likely related to the suppressive role of the LC in the generation of epileptic discharges (Fornai et al., 2011) and takes place during the phasic activation of LC neurons. At variance, changes in LC background discharge affect neuronal responses to whatever input (Devilbiss and Waterhouse, 2011). In this respect, it has been recently proposed (Aston-Jones et al., 1999; Aston-Jones and Cohen, 2005) that phasic and tonic LC discharges underline two different behavioral states. The phasic discharge can be observed during waking, in response to task-relevant stimuli. The tonic discharge is very regular, ranges between 2 Hz and 15 Hz during active waking; it decreases to less than 2 Hz in quiet waking and drops further during slow wave sleep, being abolished during REM sleep. During waking, there are periods characterized by strong phasic excitation with a low level of tonic LC activity (phasic state), that correspond to a behavioral state called "exploitation", which favors the engagement in specific tasks and optimizes performance. A high tonic discharge is associated with a reduced capability to generate burst firing (tonic state) and with a behavior called "exploration", characterized by poor task performance, task disengagement and search of alternative behavioral choices. When the phasic discharge is present, the level of tonic LC activity is moderate. Transitions between phasic and tonic patterns change the responsiveness of LC targets: in the tonic mode there is a generalized increase in responsiveness, whereas in the phasic one, the increase in responsiveness is selective for the inputs related to the specific task in which the subject is engaged (Aston-Jones and Cohen, 2005).

LC and Pupil Size

Interestingly, the LC activity can be indirectly estimated by measuring the pupil diameter (Silvetti et al., 2013; Hoffing and Seitz, 2015; Kihara et al., 2015; Einhäuser, 2016; Joshi et al., 2016; Reimer et al., 2016), since it has been shown, both in humans (Murphy et al., 2014) and in animals (Rajkowski et al., 1993, 1994) that pupil size is positively correlated to the LC activity. Pupil mydriasis is one of the most characteristic signs of arousal (Bradshaw, 1967; Bradley et al., 2008), it represents a fine indicator of brain state (McGinley et al., 2015) and, together with purposeful eyes motion, it has been taken has an indicator of persistent awakening status in the midpontine pretrigeminal preparation (Batini et al., 1959). The relation between noradrenergic LC neurons and pupil size is due to the control exerted by the LC on the preganglionic parasympathetic neurons of the Edinger-Westphal nucleus (Breen et al., 1983), which innervate the iris constrictor, inhibiting their discharge through an α2-mediated mechanism (Szabadi and Bradshaw, 1996; Samuels and Szabadi, 2008). This inhibition is necessary to increase the pupil size, since the tonic activity of the iris constrictor would prevent pupil enlargement by iris dilatator (Wilhelm et al., 2011).

Other LC Influences on the Brain

Beyond its effects on neural activity, the LC regulates other neurobiological functions (see **Figure 2B**). LC axons are in intimate contact with the astrocytes (Paspalas and Papadopoulos, 1996; Cohen et al., 1997), where they enhance the intracellular Ca^{2+} inflow (Paukert et al., 2014), which has been shown to induce dilation of blood vessels (Koehler et al., 2009; Carmignoto and Gómez-Gonzalo, 2010; Petzold and Murthy, 2011; see also Girouard et al., 2010). In this respect, LC may increase brain perfusion (Toussay et al., 2013). Moreover, the LC is necessary for blood-brain barrier integrity (Harik and McGunigal, 1984; Kalinin et al., 2006), so it may affect the composition of the cerebral environment.

Moreover, noradrenergic activation increases glutamate uptake (Hertz et al., 2010), thus modulating network activity, stimulates the astrocytic glucose metabolism (Segal et al., 1980; Sorg and Magistretti, 1991) and the activity of mitochondrial enzymes (Hertz et al., 2010). It has been suggested that the LC optimizes the metabolic and functional interactions between neurons and astrocytes in the cerebral cortex and hippocampus, as well as in the nucleus basalis of Meynert: loss of LC neurons may disrupt these interactions leading to neuronal cell death in these areas (Hertz, 1989). In view of these LC effects is not surprising that an astrocytic dysfunction has been found in major depressive disorder (Chandley et al., 2013), where the noradrenergic transmission could be inadequate (Hirschfeld, 2000).

LC also controls gene expression as its stimulation induces ipsilateral c-Fos upregulation of cortical pyramidal cells and of a larger population of interneurons (Toussay et al., 2013), while its lesion abolishes the increase in c-Fos expression elicited by restraining (Stone et al., 1993) as well as by sleep deprivation (Cirelli et al., 1996). So, the LC controls the biomolecular cell adaptation to activity changes.

Finally, LC regulates neuroinflammatory processes, by inducing the phagocytosis of β-amyloid plaque by microglia (Heneka et al., 2002; Pugh et al., 2007; Kong et al., 2010), thus maintaining an adequate β-amyloid clearance (Kalinin et al., 2007) and by inhibiting the microglia inflammatory reaction (Yao et al., 2015). It has to be underlined that neuroinflammation seems to characterize also non pathological, but stressful conditions, such as sleep deprivation (Wadhwa et al., 2017).

Given the LC control of sensorimotor processing, learning and plasticity, metabolic processes, neurovascular coupling, inflammatory processes and gene transcription, a dysregulation of its activity may contribute to cognitive and arousal dysfunction and can be associated with a variety of behavioral and cognitive disorders (Berridge and Waterhouse, 2003; Aston-Jones and Cohen, 2005; Mather and Harley, 2016). Considering that LC degeneration is the early sign of both Alzheimer's and Parkinson's disease (Zarow et al., 2003; Grudzien et al., 2007; Mravec et al., 2014), is not surprising that LC dysfunction has been proposed as causal factor for such pathologies (Braak and Del Tredici, 2011; Del Tredici and Braak, 2013; see also O'Mahony et al., 1994; Femminella et al., 2016).

ARAS Activity Regulation: Role of Sensory Afferents

According to the effects of ARAS activation on neural processing (Snow et al., 1999; Castro-Alamancos, 2002; Yu and Dayan, 2005; Devilbiss and Waterhouse, 2011; Dayan, 2012; McGinley et al., 2015), subtle changes in ARAS activity during the waking state may lead to changes in the sensory, motor and cognitive performance (Lee and Dan, 2012; McGinley et al., 2015). Although the sources of these changes in ARAS activity are largely undetermined (McGinley et al., 2015), the tonic discharge of ARAS's neurons is sustained, beyond possible pacemaker properties (Chan and Chan, 1983; Sun et al., 1988), by the afferent inputs that these structures receive. Some of the afferent inputs to the ARAS are related to the control of specific somatomotor (Kuypers, 1981), oculomotor (Huerta and Harting, 1984) or vegetative (Guyenet, 2006; Ganchrow et al., 2014) functions associated with this network. Mutual connections between the different ARAS components are likely implicated in determining the level of arousal in relation to the sleep-wake cycle (Scammell et al., 2017). Other inputs arising from higher brain structures could allow to adapt the level of arousal on the basis of the cognitive (Leichnetz et al., 1984) and emotional state of the subject. In this respect, it has to be emphasized that all ARAS components strongly interact with the limbic system (Brodal, 1981; Veening et al., 1984; Ericson et al., 1991; Semba and Fibiger, 1992; Berridge and Waterhouse, 2003; Vertes and Linley, 2008; Lee et al., 2011). Finally, inputs from peripheral receptors may set the ARAS activation according to body/environmental conditions and sustain its tonic activity, as pointed out by the initial studies on RF (Starzl et al., 1951). The afferent control of ARAS by sensory afferents has three potential consequences:

1. Afferent discharge may regulate the excitability of brain networks involved in cognitive functions, which are not directly related to their specific sensory informational content, thus modulating cognition. In the same way, vestibular and neck signals acting on spinal projecting neurons belonging to the RF (Pompeiano et al., 1984) and to the LC (Manzoni et al., 1989) modulate the discharge of motor networks controlling the postural tone. So, based on this assumption, it could be expected that sensory afferent stimulation may boost cognitive performance by increasing the ARAS/LC discharge.
2. An asymmetry in the level of specific tonic sensory signals may lead to an asymmetric ARAS activity and, in turn, to an imbalance in hemispheric excitability. There is indeed evidence that a lesion-induced hemispheric imbalance may lead to specific cognitive deficits which are abolished by a second symmetric lesion (Lomber and Payne, 1996). So, asymmetries in the level of sensory afferent inputs may potentially induce cognitive dysfunctions, which could be prevented by counteracting the afferent asymmetry. Alternatively, an asymmetric stimulation of specific sensory afferents may compensate for hemispheric imbalance (Rubens, 1985; Bottini et al., 1995; Schiff and Pulver, 1999).
3. Finally, due to the neurobiological influences that LC exerts on the brain, a disruption of sensory afferent discharge, impinging on the LC itself, may potentially influence the development of neuroinflammatory and degenerative processes in brain structures.

In the next three sections, we will review a large body of evidence supporting points 1–3, arising from experiments investigating the influences exerted on the brain by modifications of the afferent trigeminal, visceral and vestibular inputs.

TRIGEMINAL, VISCERAL AND VESTIBULAR INFLUENCES ON BRAIN FUNCTIONS THROUGH ARAS AND LC

Acute Trigeminal Influences: Effects of Afferent Stimulation

Previous studies report that chewing improves cognitive processing speed (Hirano et al., 2013), alertness (Allen and Smith, 2012; Johnson et al., 2012), attention (Tucha et al., 2004) and intelligence (Smith, 2009). Objective modifications can be observed in the reaction times (shortening; Tucha et al., 2004; Allen and Smith, 2012; Hirano et al., 2013), in the event-related potentials latencies (decreasing, see Sakamoto et al., 2009, 2015) and in the cerebral blood oxygen-dependent signal (increasing, see Hirano et al., 2013). Interestingly, the effects of chewing were not replicated by rhythmic finger motor activity or by rhythmic jaw movement in the absence of chewed material (Sakamoto et al., 2015).

So, orofacial input is particularly effective in enhancing arousal, thus boosting performance. In fact, 2 min of chewing a hard pellet may boost performance and task-induced mydriasis, while a bilateral handgrip exercises of the same duration do not (Tramonti Fantozzi et al., 2017). These data are consistent with the observation that, in the *encephale isolé* preparations, where ARAS and LC have been disconnected from the spinal cord, an additional lesion of the trigeminal, but not of the other cranial nerves afferents, triggers the transition of the desynchronized EEG activity towards a synchronized sleeping pattern (Roger et al., 1956).

However, the positive effects of chewing on cognitive performance are not observed following sleep deprivation, although, in this condition, chewing may again improve alertness and mood (Kohler et al., 2006). This result is not surprising, since sleep deprivation enhances LC activity, as documented by the c-Fos expression (Pompeiano et al., 1992). A higher LC background discharge would prevent the trigeminal-induced enhancement in phasic LC activity, which seems necessary for focusing the attention on the task, and for enhancing performance (Aston-Jones et al., 1999; Aston-Jones and Cohen, 2005).

Acute Trigeminal Influences: Effects of Balancing and Unbalancing Occlusion

Recent experiments have shown that the presence of a sensorimotor trigeminal imbalance (related to occlusal

problems)—consisting in an asymmetric activation of masseter muscles during clenching—is strongly correlated to an asymmetry in pupils size, either in the normal jaw resting position, with the arches few millimetres apart, or with the teeth in contact (De Cicco et al., 2014, 2016). Since pupil size is considered as a reliable indicator of LC activity and its asymmetry is strongly reduced following removal of the trigeminal imbalance by occlusal correction, these data suggest that the trigeminal imbalance makes the LC discharge asymmetric. Removal of the asymmetries in sensorimotor trigeminal activity, not only makes pupils symmetric, but also enhances the performance in a cognitive task and the mydriasis associated with an haptic task (De Cicco et al., 2014, 2016), thus suggesting that trigeminal-induced imbalance in LC activity (and, as a consequence in hemispheric excitability) is detrimental to the performance and to cortical arousal, whose level is indicated by task induced mydriasis (Bradshaw, 1967; Bradley et al., 2008). The effects of hemispheric imbalance on behavior are documented by the observation that unilateral brain lesions lead to severe cognitive deficits (Lomber and Payne, 1996; Kerkhoff, 2001) which can be greatly reduced by a second, symmetric lesion on the opposite side (Lomber and Payne, 1996), or by asymmetric sensory stimulation (Kerkhoff, 2001). Consistently, manipulation of the trigeminal information may either induce or relieve asymmetries in brain excitability, thus modifying the cognitive performance (De Cicco et al., 2014, 2016).

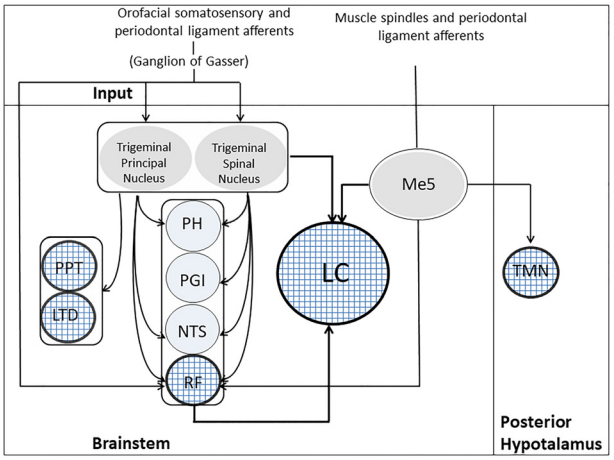

FIGURE 3 | Trigeminal Pathways to ARAS's structures. Muscle spindles and periodontal ligament receptors, through the Me5, project to five ARAS's structures, indicated by the textured circles: LC, RF, PPT, LDT and TMN. Orofacial somatosensory/periodontal ligament afferents reach the trigeminal principal/spinal nuclei and the RF through the ganglion of Gasser. The trigeminal principal/spinal nuclei project to both LC and RF, as well as to PPT and LTD. Indirect pathways from trigeminal nuclei to the LC may run through the PH, the PGI, the NTS and the RF. LC, Locus Coeruleus; LTD, laterodorsalis tegmental nucleus; Me5, mesencephalic trigeminal nucleus; NTS, nucleus of the tractus solitarius; PGI, nucleus paragigantocellularis; PH, prepositus hypoglossi; PPT, pedunculopontine nucleus; RF, reticular formation; TMN, tuberomammilary nucleus.

Trigeminal Pathways to ARAS and LC

There are several pathways that may bring trigeminal input to the ARAS and the LC: the most important are shown in **Figure 3**. First of all, primary sensory fibers, including proprioceptive jaw muscle spindles from mesencephalic trigeminal sensory nucleus (Me5) reach directly the RF (Brodal, 1981; Dessem et al., 1997). Me5 afferents transporting proprioceptive information from periodontal ligaments and muscle spindles of the oral cavity project also to hypothalamic TMN neurons (Fujise et al., 1998; Sakata et al., 2003). Secondary fibers from trigeminal nuclei reach the RF (Brodal, 1981; Shammah-Lagnado et al., 1987, 1992; Schmid et al., 2003) and, in addition, the diffuse thalamic system (Krout et al., 2002). All trigeminal nuclei, including the Me5, project to the LC (Cedarbaum and Aghajanian, 1978; Luo et al., 1991; Craig, 1992; Couto et al., 2006; Dauvergne et al., 2008). In particular, it has been claimed, on the basis of fluorogold transport from Me5 cells to neurons within the boundaries of the LC (Fujita et al., 2012), that these two structures are electrotonically coupled (Matsuo et al., 2015). Moreover, trigeminal signals may reach the LC also through the nucleus of tractus solitarius (NTS) and the RF (Zerari-Mailly et al., 2005; Schwarz and Luo, 2015).

All the fibers mentioned above terminate outside the core of the nucleus, in a region where only LC dendrites are located. So they are probably less effective in driving LC neurons than those impinging upon the cell bodies (Aston-Jones et al., 1991). Inputs to dendrites, however, could affect the electrical coupling of LC neurons, which occurs at dendritic level (Ishimatsu and Williams, 1996). The core of the LC, where cell bodies are located, receive inputs, which arises from the hypothalamic orexinergic neurons (Peyron et al., 1998), the nucleus paragigantocellularis (PGI) and the nucleus prepositus hypoglossus (PH; Aston-Jones et al., 1986), PGI sending excitatory (glutamatergic) and PH inhibitory (GABAergic) fibers (Aston-Jones et al., 1991). Both PGI and PH receive afferents from the trigeminal nuclei (Lovick, 1986; Buisseret-Delmas et al., 1999). So, through these indirect pathways the orofacial input is likely to exert its strongest influence on LC activity. The effectiveness of trigeminal input in LC activation is documented by the increase in c-Fos expression induced at this level by trigeminal stimulation (Mercante et al., 2017). Finally, the principal and the spinal trigeminal nuclei project to cholinergic PPT and LDT neurons (Semba and Fibiger, 1992).

Long-Term Effects of Trigeminal Afferents

There is evidence that, in addition to these short-term effects on performance and task-induced mydriasis, trigeminal signals may lead to long-term effects on the central nervous system (CNS), that may be helpful in preventing degradation of brain functions (see Ono et al., 2010 for reference). In particular, epidemiological studies have verified that tooth loss before 35 years of age or unilateral masticatory activity represents a significant risk factor for developing dementia or Alzheimer's disease (AD; Weijenberg et al., 2011; Okamoto et al., 2015), while masticatory rehabilitation allowed aged animals to recover spatial abilities (Mendes Fde et al., 2013). Moreover, it has been

documented, in aged animals, that bilateral molar extractions, leading to long-term masticatory dysfunction, decreases the number of pyramidal cells in the hippocampal CA1 and gyrus dentatus (Oue et al., 2013), with impairments in spatial learning and memory in water maze tests (Kato et al., 1997; Onozuka et al., 1999). These deficits seem to increase with aging and time after tooth loss (Onozuka et al., 2000) and can be replicated by soft-diet feeding (Tsutsui et al., 2007). Teeth loss also increases, at hippocampal level, the proliferation and the hypertrophy of the astrocytes, as it occurs following neuronal degeneration and senescence processes (see Onozuka et al., 2000), while decreasing c-Fos expression during spatial task (Watanabe et al., 2002), dendritic spines density (Kubo et al., 2005) and neurogenesis (Aoki et al., 2010). It is worth of note that reduction in number of c-Fos-positive cells in the hippocampal CA1 region and spatial learning impairment were partially antagonized by restoring the lost molars with artificial crowns (see Watanabe et al., 2002). In addition to teeth loss, also a soft diet may depress spatial learning in aged animal (Frota de Almeida et al., 2012), leading to changes in size and laminar distribution of astrocytes (Frota de Almeida et al., 2012) and reducing the BDNF levels (Yamamoto et al., 2008) and hippocampal neurogenesis (Yamamoto et al., 2009).

Finally, malocclusion leads to increase of apoptosis markers in the hippocampus and to accumulation of β-amyloid (Ekuni et al., 2011), which is one of the clinical symptoms associated with AD (Sisodia et al., 1990). Some authors point to stress-induced malocclusion with elevation of glucocorticoid levels (Yoshihara et al., 2009) as a causal factor for the expression of this constellation of symptoms which remind brain neurodegenerative processes. This hormonal response is abolished following destruction of the ventral ascending LC projections (Yoshihara and Yawaka, 2001), thus indicating that LC may link a disrupted trigeminal input to a glucocorticoid induced trophic dysfunction. However, a trophic dysfunction may also arise from a molarless condition-impaired LC discharge, leading to accumulation of β-amyloid, with the development of local inflammation and neurodegenerative processes (Ekuni et al., 2011), to a drop in local BDNF levels (Juric et al., 2006), to altered neuron-astrocyte interaction with metabolic dysfunctions at neuronal level (Hertz, 1989), to blood brain barrier dysfunction (Harik and McGunigal, 1984) and, finally to altered neurovascular coupling (Koehler et al., 2009; Carmignoto and Gómez-Gonzalo, 2010; Petzold and Murthy, 2011). All these factors may contribute to the degenerative processes that seem associated with impairment of the trigeminal functions.

In the light of all this body of experimental evidence, it appears that the lack of masticatory stimulation could be a major problem in the elders, where general motor activity, which may represent another way to drive LC discharge (McMorris, 2016), is generally reduced. In order to face the consequence of a deteriorated trigeminal input in the older age, strategies of "environmental enrichment" could be attempted, which improve cognition during aging (Stuart et al., 2017) and are likely to boost the LC activity (Saari et al., 1990).

Trophic Trigeminal Influences on LC

How can a disruption of trigeminal afferent input impair LC activity? Beyond the consequences of a lack of excitation on the side where the trigeminal input is decreased, trigeminal fibers may also exert a trophic action on LC neurons by carrying neurotrophic factors from the periphery. Normally, the masseter muscle and its neuro-muscular spindles synthetize and release BDNF and neurotrophin-3/glial cell line-derived neurotrophic factor (GDNF), respectively, which prevent cell body atrophy of proprioceptive Me5 (Fan et al., 2000), of LC and autonomic neurons (Arenas and Persson, 1994; Buj-Bello et al., 1995), as well as of astrocytes (Henderson et al., 1994) and regulate LC neurons differentiation (Traver et al., 2006). Thus, a reduction of trigeminal proprioceptive signals may lead to reduced levels of neurotrophic factors for LC neurons, favoring the development of their dysfunction. Such a dysfunction may also extend to the glia, due to the large electrical coupling between Me5-LC cells and local astrocytes (Alvarez-Maubecin et al., 2000; Moore and O'Brien, 2015) and to the spread of local injury to the adjacent coupled elements, via gap junctions (bystander killing, see Moore and O'Brien, 2015).

Effects of Non-trigeminal Orofacial and Visceral Inputs

It is known that some somatosensory orofacial inputs from the outer ear reach the brain through the vagus nerves (Brodal, 1981). Moreover, visceral afferents from the pharynx and the larynx travel in the glossopharyngeal and vagal nerves. These orofacial, non-trigeminal, pharyngeal and laryngeal fibers reach the trigeminal nuclei and the NTS (Brodal, 1981; Altschuler et al., 1989; Grélot et al., 1989; Chien et al., 1996), the latter receiving a wide spectrum of visceral afferents from the inner organs. It is likely that all this information may play a role similar to that of trigeminal orofacial afferents in modulating ARAS/LC excitability and cognitive functions.

Recent investigation has shown, in fact, that vagal stimulation in humans may affect mood, by decreasing depression symptoms (Milby et al., 2008), while subdiaphragmatic vagal section in animals leads to differential changes in the level of innate and learned anxiety (Klarer et al., 2014).

It is also known that and altered gut-brain axis feedback may contribute to mood disorders (Lerner et al., 2017). Stimulation of vagal afferents also improves memory consolidation in humans (Vonck et al., 2014) and animals (Clark et al., 1998), as well as hippocampal long-term potentiation (Zuo et al., 2007); moreover, it enhances the velocity of action selection during execution of sequential operations (Steenbergen et al., 2015). Interestingly, an improvement in memory has been observed also in subjects affected by AD (Vonck et al., 2014). On the other hand, vagal stimulation in humans seems to be detrimental for cognitive flexibility (Ghacibeh et al., 2006), while, in animals, vagotomy increases the ability in responding to instruction reversal (Klarer et al., 2017). Although several central visceral pathways could be involved in these vagal effects, most of the latter seems to be consistent with a vagal activation of the noradrenergic system: in fact an increase in NE release, due to LC activation, would

improve depression symptoms (Hirschfeld, 2000; see, however Liu et al., 2017) boost memory and LTP (Hansen, 2017), facilitate sequential operations (Mückschel et al., 2017), while decreasing cognitive flexibility (Beversdorf et al., 2002). The effect on mood could be in part related to an enhanced release of melatonin from the pineal gland (Zarate and Manji, 2008), elicited by LC activation (Mitchell and Weinshenker, 2010).

Vagal stimulation directly increases the activity of LC neurons (Dorr and Debonnel, 2006; Manta et al., 2009) and indirectly—via the LC (Manta et al., 2009)—also that of raphe neurons (Dorr and Debonnel, 2006). Moreover, vagal stimulation leads to an increase of c-Fos expression within the LC (Gieroba and Blessing, 1994). It is not surprising, therefore, that vagal stimulation increases the pupil diameter (Desbeaumes Jodoin et al., 2015) and enhances NE release at the level of the CNS (Hassert et al., 2004; Roosevelt et al., 2006; Follesa et al., 2007; Raedt et al., 2011). Similarly to the trigeminal system, also the viscero-sensitive system could be involved in neurodegenerative processes. The NTS, in fact, is particularly sensitive to the effects of circulating inflammatory mediators (Hermann et al., 2001; Daulatzai, 2012) and it is known that prolonged activation of the peripheral immune system may promote microglia activation and neuroinflammation within the CNS (Godbout et al., 2005; Henry et al., 2009). So, similarly to teeth removal, peripheral inflammatory processes may lead to disruption of an important pathway impinging on the LC.

On the other hand, vagal stimulation exerts important trophic effects on the brain: it has been documented, in fact, that activation of vagal afferents induces an increased production of BDNF and NGF (Follesa et al., 2007) within the brain, together with an enhancement of hippocampal neurogenesis (Revesz et al., 2008); moreover, vagal stimulation also decreases neuroinflammatory responses (Meneses et al., 2016). These actions, possibly related to LC activation (Coradazzi et al., 2016; Mello-Carpes et al., 2016), may contribute to the positive effects of vagal stimulation in AD.

Visceral Input to ARAS/LC

Visceral input has a wide access to ARAS structures: in fact, as shown in **Figure 4A**), it may reach directly the RF through primary vagal and glossopharyngeal fibers, as well as from NTS (Brodal, 1981; Ganchrow et al., 2014). Moreover, the NTS projects to the LC (Cedarbaum and Aghajanian, 1978; Van Bockstaele et al., 1999; Ruffoli et al., 2011), which may also receive the visceral input through the nucleus paragigantocellularis, targeting neuronal cell bodies (Lovick, 1986). Finally, the NTS projects to the LDT (Cornwall et al., 1990), thus influencing also the discharge of cholinergic neurons.

Vestibular Influences: Balancing the Brain

Recent evidence indicates that vestibular information could play a role in neural processing, beyond the contribution to a multisensory representation of the self and of the space (Britten, 2008; Zu Eulenburg et al., 2013; Hitier et al., 2014; Pfeiffer et al., 2014; Yoder and Taube, 2014). In fact, asymmetric labyrinthine activation ameliorates the cognitive deficits induced by lesions of the right parietal cortex (Rubens, 1985; Bottini et al., 1995; Schiff and Pulver, 1999) and of other brain regions (Schiff and Pulver, 1999), in humans. Attenuation of the symptoms was observed by either increasing the afferent vestibular discharge on the lesioned side, or by decreasing it on the contralateral side. Patients with right cortical damage are unconscious of body parts (asomatognosia) contralateral to the lesion and present spatial (hemi) neglect, i.e., failure to attend and respond to sensory stimuli contralateral to the lesion side (Kerkhoff, 2001). These alterations are not only due to the nervous tissue damage, but also to the imbalance in the activity of right and left hemispheres. It has been observed, in fact, that hemi neglect due to parietal lesions, in animals, ameliorate following a second, symmetric lesion produced in the contralateral hemisphere (Lomber and Payne, 1996). In other words, two symmetric lesions are less impairing than a unilateral one. A possible interpretation of these effects of vestibular stimulation is that these afferents exert a tonic control on the excitability of brain structures and that appropriate modulation of their discharge can decrease the inter-hemispheric imbalance, thus attenuating its neurological consequences.

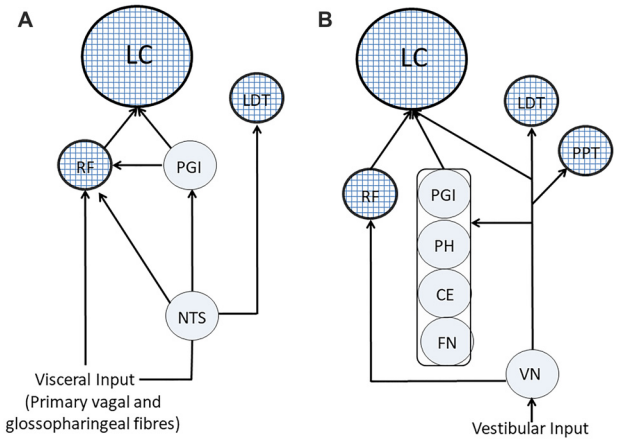

FIGURE 4 | Visceral and vestibular pathways to ARAS's structures. **(A)** Visceral pathways. **(B)** Vestibular pathways. ARAS structures are indicated by textured circles. CE, cerebellar cortex; FN, fastigial nucleus; LC, Locus Coeruleus; LTD, nucleus laterodorsalis tegmenti pontis; NTS, nucleus of the tractus solitarius; PGI, nucleus paragigantocellularis; PH, prepositus hypoglossi; PPT, pedunculopontine nucleus; RF, reticular formation; VN, vestibular nuclei.

Vestibular Input: Cognitive Effects

In normal subjects, labyrinthine stimulation may affect several cognitive functions somehow related to self and space representation, such us the localization of tactile stimuli on the hand (Ferrè et al., 2013c), the perceived size of the touched objects (Lopez et al., 2012), the perceptual somatosensory illusion of a "fake" rubber hand ownership (Lopez et al., 2010) and line bisection task (Ferrè et al., 2013b).

Moreover, vestibular deficits impair the metric properties of space representation, as inferred by mental imagery tests (Péruch et al., 2011) and are associated with personality

changes, further suggesting that vestibular sensation is implicated in the sense of self. These are depersonalization and derealization symptoms such as feeling "spaced out", "body feeling strange" and "not feeling in control of self" (Smith and Darlington, 2013). It has to be acknowledged that neural processing related to self and space representation benefit from the informational content of vestibular input and may be directly affected by its manipulation. So, the evidence that vestibular signals exert a tonic modulatory action on cognitive functions is not so compelling as for trigeminal and visceral information.

However, vestibular signals may also affect cognitive performances not directly related to self and spatial perception. Labyrinthine stimulation has been shown to ameliorate tactile allodynia, which is attributed to a hemispheric imbalance (McGeoch et al., 2009), and to improve face perception deficits in post-stroke subjects (Wilkinson et al., 2005). Moreover, it influences also the balance between novel and routine motor responses to acoustic and visual stimuli (Ferrè et al., 2013a) and speed of visual memory processing (Wilkinson et al., 2008).

Vestibular Pathways to ARAS and LC

At least in part, the effects of vestibular afferents on cognitive functions could be mediated by the central noradrenergic system arising from the LC, whose neurons are affected by the labyrinthine input (Manzoni et al., 1989; Kaufman et al., 1992), as confirmed by the observation that vestibular stimulation induces changes in pupil size (Kitajima et al., 2010, 2013), which is a reliable indicator of LC activity (Rajkowski et al., 1993, 1994; Murphy et al., 2014). It should be reminded that the vestibular influences on the LC have been proposed as the cause of the development of hemispheric specialization, since, during foetal development, the asymmetric position of the head is likely to favor activation of the left labyrinth during maternal locomotion, leading to an asymmetric development of several central structures (Previc, 1991).

As shown in **Figure 4B**, fibers arising from the spinal vestibular nucleus may directly reach the LC (Schwarz and Luo, 2015), but vestibular information can also travel along indirect pathways running through LC-projecting structures (Schwarz and Luo, 2015), such as the cerebellar cortex and the fastigial nucleus (Brodal, 1981), the PH (Belknap and McCrea, 1988) and the nucleus paragigantocellularis (Lovick, 1986). Finally, vestibular nuclei project to the RF (Brodal, 1981; Lai et al., 1999; Matesz et al., 2002) and to PPT and LDT cholinergic neurons (Semba and Fibiger, 1992; Aravamuthan and Angelaki, 2012).

TRANSLATIONAL EXPLOITATION OF TRIGEMINAL, VISCERAL AND VESTIBULAR CONTROL OF ARAS/LC ACTIVITY

The impact of trigeminal, visceral and vestibular signals on ARAS and LC may lead to interesting clinical applications. Infraorbital trigeminal branch stimulation (ITS; DeGiorgio et al., 2003, 2006) has been utilized in humans for seizure control in epileptic patients refractory to antiepileptic drug treatment. This effect depends upon the activation of LC noradrenergic system (Zare et al., 2014), whose anti-seizure effect has been described in details (Weinshenker and Szot, 2002). Similar results have been also obtained with stimulation of afferent vagal fibers (Groves and Brown, 2005), which also activate LC and raphe system through the NTS (Krahl and Clark, 2012).

Recently, the observation that ITS induces an improvement of the mood, promoted the use of the trigeminal nerve stimulation (TNS) in subjects with major depressive disorder (Cook et al., 2013), with a significant improvement of the patient's symptoms. This positive effect was attributed to the activation of the trigeminal nerve projections to LC and raphe nuclei (Cook et al., 2013), leading to an increase of serotonin and NE within the brain.

ITS was also tested in adult patients affected by fibromyalgia, a pain syndrome associated with neurological deficits in intracortical modulation as well as in young people affected by attention-deficit/hyperactivity disorder, a condition characterized by abnormal levels of inattention and/or hyperactivity/impulsivity. In the former case, TNS can improve pain and depressive symptoms (Shiozawa et al., 2014), while in the second it ameliorates attention, mood and sleep quality (McGough et al., 2015). Yet, the observed results could be related to trigeminal activation of LC/raphe nuclei, possibly driving the antinociceptive control system (Stamford, 1995) and normalizing monoamine levels in the brain. Of course, all these investigations could be extended to electrical activation of the vestibular nerve.

Another interesting field of application for peripheral activation of signals with a high impact on ARAS structures could be that of neurodegenerative disease. A recent approach to the treatment of AD has been to modulate brain activity through electrical stimulation of peripheral nerve and deep brain structures (see Laxton et al., 2014, for reference). As recently proposed, when neurons die synaptic activity on target cells is reduced, leading to upregulation of AMPA receptors, in an attempt to stabilize the global activity of the circuit (Palop and Mucke, 2010) and, as a consequence, to an increased cell excitability (Fröhlich et al., 2008). Such an increase will be coupled with a dysregulation of Ca^{2+} homeostasis due to β-amyloid (Demuro et al., 2010), leading to a greater influx of Ca^{2+} into the cell and making cell death (apoptosis) more likely. Consistently, neurons are more excitable in the brain of AD patients (Busche et al., 2008) resulting in an increased frequency of seizures. Based on these findings, it has been proposed that enhancing the circuit activity by electrical stimulation of peripheral or central structures may counteract the hypoactivity of the circuit, thus avoiding the reactive increase in excitability and the spreading of neurodegeneration (Rowan et al., 2014), assumption validated by neural network studies (Rowan et al., 2014). According to this hypothesis, long-term deep (Smith et al., 2012) or transcranial electrical (Hansen, 2012) or magnetic brain stimulation (Rabey et al., 2013) seem to improve the cognitive performance of patients, and similar results can be obtained by vagal nerve stimulation (Laxton et al., 2014). As compared to

direct brain stimulation techniques, peripheral nerve stimulation is technically easier; moreover, it may activate the same neural circuits engaged in physical and cognitive training (activities which can be of help in counteracting AD), but without being limited by patient's cooperation and motivation.

Since the LC is implicated in AD initial stages, stimulation of trigeminal proprioceptive afferents, which could be electrically coupled to LC neurons (Fujita et al., 2012; see however Tramonti Fantozzi et al., 2017), could rescue their activity and trophic condition. Indeed, chewing helps to maintain cognitive functions while masticator deficiency is associated with development of dementia (Teixeira et al., 2014). Moreover, several studies demonstrate that the loss of the molar teeth (molarless condition) may induce hippocampal senescence, characterized by a reduction of CA1 subfield neurons and by proliferation and hypertrophy of glial fibrillary acidic protein-labeled astrocytes (GFAP) in the same subfield (Onozuka et al., 1999, 2000). Also, Parkinson's disease patients could benefit from trigeminal stimulation, since LC degeneration also occurs in this pathology at early stages.

Another interesting field of application of trigeminal stimulation could be the treatment of functional impairments induced by hemispheric asymmetries. It is known that asymmetries in trigeminal (as well as vestibular) signals induced by malocclusion are detrimental for performance and their elimination by occlusal correction improves cognitive performance in patients affected by AD (De Cicco, 2012), as well as in normal subjects (De Cicco et al., 2014, 2016). An asymmetric brain activity may also arise from unilateral lesions and, in this instance, activation of vestibular afferents on the appropriate side brings to a relief of patient's symptoms (Rubens, 1985; Bottini et al., 1995; Schiff and Pulver, 1999). This effect is likely dependent upon the vestibular connections with ARAS and LC, which allow to rebalance hemispheric activity, thus prompting the use of trigeminal stimulation for the same purpose, lacking secondary undesired effects, such as vertigo. Finally, brain asymmetries may also result from environmental factors, such as exposure to ionizing radiations, shown to be associated with a higher incidence of neuropsychiatric disorders (Loganovsky et al., 2008). This is particularly relevant for subjects working in cardiac catheterization units, who are exposed to a high cumulative levels of low-doses of ionizing radiations, specifically at the left hemisphere, given the standard setup in the catheterization lab (Andreassi et al., 2016). It has been shown that this condition is associated with impairment of cognitive performance and may therefore represent a professional risk (Marazziti et al., 2015). Since irradiation affects brain activity (Loganovsky and Yuryev, 2001; Loganovsky and Kuts, 2016), it is likely that an asymmetric exposure to ionizing radiations leads to an asymmetric brain activity and, as a consequence to cognitive impairments. Even in this instance TNS, unilaterally applied could be used to reduce asymmetries in brain excitability, thus resulting in an improvement of cognitive performance.

AUTHOR CONTRIBUTIONS

VC and MPTF planned the manuscript layout, performed bibliographical research and wrote part of the manuscript. EC performed bibliographical research and wrote part of the manuscript. MB and LB performed bibliographical research and provided critical insights. UF and DM conceived and wrote the manuscript.

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frontiers in Neuroanatomy

REVIEW
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The Brainstem in Emotion: A Review

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Emotions depend upon the integrated activity of neural networks that modulate arousal, autonomic function, motor control, and somatosensation. Brainstem nodes play critical roles in each of these networks, but prior studies of the neuroanatomic basis of emotion, particularly in the human neuropsychological literature, have mostly focused on the contributions of cortical rather than subcortical structures. Given the size and complexity of brainstem circuits, elucidating their structural and functional properties involves technical challenges. However, recent advances in neuroimaging have begun to accelerate research into the brainstem's role in emotion. In this review, we provide a conceptual framework for neuroscience, psychology and behavioral science researchers to study brainstem involvement in human emotions. The "emotional brainstem" is comprised of three major networks – Ascending, Descending and Modulatory. The Ascending network is composed chiefly of the spinothalamic tracts and their projections to brainstem nuclei, which transmit sensory information from the body to rostral structures. The Descending motor network is subdivided into medial projections from the reticular formation that modulate the gain of inputs impacting emotional salience, and lateral projections from the periaqueductal gray, hypothalamus and amygdala that activate characteristic emotional behaviors. Finally, the brainstem is home to a group of modulatory neurotransmitter pathways, such as those arising from the raphe nuclei (serotonergic), ventral tegmental area (dopaminergic) and locus coeruleus (noradrenergic), which form a Modulatory network that coordinates interactions between the Ascending and Descending networks. Integration of signaling within these three networks occurs at all levels of the brainstem, with progressively more complex forms of integration occurring in the hypothalamus and thalamus. These intermediary structures, in turn, provide input for the most complex integrations, which occur in the frontal, insular, cingulate and other regions of the cerebral cortex. Phylogenetically older brainstem networks inform the functioning of evolutionarily newer rostral regions, which in turn regulate and modulate the older structures. Via these bidirectional interactions, the human brainstem contributes to the evaluation of sensory information and triggers fixed-action pattern responses that together constitute the finely differentiated spectrum of possible emotions.

Keywords: brainstem, emotion, networks, interoception, feeling, midbrain, pons, medulla

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INTRODUCTION

Emotions are mental and bodily responses that are deployed automatically when an organism recognizes that a situation warrants such a reaction (Damasio, 1994). Due to humans' intellectual capacities, human emotional reactions are not necessarily triggered by immediate (real) physical or social circumstances, but can also be precipitated by inferences, memories, beliefs or imaginings (Immordino-Yang, 2010). Although human emotions can involve complex cognitive deliberations (Immordino-Yang, 2010, 2015) their activating power fundamentally depends upon the modulation of arousal, motor control and somatosensation. Emotions are therefore regulated by a broad range of subcortical and cortical structures, with a critical role being played by subcortical nuclei in the pontine and midbrain tegmentum (Nauta, 1958; Parvizi and Damasio, 2001), as well as by autonomic and cardiorespiratory nuclei in the medulla (Edlow et al., 2016). Currently, most investigations of human emotion, especially in the neuropsychology literature, have focused on contribution of cortical rather than subcortical structures to human emotion, with a few notable exceptions (Buhle et al., 2013). Given that the brainstem plays a critical role in regulating and organizing emotion-related processing, the aim of this review is to provide a conceptual framework for affective researchers to study the brainstem's role in human emotion.

ORGANIZATION OF BRAIN REGIONS INVOLVED IN EMOTION

For the purpose of studying its role in emotion, the brainstem can be conceptualized as being composed of Ascending, Descending, and Modulatory networks. The gray matter nodes and white matter connections within each of these networks are summarized in **Table 1**, while **Figure 1** provides a schematic overview of the networks' brainstem nodes. Our description of an Ascending sensory network in the brainstem that contributes to emotion is rooted in prior work by Parvizi and Damasio (2001) and Damasio and Carvalho (2013). The Descending network is based upon the "emotional motor system" initially proposed by Holstege (2009). The Modulatory network is based upon evidence showing that multiple brainstem-derived modulatory neurotransmitters contribute to emotion and emotional behavior (Alcaro et al., 2007; Berridge and Kringelbach, 2008; Dayan and Huys, 2009).

Integration of signaling within these three networks occurs at all levels of the brainstem, while progressively more complex levels of integration occur in the thalamus, hypothalamus and cerebral cortex. This encephalization and hierarchical organization allows phylogenetically older pathways in the brainstem, which evaluate sensory information and give rise to fixed-action pattern responses, to be regulated by evolutionarily newer rostral regions (Tucker et al., 2000). It is important to emphasize here that this conceptual model is based upon limited information about the functioning of the human brainstem, and will likely require revision and further differentiation as new evidence arises (Seeley et al., 2007; Coenen et al., 2011; Hermans et al., 2014).

TABLE 1 | The three networks of brainstem structures involved in emotion processing, and their components.

Network	Important structures
Ascending (sensory)	Spinothalamic tracts; Medial forebrain bundle; Nucleus of the tractus solitarius; Parabrachial nuclear complex; Thalamic nuclei
Descending (motor)	Lateral: periaqueductal gray and its projections Medial: Caudal raphe nuclei, locus coeruleus and their projections
Modulatory	Raphe nuclei (serotonergic) Locus coeruleus (noradrenergic) Ventral tegmental area (dopaminergic) Pedunculopontine and laterodorsal tegmental nuclei (cholinergic)

ASCENDING NETWORK

Damasio's (1996) Somatic Markers Hypothesis suggests that emotion processing incorporates somatosensory and visceral feedback from the periphery, either directly or through intervening sensory representations in caudal structures. Multiple representations of the body state in the brainstem and in the insular cortices are believed to enable simulation of future actions and sensations to guide decision making, as well as to contribute to empathy and theory of mind in humans. Self-awareness may arise from successive temporal representations of the body with increasing levels of detail (Craig, 2003a). Even the simple sensory representations of the body in the brainstem nuclei can alter affective experience, as demonstrated by studies showing that subtle modulation of a subject's facial expressions can change self-reported affect (Harrison et al., 2010).

Interoception, which is the sense of the internal condition of the body, and emotional feeling, may share a common route through the brainstem to the anterior insular cortex (Craig, 2003a; Drake et al., 2010). The interoceptive system, represented in the cortex by the insula and adjacent regions of the frontal operculum, is particularly important for the internal simulation of observed emotion in humans (Preston et al., 2007; Pineda and Hecht, 2009) and for the experience of complex social emotions (Immordino-Yang et al., 2009, 2014, 2016). The other body map in the somatosensory cortex, which is built from dorsal column inputs and segments of the anterolateral pathway, contributes to affective understanding by simulation of facial expressions (Pineda and Hecht, 2009), analogous to the proposed function of primate mirror neurons in perception/action coupling (Rizzolatti and Craighero, 2004).

The neuroanatomic basis for the Ascending sensory network and the mechanisms by which it modulates human emotion remain poorly understood. Although the structural and functional properties of these ascending pathways have been studied extensively in rodents and non-human primates using premortem tract-tracing and invasive electrophysiological studies, these techniques cannot be applied in humans. Recent studies using diffusion tractography and resting-state

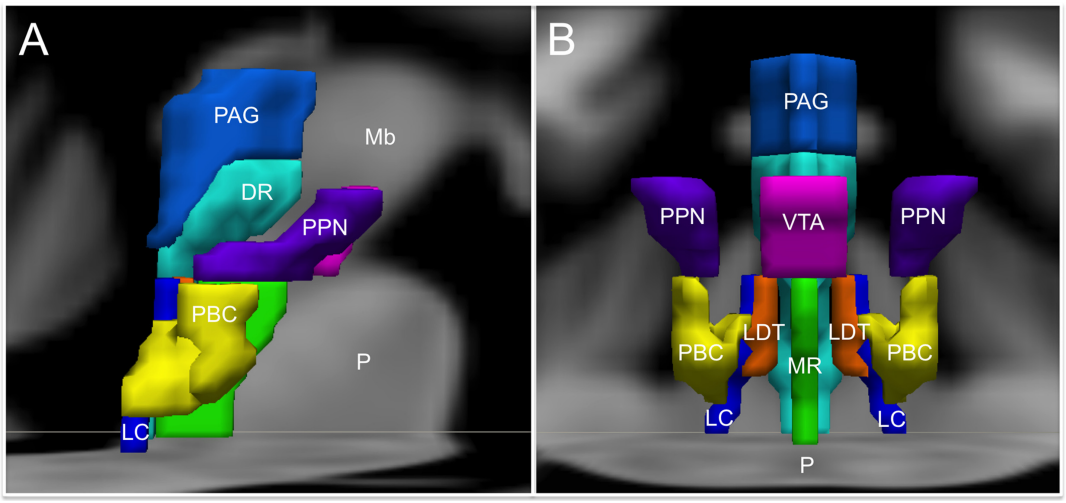

FIGURE 1 | Brainstem nuclei involved in human emotion. (A) Sagittal view and **(B)** Coronal view. DR, Dorsal Raphe; LC, Locus coeruleus; LDT, Laterodorsal tegmental nucleus; Mb, Midbrain; MR, Median raphe; P, Pons; PAG, Periaqueductal gray; PBC, Parabrachial nuclear complex; PPN, Pedunculopontine nucleus; VTA, Ventral tegmental area. The substantia nigra and the nucleus of the tractus solitarius are not shown to optimize visibility of the other structures.

functional connectivity techniques in humans have found that forebrain regions involved in regulation of mood and affect are interconnected not only with mesencephalic and pontine arousal nuclei, but also with medullary cardiorespiratory and autonomic nuclei through the medial and lateral forebrain bundles (Vertes, 2004; Edlow et al., 2016). **Figure 2** provides an overview of the main structures in the Ascending network.

It is well established that sensations from the human body are carried in two major ascending pathways in the brainstem – the dorsal columns of the spinal cord, which continue as the medial lemnisci, carry discriminatory sensation, deep touch and proprioception; the anterolateral pathway, composed of the spinothalamic tracts, carries nociceptive and temperature-related signals (Nogradi et al., 2000-2013).

The Anterolateral Pathway

The nociceptive fibers in the anterolateral pathway give off collaterals at every level that converge with projections from visceral sensory neurons in the brainstem, thereby ensuring close coordination of pain and autonomic processing (Craig, 2003b). The pathway begins with small-diameter fibers that transmit signals of fast and slow pain, chemical changes, temperature, metabolic state of muscles, itch, and sensual or light touch to lamina I of the spinal cord, from where ascending projections arise. In the caudal brainstem, these projections target the nucleus of the tractus solitarius in the medulla (**Figure 2**), which is also innervated by visceral and taste sensations through the vagus, glossopharyngeal and facial nerves.

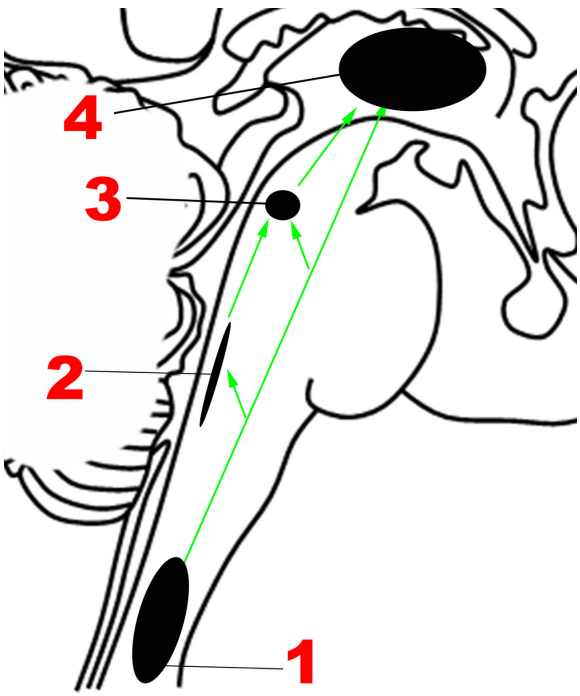

FIGURE 2 | Major structures involved in the Ascending network. (1) Spinothalamic tracts. (2) Nucleus of the tractus solitarius. (3) Parabrachial nuclear complex. (4) Thalamus. Green arrows: Ascending projections.

The Parabrachial Complex

Tract-tracing studies in rodent models have revealed that ascending projections from the nucleus of the tractus solitarius travel to the parabrachial complex (**Figures 1**, **2**) in the upper pons (Herbert et al., 1990), which also receives direct projections from lamina I neurons (Craig, 2003b), in addition to other inputs such as balance (Balaban, 2002). Rat studies suggest that the parabrachial complex integrates multiple types of converging sensory inputs and in turn projects to rostral regions such as the thalamus, hypothalamus, basal forebrain and amygdala, and may

play an important role in arousal (Fuller et al., 2011; Edlow et al., 2012). The upper brainstem, where the parabrachial complex lies, is therefore the most caudal structure where a topographically complete map of the body can be assembled that includes all manner of interoceptive information (Damasio and Carvalho, 2013). There is also ongoing investigation of the role played by the superior colliculus, a structure in the dorsal aspect of the upper brainstem, in sensory and emotional processing in humans, but the available evidence is sparse (Celeghin et al., 2015).

The Thalamus

Immediately rostral to the upper brainstem is the thalamus, and the spinothalamic tracts, as their name indicates, end in the thalamus. A subset of thalamic nuclei function as relay structures between the emotional brainstem and rostral brain structures. The ventral posteromedial nuclei of the thalamus, which receive projections from the parabrachial complex and other parts of the anterolateral pathway, project to the insular cortex, particularly the mid/posterior dorsal part. Craig and colleagues suggested that the posterior part of the ventral medial nucleus of the thalamus, or VMPo, was uniquely involved in pain processing, particularly in primates (Craig, 2003a), but other authors had questioned the separate existence of this nucleus (Willis et al., 2002).

The intralaminar nuclei of the thalamus receive non-topographical sensory input from the spinal cord, which are in turn projected to the orbitofrontal and anterior cingulate cortices. The intralaminar nuclei are involved in orienting and attention, while arousal and visceral sensation are subserved by the midline nuclei (Morgane et al., 2005). In primates a direct pathway from lamina I to the anterior cingulate through the medial dorsal nucleus is also present (Craig, 2003a), and it has been suggested that these pathways may mediate the affective aspect of pain (Tucker et al., 2005). Indeed, the mediodorsal nucleus progressively increases in cytoarchitectonic complexity in higher animals, and is also known to project to the frontal and prefrontal cortices (Morgane et al., 2005). Thus, the thalamus contains multiple structures that appear to play a role in transmitting the signals essential for emotion processing from the brainstem to the forebrain.

Summary statement: Representations of the body of varying degrees of complexity that exist at multiple levels in the Ascending network, including the nucleus of the tractus solitarius and the parabrachial nucleus, are believed to be give rise to the "feeling" of an emotion.

DESCENDING NETWORK

The chief descending pathway in the human brainstem is composed of large, myelinated axons of the corticospinal tracts, transmitting motor impulses to the anterior horn cells of the spinal cord and thereafter to skeletal musculature (Nogradi and Gerta, 2000–2013). In addition, the midbrain and pontine tegmentum, as well as the medulla, contain several structures that serve as the output centers for motor and autonomic regulatory systems, which in turn regulate the bodily manifestations of the "emotion proper" (Damasio, 1994). Holstege (2009) considered the interconnected network of descending fibers and effector regions in the brainstem an "emotional motor system," distinct from the corticospinal somatic motor pathway, each of which they divided into lateral and medial parts [**Figure 3**, adapted from (Holstege, 2016)].

The brainstem, as noted previously, contains a hierarchy of circuits linking ascending sensory neurons and descending effector neurons. Evidence from rat and cat studies indicates that the lower-level circuits enable quick stereotypical responses to stimuli, while the higher-level involvement of rostral centers allows for complex motor and autonomic activity and action specificity (Bandler et al., 2000; Gauriau and Bernard, 2002). This close relationship between sensory and effector networks in emotion processing is best illustrated by the close overlap seen between sites involved in emotional vocalization and pain processing in animals. Both physical and psychological pain (caused by separation from caregivers, for example) can produce distress vocalizations in animals, with the caudal brainstem containing multiple regions that control the respiratory and phonetic changes of vocalization (Tucker et al., 2005) and cardiorespiratory function during emotion (Lovick, 1993; Rainville et al., 2006; Edlow et al., 2016). The rostral nuclei are able to modulate the activity of caudal nuclei that control cardiorespiratory control and vocalization in a coordinated manner that makes the resultant action more complex and nuanced.

Lateral Part of the Emotional Motor System

The emotional motor system's lateral part consists of projections primarily from the periaqueductal gray, as well as more rostral structures such as the amygdala and hypothalamus, to the lateral tegmentum in the caudal pons and medulla (**Figures 3**, **4**). This lateral part of the emotional motor system is involved in specific motor actions invoked in emotions, as well as in the control of heart rate, respiration, vocalization, and mating behavior (Holstege, 2009). Studies in multiple animal models as well as in humans have revealed that the periaqueductal gray (**Figures 1**, **4**) is a major site of integration of affective behavior and autonomic output, with strong connections to other brainstem structures (Behbehani, 1995).

Several fixed patterns of behavior, particularly those related to responding to external threats, with accompanying autonomic changes, are organized in the different columns of the periaqueductal gray in rats (Brandao et al., 2008). The lateral/dorsolateral column receives well-localized nociceptive input (superficial 'fast' pain, as might be expected from bites or scratches) and is believed to organize fight-or-flight reactions. When stimulated this column produces emotional vocalization, confrontation, aggression and sympathetic activation, shown by increased blood pressure, heart rate, and respiration. Many of these responses are mediated by descending projections to the paragigantocellularis lateralis nucleus in the rostral ventrolateral medulla (respiratory rhythm), the dorsal motor nucleus of the vagus (heart rate and rhythm), and caudal raphe

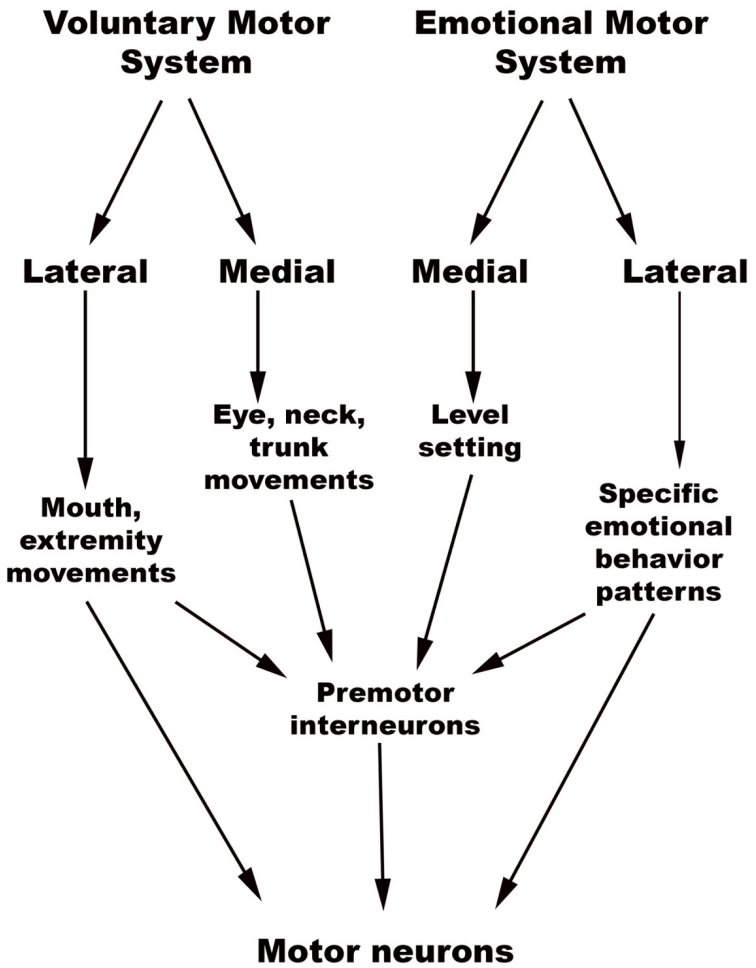

FIGURE 3 | Holstege's conception of the Emotional and Somatic motor systems. (Adapted from Holstege, 2016).

(cardiorespiratory integration; Lovick, 1993; Edlow et al., 2016). Within this dorsolateral/lateral column itself, there are two parts. The rostral part is responsible for power/dominance (producing a "fight" response), while the caudal part invokes fear (producing a "flight" response) with blood flow to the limbs (Sewards and Sewards, 2002).

The ventrolateral column of the periaqueductal gray receives poorly localized "slow, burning" somatic and visceral pain signals, and on stimulation produces passive coping, long-term sick behavior, freezing with hyporeactivity and an inhibition of sympathetic outflow (Parvizi and Damasio, 2001; Craig, 2003b; Brandao et al., 2005; Benarroch, 2006). In this way, it is likely involved in background emotions such as those that contribute to mood. Rat studies have further revealed that lesions of the dorsolateral periaqueductal gray reduce innate defensive behaviors, while lesions of the caudal ventrolateral part reduce conditioned freezing and increase locomotor activity (Brandao et al., 2005). When the predator is far away, the ventromedial prefrontal cortex and the hippocampus, through the amygdala, activate midbrain structures centered around the ventrolateral periaqueductal gray, which results in freezing (Tucker et al., 2000). In the "circa-strike" stage when the predator is imminent, forebrain pathways are silenced, and the dorsolateral periaqueductal gray is activated, resulting in fight-or-flight reactions.

The Periaqueductal Gray in Human Emotion

Though the reactions detailed above are almost certainly incorporated into human emotion, the precise mechanisms have not been elucidated. One study involving high-resolution MRI of the human periaqueductal gray indicated that this structure has discrete functional subregions that parallel the divisions seen in animals – aversive stimuli caused activation in the ventrolateral regions of the caudal periaqueductal gray and in the lateral/dorsomedial regions of the rostral periaqueductal gray (Satpute et al., 2013). The periaqueductal gray threat response system is likely co-opted in the pathophysiology of conditions such as panic disorder and generalized anxiety disorder. Blood flow analysis suggests that the inhibitory influence of the cortex over the fight-or-flight mechanisms

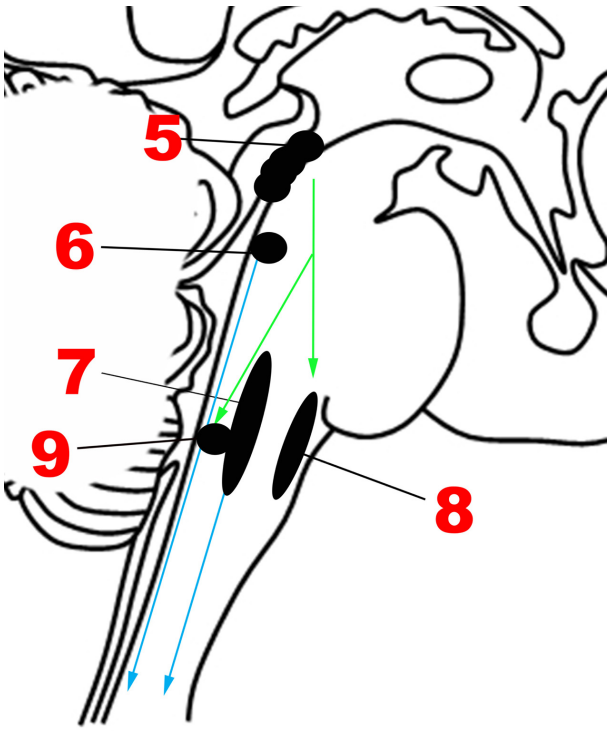

FIGURE 4 | Major structures involved in the Descending network. (5) Periaqueductal gray. (6) Locus coeruleus. (7) Caudal raphe nuclei. (8) Rostral ventrolateral medullary nuclei. (9) Dorsal motor nucleus of the vagus nerve. Green arrows: Descending projections from periaqueductal gray. Blue arrows: Descending projections from the caudal raphe and locus coeruleus.

in the periaqueductal gray is reduced in panic disorder (Del-Ben and Graeff, 2009). Functional MRI has also revealed activation of the human periaqueductal gray in complex emotions such as frustration (Yu et al., 2014), admiration and compassion (Immordino-Yang et al., 2009), in addition to more immediate threat responses (Lindner et al., 2015).

Medial Part of the Emotional Motor System

The medial part of the emotional motor system (**Figures 3**, **4**) consists of descending projections from the reticular formation that are involved in level-setting and modulatory functions (Holstege, 2009). Once again, the vast majority of the research on this subject has been in animals. The caudal third of the locus coeruleus (Sasaki et al., 2008) and the caudal raphe nuclei both send projections downward to the spinal cord, as depicted in **Figure 4**, and are responsible for descending pain modulation (Renn and Dorsey, 2005). The effect of norepinephrine from the locus coeruleus is mostly antinociceptive, while serotonin from the raphe nuclei can have varying effects depending upon the type of receptor activated (Benarroch, 2008). In rats, it has been shown that the midbrain tectum and the dorsal/lateral periaqueductal gray indirectly produce the analgesia that occurs in fear (Coimbra et al., 2006), through a primarily non-opioid mechanism involving GABAergic and serotonergic neurons (as opposed to the ventrolateral periaqueductal gray that produces a long-lasting opioid mediated analgesia; Gauriau and Bernard, 2002). It is likely that this system of fear suppressing the pain system is still present in humans, allowing us to act and move rapidly in situations of threat (Mobbs et al., 2007).

In addition to nociceptive modifications, the medial part of the emotional motor system is also involved in level-setting for arousal levels and muscle function – studies on rodents and monkeys indicate that this is accomplished through norepinephrine secretion from the locus coeruleus (Aston-Jones and Cohen, 2005; Lang and Davis, 2006) and cholinergic projections from the pedunculopontine tegmental nucleus in the upper pons (Bechara and van der Kooy, 1989; Homs-Ormo et al., 2003). Further detail regarding these important structures is provided in the section below on the Modulatory network.

Summary statement: The Descending network, otherwise referred to here as the emotional motor system, has a lateral part that triggers patterned emotional behaviors, while the medial part is responsible for level-setting in sensory and arousal systems that might be important in emotionally charged situations.

MODULATORY NEUROTRANSMITTER NETWORK – VALENCE, AROUSAL, AND REWARD

Since a major characteristic of an adaptive emotional behavioral response is flexibility, a network that modulates the autonomic, motor, affective and memory changes brought about by different stimuli is needed. The chief upper brainstem structures involved in this modulation are the neurotransmitter pathways arising from the upper raphe nuclei (serotonergic), the ventral tegmental area-substantia nigra pars compacta complex (dopaminergic), and the upper locus coeruleus (noradrenergic), which project widely throughout the hypothalamus, cortex and other parts of the forebrain. In addition, the laterodorsal and the pedunculopontine tegmental nuclei are sources of cholinergic fibers, which stimulate cortical activation through the thalamus. These structures are depicted in **Figures 1**, **5**. Ascending projections from the brainstem to subcortical and cortical structures communicate the states of brainstem structures to more rostral regions of the nervous system, where these states contribute to affective experience. Since these pathways are involved in arousal and in the maintenance of consciousness (Jones, 2003), they are sometimes called the Ascending Reticular Activating System or Ascending Arousal Network (Moruzzi and Magoun, 1949; Edlow et al., 2012). The following sections on the various pathways that comprise the Modulatory network are in large part descriptions of the Ascending Reticular Activating System, albeit with a focus on how these relate to emotion.

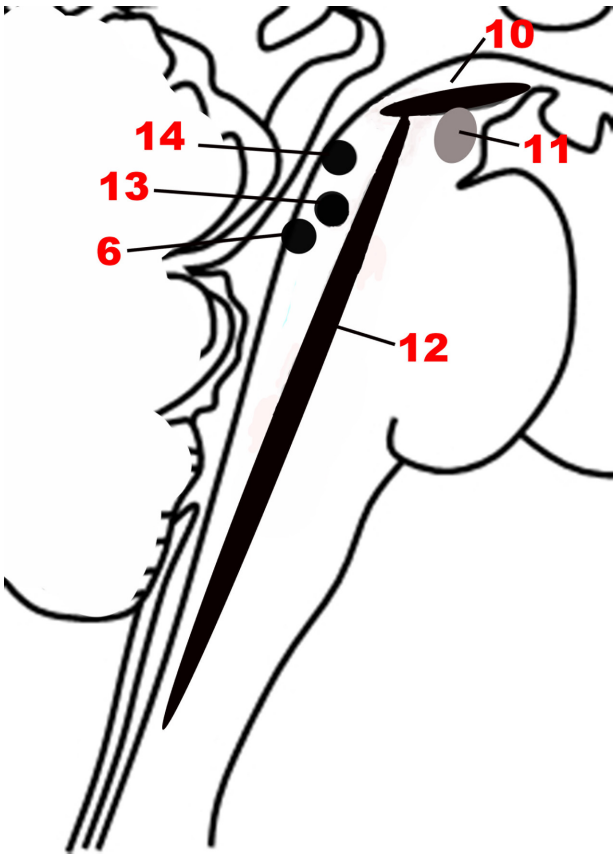

FIGURE 5 | The nuclei of the Modulatory network. (10) Substantia nigra. (11) Ventral tegmental area. (12) Raphe nuclei. (6) Locus coeruleus. (13) Pedunculopontine nucleus. (14) Laterodorsal tegmental nucleus.

The Valence-Arousal Model of Emotion and Its Critiques

The modulation of affective states by these upper brainstem-based pathways has been expressed through the two domains of valence and arousal. According to the circumplex model of emotions, each basic emotion is postulated to be a combination of these two domains, in differing degrees (Russell, 1980; Zald, 2003; Posner et al., 2009). In humans, valence correlates with pleasantness ratings, heart rate, and facial muscle activity, while arousal correlates with skin conductance, interest ratings and viewing time for stimuli (Lang and Davis, 2006). Both valence and arousal have significant impact on an organism's relationship with the environment, influencing, for example, the allocation of attention and long term memory formation (Arbib and Fellous, 2004).

Recent work, especially in the neuroimaging literature, has raised questions about whether complex neurological processes like emotions can actually be represented by reducing to dimensions of valence and arousal. Kragel and LaBar (2016), in an interesting review of the nature of brain networks that subserve human emotion, argue that each emotion uniquely correlates with activation of a constellation of cortical and subcortical structures (Kragel and LaBar, 2016), and that the current neuroimaging data do not support the valence-arousal model of emotions. They focused on fMRI studies which have applied novel statistical methods collectively known as multivoxel pattern analysis to identify mappings between mental states and multiple measures of neural activity. The mainstay of earlier neuroimaging research on emotion was univariate pattern analysis, but multivariate analyses have the advantages of higher sensitivity, and the ability to detect counterintuitive relationships because of the lack of reliance on *a priori* hypotheses. These approaches also have the advantage of overcoming the assumption that dedicated modules or homogeneous neural units subserve each emotion, because they can investigate various neuronal populations at much larger spatial scales.

Kragel and LaBar (2016) suggest that while the use of machine learning approaches to large neuroimaging datasets is likely to expand in the near future, it might be premature to draw conclusions about neural substrates underlying each emotion, because the current studies using multivariate analyses have not all been consistent with one another. These differences may be coming from technical variations in the methods used to induce and assess the emotion and associated neural activations, but might also represent fundamental variations in the circuitry employed in different individuals, or even a lack of emotional "essences" that can be studied in a standardized manner across people and cultures. While this is a valid critique, we believe that the older valence-arousal classification still holds value in furthering our understanding of brainstem contributions to emotions and especially to basic emotions shared with intelligent animals. This debate may eventually be resolved with technical advances in functional neuroimaging and multidisciplinary approaches to studying emotional experiences (Immordino-Yang and Yang, 2017, in press).

DOPAMINE AND REWARD PATHWAYS

Emotional valence is closely tied to the concept of reward and punishment (Dayan and Huys, 2009). Rewards, both natural (such as that induced by social play in animals) and drug-induced, include both hedonic and motivational aspects (Trezza et al., 2010). While the core hedonic status, as demonstrated by consummatory pleasure and facial expressions, is believed to be primarily based on opioid transmission, motivation is more dependent on dopamine transmission (Alcaro et al., 2007). The separation between the motivation and liking systems may have allowed the same motivational circuitry to be used in positive and negative events (Berridge and Robinson, 2003).

Anatomy

Brainstem dopaminergic neurons in mammals are located in the midbrain, and are typically divided into three contiguous groups: the retrorubral field, the substantia nigra pars compacta, and the ventral tegmental area (**Figures 1**, **5**). There are two major ascending dopaminergic pathways arising from these clusters: the nigrostriatal pathway from the substantia nigra pars compacta, and the mesocorticolimbic pathway from the ventral

tegmental area (Arias-Carrion and Poppel, 2007). The medially situated mesocorticolimbic pathway is evolutionarily older than the laterally situated nigrostriatal pathway (Alcaro et al., 2007). The dopaminergic neurons of the ventral tegmental area are subject to feedback inhibition from the cortex and the striatum. The pedunculopontine nucleus also sends ascending projections that have been shown to affect tonic dopamine release and arousal in studies on rats and monkeys (Sesack et al., 2003; Xiao et al., 2016).

Function

The mesocorticolimbic pathway is thought to be part of a larger, general-purpose appetitive foraging system in animals that enables establishment of adaptive expectations about the configurations and reward-availability in the environment, with dopamine inducing a "seeking" disposition toward the environment (Alcaro et al., 2007). This seeking disposition itself may have hedonic properties independent of reward attainment. Studies on rats and other models indicate that dopaminergic neurons in the ventral tegmental area show both tonic activity, maintaining a baseline level of dopamine in the brain, and phasic burst firing in response to certain cues (Grace, 1991). The two are believed to antagonize each other (Ikemoto, 2007). Unpredicted rewards, prediction errors (Song and Fellous, 2014), novel stimuli (Bunzeck and Duzel, 2006), physically salient stimuli, motivational/affective salience, and attention shifts related to approach behavior are all potential causes of altered dopaminergic firing based on studies in rats, monkeys and humans, although only a subpopulation of the neurons in the ventral tegmental area may be activated in each case (Schultz, 2010). Tonic dopamine release, on the other hand, promotes arousal in almost all mammals, and this is likely achieved by D2-receptor-mediated inhibition of cortical and limbic top-down control over subcortical structures (Alcaro et al., 2007; Song and Fellous, 2014).

An appetitive/aversive opponency is thought to exist between the serotonin and dopamine pathways, with serotonin antagonizing several energizing and appetitive effects of dopamine (Dayan and Huys, 2009). One series of experiments on rats showed that single bursts of norepinephrine release from the locus coeruleus activated dopaminergic firing (Grenhoff et al., 1993), but in depressive mood states, sustained burst firing of locus coeruleus neurons was seen, which caused suppression of dopamine release (Grenhoff et al., 1993; Weiss et al., 2005). Significant attention has been focused on the role of dopamine in the motivational deficits seen in depression, schizophrenia, Parkinson's disease and other disorders (Salamone et al., 2016), and the antidepressant bupropion thought to exert its effects through inhibition of the reuptake of both norepinephrine and dopamine (Patel et al., 2016).

Summary statement: Dopaminergic neurons from the ventral tegmental area show both tonic and phasic firing patterns, are involved in reward, motivation, and arousal, and malfunction of these pathways likely contributes to motivational deficits in depression.

SEROTONERGIC PATHWAYS AND THE RAPHE NUCLEI

Anatomy

The cell bodies of all the serotonergic neurons in the human brain lie in the raphe nuclei (**Figures 1**, **5**). They are clustered along the midline throughout the brainstem. The rostral group lies in the midbrain and upper pons (caudal linear, dorsal raphe, and median raphe nuclei), while the caudal group lies in the lower pons and medulla (raphe magnus, raphe obscurus, and raphe pallidus nuclei). The rostral raphe nuclei mainly send ascending projections, while the caudal raphe send descending projections as discussed above (Hornung, 2003).

Function

Serotonin modulates the sensitivity of the fear/defense circuitry and the magnitude of these responses in response to various stimuli. Inescapable shock, for instance, produces inhibition of the fight-flight defensive response and activation of the fear-anxiety response in rats (Maier and Watkins, 2005). This might be through its suppression of panic and escape reactions encoded in the dorsal periaqueductal gray (Zangrossi et al., 2001). Serotonin is also involved in regulation of social behaviors such as aggression, status-seeking and affiliation (Arbib and Fellous, 2004; Gobrogge et al., 2016). It is believed to enable prosocial and agreeable behavior in humans as well as other animals (Moskowitz et al., 2003). A correlation between anxiety, depression and serotonin is suggested by the effectiveness of Selective Serotonin-Reuptake Inhibitor (SSRI) drugs in mood disorders (Adell, 2015). The dorsomedial part of the dorsal raphe is particularly important for anxiety-related processing in humans, receiving innervations from several forebrain structures implicated in anxiety, including the bed nucleus of the stria terminalis (Lowry et al., 2008).

It must be noted that studies on the role of serotonin in affective control have yielded contradictory results (Dayan and Huys, 2009). Though serotonin projections to the amygdala enhance anxiety, those to the hippocampus are associated with depression and the retrieval of fear memories, and are known to contribute to hyperalgesic effects in times of stress (Dayan and Huys, 2009; Ohmura et al., 2010). One possibility, as noted by Gold (2015), is that the level of arousal may be an important factor in determining how abnormalities in serotonergic signaling manifest themselves. The chief distinction between melancholic or typical depression and atypical depression is that the former is worst in the morning, when arousal systems are at their maxima, while the latter is the worst in the evenings, when arousal systems are winding down (Gold, 2015). Another explanation is that the tremendous diversity in the types of serotonin receptors allows it to exert varying effects in relation to emotion and mood (Meneses and Liy-Salmeron, 2012).

Summary statement: Serotonin from the raphe nuclei appears to perform different functions in anxiety, stress, depression, and social behavior, likely because it acts through a diverse set of receptors, and its role may vary with the level of arousal.

NOREPINEPHRINE AND THE LOCUS COERULEUS

Anatomy

Studies in the monkey have revealed that the locus coeruleus (**Figures 1**, **4**, **5**) is innervated by the amygdala, anterior cingulate and orbitofrontal cortices, which are rostral centers involved in evaluating the motivational significance of a stimulus, as well as the raphe, which transmit viscerosensory stimuli from the nucleus of the tractus solitarius (Aston-Jones and Waterhouse, 2016). Thus the locus coeruleus, which is activated by stress, can integrate both external sensory and visceral signals and influence several effector targets, including the arousal pathways and the adrenal medulla (Sara, 2009).

Function

The role of norepinephrine is understood to be twofold. It maintains a basal level of neuronal activity in the forebrain for the acquisition of sensory input (alertness), and also contributes to the level-setting in circuits involved in gathering and processing of salient, emotionally relevant information in both humans and animals (van Stegeren, 2008; Espana et al., 2016). Additionally, monkey experiments have indicated that norepinephrine and dopaminergic pathways may play a synergistic role in learning (Aston-Jones and Cohen, 2005). Studies on monkeys and fMRI investigations on humans suggest that arousal levels, primarily as determined by norepinephrine signaling, may gate learning, determining which events are prioritized for encoding in memory, and which are allowed to be forgotten (Mather and Sutherland, 2011). Beta-adrenoceptors appear particularly important in suppressing memory for less emotionally salient stimuli, and there may be an interaction of the norepinephrine signaling with sex hormones, especially in women (Clewett, 2016).

Summary statement: Norepinephrine pathways from the locus coeruleus are important in maintaining arousal, and also in level-setting for gathering sensory information and storing emotional memories.

CHOLINERGIC PATHWAYS IN THE BRAINSTEM

Anatomy

The pedunculopontine nucleus and the laterodorsal tegmental nucleus (**Figures 1**, **5**) are the main cholinergic cell groups in the human brainstem. They provide the major cholinergic innervations of the thalamic relay nuclei and the reticular nucleus of the thalamus (Mesulam, 1995; Saper et al., 2005). Cortical structures, on the other hand, receive cholinergic innervations from cell groups outside the brainstem, in the basal forebrain (Mesulam, 2004).

Function

Hyperpolarization of the GABAergic neurons in the reticular nucleus of the thalamus by cholinergic projections from the brainstem ultimately results in disinhibition of thalamic nuclei, and thereby influence level of arousal by gating of connections between the thalamic relay nuclei and cortical regions (Mesulam, 1995; Saper et al., 2005). Brudzynski (2014) suggests, based on anatomical studies in cats and rats that the ascending cholinergic projections from the laterodorsal tegmental nucleus to the forebrain and diencephalon form a mesolimbic pathway related to aversive emotional states, parallel to the mesocorticolimbic dopamine pathway that plays a role in motivation and positively valenced states. Stimulation of this pathway has been shown to produce an aversive response with distress vocalizations in these animal models, suggesting that this pathway serves as a "physiological, psychological, and social arousing and alarming system." The pedunculopontine and laterodorsal nuclei have also been found to project extensively to the ventral tegmental area and substantia nigra pars compacta in a rat model, and are involved in reward processing through their effects on the dopaminergic pathways (Xiao et al., 2016).

Summary statement: The poorly studied cholinergic pathways in the brainstem are part of the ascending reticular activating system, and are thought to influence emotion primarily through their modulation of dopaminergic signaling.

OTHER TRANSMITTERS IN EMOTION

GABAergic mechanisms serve to limit the arousal caused by the neurons of the Ascending Reticular Activating System (Lu and Greco, 2006). Histamine from the tuberomamillary nucleus in the hypothalamus is believed to increase neocortical arousal, and is involved in the modulation of other neurotransmitter pathways (Brudzynski, 2014). Hypocretin/orexin neurons arising from the hypothalamus project widely to various targets, including the limbic areas, and apart from modulating arousal, animal studies have also implicated them in fear and anxiety, reward processing (through projections to the Ventral Tegmental Area) and stress (Flores et al., 2015). Endorphins, endocannabinoids, and oxytocin are some other transmitter pathways known to play a role in emotion and valence. These pathways are not considered in greater detail here since their major source structures do not localize primarily to the brainstem tegmentum.

CONCLUSION AND FUTURE DIRECTIONS

The brainstem contains several structures that are likely of critical importance in the generation and experience of emotion. Most prior research on human emotion has focused on cortical mechanisms, largely because of the complexity of the brainstem coupled with the difficulty of analyzing brainstem functioning using current technologies. We have provided a conceptual overview of how tegmental structures of the brainstem are involved in emotion-related processes. Future research on the structural and functional connectivity of the human brainstem is needed to further understand its role in emotion. Such

work will undoubtedly contribute to a more enriched and nuanced understanding of the neurobiology of human emotion in psychology and in affective neuroscience.

AUTHOR CONTRIBUTIONS

Conceptualization was by AV, BE, and MHI-Y. AV contributed to writing and framing the manuscript. Critical revision and guidance was provided by BE and MHI-Y. Figures were made by AV and BE.

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frontiers in Neuroanatomy

REVIEW
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Reticular Formation and Pain: The Past and the Future

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The involvement of the reticular formation (RF) in the transmission and modulation of nociceptive information has been extensively studied. The brainstem RF contains several areas which are targeted by spinal cord afferents conveying nociceptive input. The arrival of nociceptive input to the RF may trigger alert reactions which generate a protective/defense reaction to pain. RF neurons located at the medulla oblongata and targeted by ascending nociceptive information are also involved in the control of vital functions that can be affected by pain, namely cardiovascular control. The RF contains centers that belong to the pain modulatory system, namely areas involved in bidirectional balance (decrease or enhancement) of pain responses. It is currently accepted that the imbalance of pain modulation towards pain facilitation accounts for chronic pain. The medullary RF has the peculiarity of harboring areas involved in bidirectional pain control namely by the existence of specific neuronal populations involved in antinociceptive or pronociceptive behavioral responses, namely at the rostroventromedial medulla (RVM) and the caudal ventrolateral medulla (VLM). Furthermore the dorsal reticular nucleus (also known as subnucleus reticularis dorsalis; DRt) may enhance nociceptive responses, through a reverberative circuit established with spinal lamina I neurons and inhibit wide-dynamic range (WDR) neurons of the deep dorsal horn. The components of the triad RVM-VLM-DRt are reciprocally connected and represent a key gateway for top-down pain modulation. The RVM-VLM-DRt triad also represents the neurobiological substrate for the emotional and cognitive modulation of pain, through pathways that involve the periaqueductal gray (PAG)-RVM connection. Collectively, we propose that the RVM-VLM-DRt triad represents a key component of the "dynamic pain connectome" with special features to provide integrated and rapid responses in situations which are life-threatening and involve pain. The new available techniques in neurobiological studies both in animal and human studies are producing new and fascinating data which allow to understand the complex role of the RF in pain modulation and its integration with several body functions and also how the RF accounts for chronic pain.

Keywords: serotonin, noradrenaline, analgesics, opioids, emotions, cognition, connectome

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Abbreviations: AT_1, angiotensin type 1 receptors; DNIC, diffuse noxious inhibitory control; DRt, dorsal reticular nucleus; fMRI, functional magnetic resonance imaging; HSV-1, Herpes Simplex virus type 1; LC, locus coeruleus; LRt, lateral reticular nucleus; NRM, nucleus raphe-magnus; NTS, nucleus of the solitary tract; PAG, periaqueductal gray; RF, reticular formation; RVM, rostroventromedial medulla; Sp5C, spinal trigeminal nucleus; TRPV1, vanilloid receptor type 1; VLM, caudal ventrolateral medulla; VLMlat, caudalmost part of the VLM; WDR, wide-dynamic range.

INTRODUCTION

The involvement of the brainstem reticular formation (RF) in the transmission and modulation of pain is well established. A long path has been covered since the initial anatomical studies demonstrating that the RF projects to the thalamus, passing by the functional approaches showing that manipulation of the RF changes behavioral nociceptive responses and going through imaging studies in humans indicating activation of the RF in response to pain. The study of the role of the RF in pain is challenging due to anatomical and functional reasons. Anatomically, the RF is defined as an aggregation of neurons with several morphological configurations and without distinct connection pattern. Functionally, and besides the sensory component of pain, the RF is involved in a plethora of functions which include arousal, motor reactions, cardiovascular control and visceral functions. This anatomofunctional complexity of the RF deviated neuroscientists from a global study of the involvement of the RF in pain processing and most studies have been directed to specific areas of the RF. Taking into account the concept that pain control cannot be studied apart from other brain functions and also the intrinsic feature of the RF as the brain network, by excellence, it is possible that the RF represents an outstanding example of the "dynamic pain connectome" (Kucyi and Davis, 2015, 2016).

Based on our own experience in the study of the involvement of specific areas of the brainstem RF in pain transmission and modulation in animal models of pain, in this article we critically review the participation of the medullary RF in pain modulation. The medulla oblongata contains three areas of the RF from which a wide variety of data have recently been collected, namely the rostroventromedial medulla (RVM), the caudal ventrolateral medulla (VLM) and the dorsal reticular nucleus (also known as the subnucleus reticularis dorsalis; DRt). After reviewing the anatomofunctional features of the involvement of each area in pain transmission and modulation, we then discuss how the triad RVM-VLM-DRt plays a key role as a gateway to allow pain modulation from the brain to target the spinal cord, i.e., top-down modulation (**Figure 1**). The role of the RVM-VLM-DRt triad in pain processing is proposed as a homeostatic neuronal circuit that allows adequate body reactions in life-threatening events, such as escaping a predator or an intense acute pain (McNaughton and Corr, 2004; Mobbs et al., 2007). Additionally the triad RVM-VLM-DRt is also proposed to be involved in situations in which adequate emotional responses to pain are necessary (Craig, 2003).

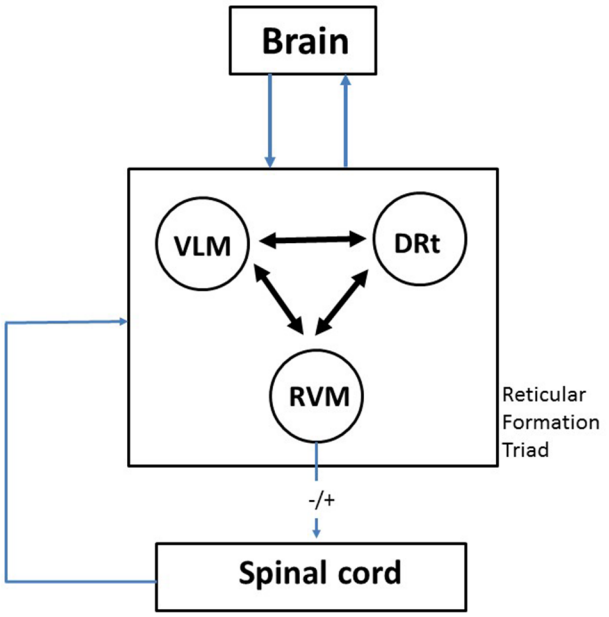

FIGURE 1 | Diagram depicting the crucial role of the medullary reticular formation (RF) triad rostroventromedial medulla (RVM)-ventrolateral medulla (VLM)-dorsal reticular nucleus (DRt) as a gateway between the brain and the spinal cord. The three components of the triumvirate are reciprocally connected in a way which allows integration of responses which are related to pain, namely cardiovascular control and motor reactions. This probably represents a phylogenetically conserved mechanism that subserves the classical "fight or flight" response, activated by acute pain. The emotional and cognitive control exerted by higher brain centers upon the RVM-VLM-DRt triad may account to explain how the emotions and attention affect pain responses. By allowing to balance inhibition and facilitation (−/+) of nociceptive transmission at the spinal cord, the RVM-VLM-DRt medullary triad provides the mechanism to decrease or increase pain. A more detailed description of the neuronal circuits involving one component of the triad—the DRt—is shown in **Figure 2**, namely in what concerns its brain connections and their neurochemical characterization.

ASCENDING AND DESCENDING PATHWAYS

The involvement of the spinoreticulothalamic pathway as a major ascending pathway for nociceptive transmission to the brain is well established. Overall this multisynaptic pathway originated from neurons mainly located in the spinal cord laminae IV–V and VII–VIII targets areas of the medullary and pontine RF which have collaterals of the spinothalamic tract (Lu and Willis, 1999). A role for the RF as a relay to the medial thalamus has emerged and herein conferred the RF an interesting perspective as to its involvement in the motivational-affective components of pain (Willis and Westlund, 1997; Almeida et al., 2004). As to the areas of the RF directly receiving nociceptive information from the spinal cord, our research group performed extensive neuroanatomical tract-tracing studies showing that spinal neurons projecting to the VLM or to the DRt are strongly activated in response to several types of nociceptive stimuli (Tavares et al., 1993; Almeida and Lima, 1997; Castro et al., 2006).

The brainstem RF is not only targeted by nociceptive input from the spinal cord but is also actively involved in modulation of nociceptive transmission from the spinal cord. The association of the RF to descending pain modulation is almost as ancient as the discovery that the periaqueductal gray (PAG) is involved in stimulation-produced analgesia (Heinricher et al., 2009). The PAG plays a critical role in conveying the modulatory influences from higher brain centers involved in aspects of pain

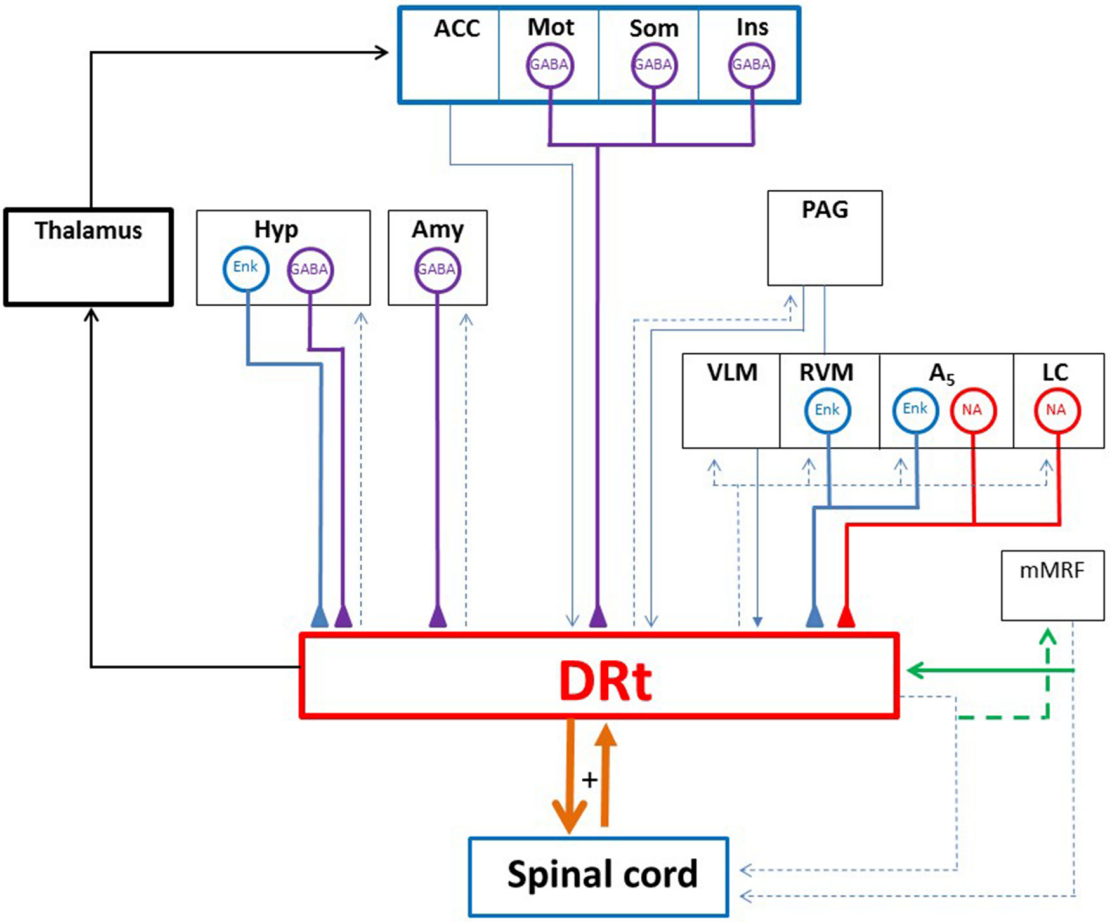

FIGURE 2 | Diagram depicting the DRt connections with the spinal cord and several brain areas. The DRt is involved in a feedback reciprocal loop with the spinal cord (*thick orange lines*) which is involved in pain facilitation. Through its projections to the lateral ventromedial thalamus the DRt participates in a reticulo-thalamo-cortical ascending nociceptive pathway (*thick black lines*). The DRt receives afferent inputs from: (i) higher centers namely the anterior cingulate cortex (ACC), the motor (Mot), somatosensory (Som) and insular (Ins) cortices, the hypothalamus (Hyp) and the amygdala (Amy); and (ii) several brainstem areas namely the periaqueductal gray (PAG), the locus coeruleus (LC), the A_5 noradrenergic cell group along with the two components of the medullary triad (RVM and VLM). The DRt is a major relay for descending pain facilitatory inputs from the ACC, the Hyp and the noradrenergic LC and A_5 areas. The neurochemical characterization of DRt afferents showed that afferents originated in the Mot, Som and Ins cortices as well as the Hyp and the Amy are GABAergic (GABA; *thick purple lines*). Hyp afferents and brainstem afferents located at the RVM and the A_5 area are enkephalinergic (ENK; *thick blue lines*). The LC and the A_5 noradrenergic cell group constitute the main source of noradrenaline (NA; *thick red lines*) released at the DRt. A peculiar reciprocal network is established between the DRt and the medial medullary RF (mMRF) through collaterals (*green lines*) of spinally descending axons. Such an arrangement was described in the cat and is thought to be involved in noxious sensing and nocifensive behavior. Blue thin lines represent neurochemically uncharacterized DRt afferents and dashed lines represent DRt or mMRF efferents.

responses such as cognition and emotion, including the insular and prefrontal cortices and the amygdala (Tracey and Mantyh, 2007). The PAG collects the modulatory influences from these areas and uses the RVM as a relay to indirectly target the spinal cord. Another contrasting and yet important aspect of the participation of the brainstem RF in descending pain modulation derives from the direct arrival of nociceptive input from the spinal cord, namely to the VLM and DRt, described in the next section. In contrast to the RVM, which is does not receive afferents from the spinal cord, the participation of the VLM and DRt in reciprocal loops established with the spinal cord and better analyzed in the next section, allows the RF to perform a fine tuning of nociceptive signals traveling from the spinal cord to the brain.

AN IMPORTANT RF TRIAD IN THE MEDULLA OBLONGATA

In this section we will summarize the anatomical and functional data that support an involvement of the RVM, VLM and DRt in the transmission and modulation of nociceptive information. The specific features of each area will be outlined in each subsection according to the following organization:

(i) anatomical location; (ii) connections with the spinal cord; (iii) nociceptive control; and (iv) local neurochemical systems.

The Rostroventromedial Medulla

Anatomical Location

The RVM includes the midline located nucleus raphe-magnus (NRM) and the adjacent RF in the vicinity of the nucleus reticularis gigantocellularis. In what concerns its involvement in pain modulation, these anatomical components of the RVM are frequently considered together inasmuch that local modulation techniques, such as electrical or chemical stimulation, do not allow a precise discrimination of the NRM from the adjacent RF. Rostrocaudally, the RVM extends from the pontomedullary junction to the level of appearance of the pyramidal decussation.

Connections with the Spinal Cord

The ascending projections from the spinal cord to the RVM are very scarce. The descending input from the RVM to the spinal cord is prominent and targets almost all spinal segments, reaching mainly the dorsal horn (laminae I–V) but also lamina X (Millan, 2002). As to the way in which RVM fibers are organized in spinal neuronal circuits, it was proposed that the descending input from the RVM directly targets spinal neurons involved in ascending transmission of nociceptive input (Urban and Gebhart, 1999). These data are based in the use of tract-tracing techniques and pharmacological manipulation of the spinal cord circuits. According to recent opto/chemogenetic manipulations of the RVM, another arrangement may exist namely by GABA-mediated inhibition of inhibitory spinal interneurons that presynaptically inhibit primary afferent fibers (Francois et al., 2017). This demonstrates that the new techniques used to manipulate neuronal circuits, namely optogenetic manipulation, will allow to uncover the complexity of top-down modulation.

Nociceptive Control

The involvement of the RVM as a crucial relay station from the pain modulatory actions arising from the PAG is well established (Fields et al., 1991). For comprehensive reviews see, for example Heinricher et al. (2009) and Ossipov (2012); for a recent update see Heinricher (2016). In animal studies it was shown that the RVM has a peculiar bidirectional role in pain control at the spinal cord namely by balancing inhibitory (antinociceptive) and facilitatory (pronociceptive) effects at the spinal cord. One of the best established neurobiological mechanism of bidirectional control from the RVM is the existence in the RVM of two classes of neurons. OFF-neurons are involved in pain inhibition and their electrophysiological responses pause when the animal exhibits a nocifensive behavior. On the contrary, ON-neurons are involved in pain facilitation as their electrophysiological activity increases just prior to the appearance of a nociceptive withdrawal reflex. Besides the two types of neurons, the RVM also harbors NEUTRAL neurons which exhibit an electrophysiological activity that is not related to identified animal behavior. The coexistence of antinociceptive and pronociceptive systems at the human RVM was only recently studied. This delay in evaluating the putative translational perspectives of the existence of OFF- and ON- neurons is mainly due to the limitations of imaging techniques in the study of small regions, such as the RVM. Initial imaging studies which managed to evaluate RVM activation in healthy volunteers confirmed activation of this RF region in response to nociceptive stimulation of healthy volunteers (Fairhurst et al., 2007; Eippert et al., 2009). More recently, and by using a brainstem optimized whole brain imaging protocol, it was possible to demonstrate distinct clusters of activity in the RVM of healthy volunteers, which match the antinociceptive and pronociceptive activities of OFF-and ON-neurons, respectively (Brooks et al., 2017). The translational perspectives of this recent imaging study need to be evaluated in the future, namely to analyze if activation of pronocicetive components of the RVM increases in chronic pain patients, in a manner similar to the well-established results in animal models (Carlson et al., 2007; Gonçalves et al., 2007; Silva et al., 2013).

Local Neurochemical Systems

The role of serotonin in local control of RVM neurons has been demonstrated. In fact, and besides targeting the spinal cord, serotoninergic RVM neurons have been shown to modulate the activity of RVM neurons (Potrebic et al., 1994; VanderHorst and Ulfhake, 2006). The RVM also contains opioid-sensitive neurons since the activity of OFF-neurons is activated by mu-opioid agonists whereas the opposite occurs with ON-neurons (Heinricher et al., 1992; Heinricher and Fields, 2013). The local neurochemical control appears to be more complex and involve GABA-mediated inhibition which is triggered by opioids. Local cholecystokinin receptors (CCK2) are also relevant and provide additional possibilities of a fine tuning of neurochemical control at the RVM. An additional neurochemical system was recently unraveled at the RVM. The endovanilloid system is remotely activated from the PAG since agonists of the vanilloid receptor type 1 (TRPV1) injected into the PAG induce glutamate-mediated activation of the activity of OFF neurons (Starowicz et al., 2007; Palazzo et al., 2010). The endovanilloid system at the RVM appears to be inactivated in non-noxious conditions or during acute pain (Silva et al., 2016b). The system is only activated at the RVM in situations of chronic pain, namely during neuropathic conditions (see below in "Chronic Pain as a Trigger of Neuroplasticity at the RVM-VLM-DRt Triad" Section).

The Caudal Ventrolateral Medulla

Anatomical Location

The caudal VLM is located in the ventrolateral quadrant of the caudalmost aspect of the medulla oblongata in several species including man, rat, mouse, cat and monkeys. The VLM extends from the spinomedullary junction up to the level of the rostral border of the area postrema. The caudalmost part of the VLM contains an area designated as VLMlat, which is located between the spinal trigeminal nucleus (Sp5C) and the lateral reticular nucleus (LRt) and appears to play

a specific role in pain modulation (Tavares and Lima, 2002, 2007). The VLM also includes the LRt, which is a nucleus with a specific pattern of connections and has been known for its involvement in motor responses (Alstermark and Ekerot, 2013).

Connections with the Spinal Cord

The ascending projections from the spinal cord to the VLM are anatomically segregated, with the VLMlat receiving mainly afferents from the superficial dorsal horn, namely from noxious-responding neurons located in lamina I (Tavares et al., 1993). An important contingent of VLMlat-projecting neurons is located at lamina II, which appears to be a special feature of this spinofugal pathway (Lima and Coimbra, 1991; Lima et al., 1991). On the contrary, the LRt is targeted from fibers originated from deeper layers in the spinal cord, namely from laminae IV–V, which specifically terminate in the lateral part of the LRt, and from lamina VII which terminate at the medial part of the nucleus (Lima and Coimbra, 1991; Lima et al., 1991).

Our research group performed several anatomofunctional studies to evaluate the areas of termination of descending pathways from the VLM to the spinal cord, taking into account the anatomical segregation reported above as to the spino-VLM pathway. The descending VLMlat-spinal pathway targets lamina I, IV–V and X and appears to course through the dorsolateral funiculus (Tavares and Lima, 1994; Tavares et al., 1996). A reciprocal loop between lamina I and the VLMlat was further characterized at the ultrastructural level (Tavares and Lima, 2002). Terminal boutons from lamina I establish mainly asymmetrical contacts with VLMlat neurons that project to the spinal cord, suggesting that the ascending input from the spinal cord directly activates VLMlat neurons. In the spinal cord, descending projections from the VLMlat establish both asymmetrical and symmetrical synapses (Tavares and Lima, 2002). Collectively the data suggest that the arrival of noxious input from the spinal cord triggers activation of VLMlat neurons. Detailed electrophysiological mapping of the VLM has shown that it harbors inhibitory neurons (OFF-like neurons) along with excitatory cells (ON-like cells; Pinto-Ribeiro et al., 2011) which, along with our ultrastructural data (Tavares and Lima, 2002), indicates that the descending modulation from the VLM may include facilitatory modulation, along with the well-established inhibitory effects.

Nociceptive Control

The anatomical data reviewed above are easy to conciliate with functional findings showing that the magnitude and duration of behavioral nociceptive responses is more intense when the VLMlat is stimulated (Gebhart and Ossipov, 1986) in comparison with stimulations directed to more medial VLM areas. The VLMlat contains neurons that respond to noxious activation of the joints (Pinto et al., 2007). Incidentally, it must be noted that the magnitude of activation of VLMlat neurons, as measured by the expression of the c-*fos* protooncogene, is directly correlated with the magnitude of activation of lamina I neurons (Pinto et al., 2006), which reinforces the functional relevance of the above mentioned lamina I-VLMlat-lamina I loop. Recent studies showed that VLMlat neurons respond to diffuse noxious stimulation of the muscles (Panneton et al., 2015) and a new role for the VLMlat as a component of the classical spinoreticulothalamic pathway has emerged in which this RF region is proposed to have a key relay role. The poor activation of more medial components of the VLM, namely the LRt, in response to noxious stimulation along with the role of the LRt in motor control suggests that the VLM may be specially positioned to provide an integrated response to an acute stimulus, namely to the classical "fight or flight" response. In fact, a recent study proposed that the spino-LRt-cerebellar pathway provides an adequate motor response in response to noxious peripheral stimulation (Huma et al., 2015). An additional component of this response involves cardiovascular parameters. The VLMlat is activated in response to increases in blood pressure (Tavares et al., 1997a; Lima et al., 2002). Increases in blood pressure are also a feature of the "fight or flight" response. Collectively, the data suggest that the VLM is an integrative center which is involved in producing the adequate pain, motor and cardiovascular responses necessary to face threatening events.

Local Neurochemical Systems

The local neurochemical circuits relevant to control the functions of the VLM in pain modulation appear to involve noradrenaline and angiotensin II. As to the former, administration of noradrenaline or the α_2-adrenoreceptor agonist clonidine into the VLM inhibit local neurons and produce hyperalgesia (Cahusac and Hill, 1983; Ossipov and Gebhart, 1986). Angiotensin II injected into the VLM also induces hyperalgesia which is mediated by local angiotensin type 1 receptors (AT_1 receptors; Marques-Lopes et al., 2010). By a selective manipulation of the noradrenergic projections from the pontine A_5 noradrenergic cell group to the VLMlat, it was proposed that VLMlat neurons expressing AT_1 receptors activate A_5 noradrenergic neurons which will inhibit nociceptive transmission at the spinal cord. That triggering action of the VLM is inhibited by collaterals of the descending A_5-spinal pathway (Tavares et al., 1997b). Another important neurochemical control system at the VLMlat is mediated by opioids. At the VLM, μ-opioid receptors are expressed mainly by VLMlat neurons that do not project to the spinal cord (Pinto et al., 2008b) and overexpression of opioids at the VLM induces antinociceptive effects (decreased behavioral nociceptive responses) and lower nociceptive spinal neuronal activation (Martins et al., 2011).

The Dorsal Reticular Nucleus

Anatomical Location

The DRt is located in the caudal-most aspect of the medulla oblongata in several species including man, rat, cat and monkey. The DRt extends from the spinomedullary junction up to the level of the rostral border of the area postrema. It confines with the cuneate nucleus and the nucleus of the solitary tract

(NTS), dorsomedially, the Sp5C, laterally and the VLM, ventrally (Andrezik and Beitz, 1985; Newman, 1985).

Connections with the Spinal Cord

In what concerns the connections with the spinal cord, our research group has shown that DRt neurons are reciprocally connected spinal lamina I neurons forming a reverberative nociceptive circuit (**Figure 2**). The DRt receives bilateral projections from spinal cord laminae I, IV–VII and X with an ipsilateral predominance of those originated in the dorsal horn (Lima, 1990; Villanueva et al., 1991). Spino-DRt pathways travel though the dorsal columns, with the projections originated in the superficial dorsal horn traveling through the dorsal funiculus and those originated in the deep dorsal horn traveling in the dorsolateral fasciculus (Lima, 1990; Almeida et al., 1995; Lima and Almeida, 2002). The lateral aspect of the ventrolateral quadrant also constitute an ascending tract used by spino-DRt pathways, most likely originated from the deep dorsal horn, since lesions including the most lateral parts of the ventral funiculus prevented the activation of DRt neurons, contrary to lesions including the dorsal funiculus where fibers originated from superficial dorsal horn neurons run (Bing et al., 1990). DRt neurons project to the superficial and deep dorsal horn (Bernard et al., 1990; Tavares and Lima, 1994; Villanueva et al., 1995). DRt-spinal pathways reach the spinal cord through the dorsolateral funiculi (Villanueva et al., 1995).

The termination of DRt neurons in the same laminae containing spinal neurons projecting to the DRt predicts the existence of reciprocal circuits between the spinal cord and the DRt. A reciprocal excitatory loop between the DRt and superficial dorsal horn neurons was demonstrated. At the superficial dorsal horn, excitatory contacts, demonstrated at the ultrastructural level, occur between DRt descending fibers impinging upon lamina I neurons, which project back to the DRt. At the DRt, excitatory contacts were also demonstrated between fibers ascending from spinal lamina I neurons and local neurons (Almeida et al., 1993, 2000). Lamina I neurons projecting to the DRt convey nociceptive inputs to the DRt as they express c-Fos, a marker of spinal neuronal nociceptive activation (Hunt et al., 1987), upon noxious stimulation (Almeida and Lima, 1997). This reciprocal excitatory loop likely forms a reverberating circuitry feeding both the spinal cord and the DRt with nociceptive information and ultimately amplifying it. The deep dorsal horn and the DRt are also likely linked by reciprocal links as suggested by anatomical and electrophysiological data. The latter showed that activation of the DRt by glutamate injection exerts excitatory influences on deep dorsal horn neurons as observed by the long lasting increase of the responses of wide-dynamic range (WDR) neurons to noxious electrical stimulation of the sciatic nerve (Dugast et al., 2003). On the contrary, blocking the ipsilateral DRt by lidocaine produces an immediate decrease of C-fiber-evoked responses to sciatic nerve stimulation postdischarge activity of WDR neurons (Lima and Almeida, 2002).

Nociceptive Control

The DRt has been assigned a peculiar role in pain processing, with most data gathered in the rat and cat. In humans, the recent developments of functional magnetic resonance imaging (fMRI), allowing to investigate more accurately the spinal cord as well as the brainstem, also confirm the involvement of the DRt in pain processing (Rempe et al., 2014, 2015; Youssef et al., 2016). Electrophysiological *in vivo* recordings performed in the rat identified two neuronal subpopulations at the DRt, total nociceptive convergent neurons which are exclusively activated by noxious stimuli conveyed by Aδ- and C-fibers from the entire body, and partial nociceptive convergent neurons, which are activated by both noxious and innocuous stimuli and are targeted by C-fibers innervating only restricted areas of the body and by Aδ-fibers originated in extensive peripheral areas (Villanueva et al., 1988, 1989). In addition to responding to and encoding cutaneous and visceral noxious inputs, DRt neurons responded in a manner proportional to the intensity of stimuli (Villanueva et al., 1989; Roy et al., 1992). These electrophysiological properties were also found in DRt neurons of the monkey (Villanueva et al., 1990) and cat (Soto et al., 2008). Recent electrophysiological *in vivo* and *in vitro* studies performed in the cat and the rat, respectively, showed that a large fraction of DRt neurons presented spontaneous activity (Soto and Canedo, 2011; Sousa et al., 2014). Additionally, DRt neurons show windup (Villanueva et al., 1988), a phenomena that consists of a gradual build-up in neuronal excitability, generated in response to low frequency C-fiber afferent input or generated supraspinally (Soto et al., 2008; Soto and Canedo, 2011). This physiological property of DRt neurons is important inasmuch as wind-up has been interpreted as a system for the amplification of pain which could lead to the development of central sensitization (Herrero et al., 2000).

The DRt facilitates acute and short-lasting inflammatory pain (Almeida et al., 1996, 1999; Ambriz-Tututi et al., 2013; Martins et al., 2013, 2015a). Stimulation of the DRt by local administration of glutamate increased nociceptive acute behavioral responses while DRt lesion by local administration of quinolinic acid had the opposite effect (Almeida et al., 1996). Chemical or electrical lesioning of the DRt decrease inflammatory pain behaviors (Almeida et al., 1999). Spino-DRt-spinal excitatory loops are likely involved in the mediation of the pain facilitatory actions observed after formalin injection since both DRt lesioning or blockade of glutamate receptors at the DRt decreases formalin-induced pain behaviors and c-Fos expression in superficial and deep dorsal horn laminae of the spinal cord (Almeida et al., 1999; Ambriz-Tututi et al., 2013).

The DRt is also involved in diffuse noxious inhibitory control (DNIC), a mechanism of top-down inhibitory control of spinal dorsal horn neurons (Bouhassira et al., 1992b). DNIC is a paradigm in which one noxious stimulus is used as a conditioning stimulus to induce reduction in pain perception by another stimulus. DNIC is triggered exclusively by a conditioning noxious stimulus, conveyed by peripheral Aδ- and C-fibers, and inhibit trigeminal and spinal convergent WDR neurons (Le Bars et al., 1979; Dickenson et al., 1980). DNIC is sustained by a complex loop, involving supraspinal structures, whose ascending and descending pathways travel through the ventrolateral and dorsolateral funiculi, respectively (Villanueva and Le Bars, 1995). Early electrophysiological and lesioning studies established that

pathways originated from the DRt are involved in DNIC (Bouhassira et al., 1990, 1992a,b, 1993). It was suggested that descending inhibitory inputs from the DRt constituted a separate type of inhibitory control, and it was hypothesized that DNIC triggered from the DRt could be part of a mechanism involved in the extraction of nociceptive information by depressing background body sensory activity (Le Bars, 2002).

The DNIC inhibitory control also occurs in humans (Yarnitsky, 2010; van Wijk and Veldhuijzen, 2010). More insights into DNIC have been gathered namely that it recruits cortical networks, which may explain why pain expectation, which relies on cortical structures, prevents DNIC (Goffaux et al., 2007). In line with this, a recent fMRI study performed in humans suggest that a lack of DNIC results from increased control of the DRt from the cortex (Youssef et al., 2016). Another interesting finding likely helping to comprehend DNIC modulation, is that DNIC involves the activation of opioid receptors at the DRt (de Resende et al., 2011). Interestingly, our work shows that opioids inhibit DRt descending facilitation, it is therefore likely that the expression of DNIC might also rely on a balance between descending inhibition and facilitation, with notably descending facilitation overpowering descending inhibition. Another evidence that pain facilitation likely opposes the effects of DNIC comes from a study showing that chronic treatment with morphine, which induces paradoxical opioid-induced hyperalgesia (Lee et al., 2011), was shown to eliminate DNIC by activating pain facilitatory neurons in the RVM (Okada-Ogawa et al., 2009). Additionally, several other recent studies show that descending spinal inhibitory noradrenergic pathways are necessary for the expression of DNIC (Bannister et al., 2015; Peters et al., 2015). Finally, DNIC is decreased in patients and animal models with chronic pain and studies using the rat show that pharmacologically increasing noradrenaline, which is deficient at the spinal cord during neuropathic pain (Hughes et al., 2013), as well as blocking serotoninergic descending facilitation mediated by 5-HT3 receptors, restores DNIC in neuropathic animals (Bannister et al., 2015). Taking these recent findings into account and given the fact that chronic pain results from an imbalance between descending inhibition and facilitation with the imbalance towards increasing descending facilitation (Ossipov et al., 2014), assessing DNIC in patients could potentially constitute a relevant tool to monitor alterations in the endogenous pain modulatory pathways (van Wijk and Veldhuijzen, 2010), which could potentially help identify patients at risk for development of chronic pain and ultimately help find better pain treatments.

Local Neurochemical Systems

Pain facilitation from the DRt is modulated by several neurotransmitters such as glutamate, opioid peptides, noradrenaline and GABA. The excitatory amino acid glutamate plays a key role in the pronociceptive actions of the DRt during the formalin test since the blockade of AMPA/KA, NMDA and mGlu1 glutamate receptors by the local administration of the respective antagonists significantly reduced formalin-induced pain behavior which was accompanied by a reduction of c-Fos expression at both the superficial and deep dorsal laminae (Ambriz-Tututi et al., 2013). The tonic activity of glutamate at the DRt likely results from the sustained peripheral afferent input, induced by formalin injection, leading to increased activation of spino-DRt-spinal reverberative circuits (Almeida et al., 1993, 2000). Noradrenaline is also involved in the mediation of pronociception from the DRt. Indeed, noradrenaline release at the DRt, measured by *in vivo* microdialysis, increases during the formalin test (Martins et al., 2013). The reduction of noradrenaline release at the DRt by genetic manipulation of DRt-noradrenergic afferents significantly attenuated pain behavior in the formalin test while increasing local extracellular levels of noradrenaline, by inhibiting its recapture, produced the opposite effect (Martins et al., 2013). The genetic manipulation of DRt-noradrenergic afferents was performed by a viral vector derived from the Herpes Simplex virus type 1 (HSV-1) which is retrogradely transported from the DRt to its noradrenergic afferents, namely the locus coeruleus (LC) and A_5 noradrenergic cell group (**Figure 2**), where it selectively reduces noradrenaline synthesis (Martins et al., 2010). The pain facilitatory actions of noradrenaline at the DRt are mediated through activation of α_1-adrenoreceptors (Martins et al., 2013).

Opioid peptides were shown to inhibit DRt pain facilitation as shown by HSV-1-mediated overexpression of enkephalin at the DRt, which produced anti-hyperalgesia in a model of acute pain (Martins et al., 2008). Opioids likely act through direct inhibition of DRt spinally projecting neurons since these neurons express μ-opioid receptors (Pinto et al., 2008a,b). Opioids also act the DRt through additional inhibitory mechanisms, likely by disinhibiting enkephalinergic interneurons which receive input from GABAergic interneurons expressing μ-opioid receptors and being presynaptically inhibited by δ-opioid expressing-fibers (Pinto et al., 2008a). Opioid peptides responsible for the anti-hyperalgesic action found in our studies are mostly released from local interneurons but also from DRt afferent sources namely the RVM, the A_5 noradrenergic cell group and the hypothalamus (**Figure 2**; Martins et al., 2008).

The inhibitory neurotransmitter GABA is involved in the mediation of pronociception from the DRt. Our recent studies show that GABA release at the DRt, measured by *in vivo* microdialysis, increases during the formalin test and that it increases DRt pain facilitation through activation of $GABA_B$ receptors (Martins et al., 2015a). Indeed, $GABA_B$ receptors knock-down at the DRt, mediated by lentiviral vectors, or the pharmacological blockade, via the local administration of a $GABA_B$ antagonist, significantly attenuated formalin-induced pain behavior while the local administration of a $GABA_B$ agonist induced the opposite (Martins et al., 2015a). The effect of GABA is likely due to disinhibition of the DRt spinally projecting neurons since a large proportion of $GABA_B$ receptors are expressed by local opioidergic neurons inhibiting DRt spinally projecting neurons (Pinto et al., 2007, 2008a; Martins et al., 2008). GABA might be released from local interneurons but also from insular, somatosensory and motor cortices (**Figure 2**) which represent the most important afferent pathways to the DRt and they are GABAergic (Martins et al., 2015a).

THE RVM-VLM-DRt TRIAD AS A KEY GATEWAY FOR TOP-DOWN PAIN MODULATION

The three components of the triad of RF medullary addressed in this review (RVM, VLM and DRt) are interconnected (**Figure 1**). The VLM↔DRt reciprocal connection is likely to have functional implications since the magnitude of nociceptive neuronal activation in two regions in response to nociceptive stimulation is positively correlated (Pinto et al., 2006). Similar correlation studies should be enlarged to the RVM.

The RVM-VLM-DRt triad is in a privileged position to collect input from higher brain centers and convey this modulation to the spinal cord (**Figure 1**). The RVM conveys input from the PAG which, on its turn, is an area that integrates modulatory influences arising from the areas involved in cognitive and emotional aspects of the pain response, such as the frontal cortex and the amygdala (Tracey and Mantyh, 2007). Furthermore, the RVM-VLM-DRt triad has the unique feature of being involved in bidirectional pain modulation, namely by balancing pain inhibition and pain facilitation (**Figure 1**). The triad is acted by the main relevant neurotransmitters involved in pain modulation, namely opioids, noradrenaline and serotonin, which has a putative translational value since most pain killers act at the opioidergic and monoaminergic systems. We therefore propose that the triad RVM-VLM-DRt is a key gateway in top-down directional pain modulation (**Figure 1**).

Due to our expertise in the study of the DRt and its peculiar role in pain facilitation, this part of the triad should be analyzed in detail. The DRt is a major integrative relay for ascending nociceptive information participating in a reticulo-thalamo-cortical pathway (**Figure 2**) through which the DRt may allow any signal of pain to again access to widespread areas of the neocortex and thus help prime the cortex for attentional reactions and/or the coordination of motor responses (Bernard et al., 1990; Villanueva et al., 1998; Monconduit et al., 1999, 2002; Desbois and Villanueva, 2001). The DRt receives projections from several brain areas predominantly from several cortical areas, the hypothalamus, the amygdala and brainstem areas involved in descending pain modulation (**Figure 2**). Besides the RVM and VLM, the DRt receives projections from the PAG, and a strong noradrenergic input from the pontine noradrenergic cell groups LC, A_5 and A_7 (Almeida et al., 2002).

The DRt is also a major integrative relay for descending nociceptive modulation from several brain areas including the cortex (Zhang et al., 2005), the hypothalamus (Amorim et al., 2015) and the brainstem (Martins et al., 2013; Velo et al., 2013; Leiras et al., 2016). The DRt was shown to relay descending pain facilitation from the anterior cingulate cortex (ACC; Zhang et al., 2005). Additionally, the DRt receives dense innervation from cortical areas, namely from the motor (M1 and M2), somatosensory (S1 and S2) and insular cortex (Desbois et al., 1999; Almeida et al., 2002). Our recent work demonstrated that a high percentage of the cortical projections to the DRt were GABAergic (**Figure 2**) and the release of GABA at the DRt enhanced DRt pain facilitation (Martins et al., 2015a). Therefore, it is likely that the DRt might relay descending facilitatory actions triggered from these cortical areas. Moreover, given the involvement of some of this cortical areas in the emotional and cognitive control, the GABAergic projections from these areas may represent a neuronal circuit that accounts for pain enhancement during imbalance of emotional and cognitive conditions. The DRt also relays descending nociceptive facilitation induced by galanin in the dorsomedial nucleus of the hypothalamus (Amorim et al., 2015). Our recent functional studies show that the DRt acts as an important relay station in the mediation of descending facilitation from noradrenergic areas, namely the LC and A_5 noradrenergic groups (Martins et al., 2013). The LC exerts a bidirectional role on pain modulation (Hickey et al., 2014) and its pain facilitatory role is relayed by the DRt (Martins et al., 2010, 2013, 2015b).

The DRt is also involved in a network of reciprocal connections established between collaterals of DRt and medial medullary reticular spinally-projecting neurons (**Figure 2**; Leiras et al., 2016). Medial medullary reticular neurons are involved in nociceptive and escape motor responses (Casey and Morrow, 1989), it is therefore likely that the DRt might integrate noxious sensing and nocifensive behavior through the collaterals of descending axons with medullary reticulospinal neurons.

The extensive ascending and descending connections of the RVM-VLM-DRt triad allow it to modulate the spinal nociceptive signals and integrate those signals with environmental and emotional conditions providing adequate and adaptable pain responses while preserving the homeostasis of the organism. Based on the phylogenetically conserved nature of the RF, we propose that the RVM-VLM-DRt triad is a conserved part of the brain and represents a vital gateway which evaluates and controls pain responses and triggers adequate and integrated responses. In a very recent review, a role for the brainstem RF in emotional control has been discussed in detail (Venkatraman et al., 2017). The brainstem has been proposed the key region to provide the effective link between sensory and effectors networks in processing emotions. By harboring components of the emotional motor system, the RVM-VLM-DRt triad can provide both the rapid "fight or flight" responses necessary to provide a rapid and specific body reaction but also the responses adequate to process "slow burning" somatic and visceral pain signals (Parvizi and Damasio, 2001; Craig, 2003). Based on the intrinsic interconnections of the RVM-VLM-DRt triad, it may also represent, by excellence, an example of the structural connectome of the central homeostatic network (Edlow et al., 2016), namely in its involvement in pain responses. Pain is a main challenger of the body homeostasis, both in acute pain when cardiovascular and motor reactions should be rapidly adjusted, and in chronic pain, when anxiety, depression and even neurodegeneration can occur.

CHRONIC PAIN AS A TRIGGER OF NEUROPLASTICITY AT THE RVM-VLM-DRt TRIAD

The data gathered in several animal models point to a participation of the RVM-VLM-DRt triad in chronic pain.

It remains to be ascertained if that RF triad plays a similar role in the human brain but the still low resolution of imaging techniques does not allow detailed studies of those medullary regions. The interesting perspectives derived from recent imaging studies (Brooks et al., 2017) is likely to provide important data in a near future.

During chronic inflammatory pain several studies indicate that the activity of the triad is altered. Increased activity at the VLM and DRt were detected in a functional study based in the consumption of 2-D-glucose in chronic monoarthitic rats (Neto et al., 1999). In the specific case of the DRt, this could be related both to increased spinal excitatory inputs to the DRt in addition to a loss of inhibitory opioidergic tone into the DRt. The former was suggested to occur due to an imbalance between excitatory and inhibitory actions exerted upon spino-DRt neurons. Spinal dorsal horn neurons projecting to the DRt are under direct inhibitory GABAergic modulation, as they express mainly $GABA_B$ receptors (Castro et al., 2006) but in the spinal dorsal horn of monoarthritic animals there are lower percentages of $GABA_B$ expressing neurons and higher percentages of NK1 neurons (Castro et al., 2005) which are being subjected to excitatory actions exerted by substance P. A loss of inhibitory opioidergic tone likely occurs at the DRt since monoarthritic rats show decreased expression levels of of μ- and δ-opioid receptors at the DRt (Neto et al., 2008; Pinto et al., 2008a). These hypotheses are consistent with the results of a recent study showing the involvement of the DRt in formalin-induced secondary allodynia and hyperalgesia through a tonic glutamatergic excitatory tone (Ambriz-Tututi et al., 2013). Indeed, the administration of glutamate antagonists into the DRt reduced both secondary allodynia and hyperalgesia and their development was prevented by pretreatment with glutamate antagonists injected into the DRt before formalin injection (Ambriz-Tututi et al., 2013). The results of this study further support a key role for glutamate receptors into the DRt in the development of formalin-induced secondary allodynia and hyperalgesia.

Neuropathic pain is associated with spinal neuronal sensitization (Laird and Bennett, 1993), an activity-dependent expression of hypersensitivity of spinal neurons. Electrophysiological studies suggest a contribution of the DRt to the maintenance of spinal sensitization during neuropathic pain (Sotgiu et al., 2008). The recent studies performed by our group implicate the noradrenergic modulation of the DRt in the enhancement of DRt pain facilitation during neuropathic pain. We showed that nociceptive stimulation increased noradrenaline release at the DRt which enhanced pain facilitation from the DRt through activation of α_1-adrenoreceptors (Martins et al., 2015b). Reducing noradrenaline release at the DRt by using a viral vector derived from the HSV-1 which selectively reduced noradrenaline synthesis in noradrenergic DRt afferents, significantly attenuated the behavioral manifestations of neuropathic pain for nearly 2 months (Martins et al., 2010). Our studies also show an impairment of the feedback inhibitory function of α_2-adrenoreceptors at the DRt during neuropathic pain, which likely further contributes to enhance the noradrenergic input to the DRt during neuropathic pain (Martins et al., 2015b). The increased noradrenergic neurotransmission at the DRt, enhancing pain facilitation from this area, raises an important issue related to the treatment of neuropathic pain with antidepressants inhibiting noradrenaline reuptake. Noradrenaline reuptake inhibitors are known to produce analgesia through a spinal action but our results also show that they may enhance pain facilitation from the brain counteracting thus their analgesic effects at the spinal cord.

The occurrence of irreversible changes during the installation of chronic pain should also be considered since it was demonstrated that the RVM suffers neurodegeneration during the installation of neuropathy. In initial phases of neuropathy, the RVM exhibits plastic changes in what concerns its involvement in descending modulation namely by increases in the activity of pain facilitatory ON-neurons and opposite for OFF-neurons, along with an enhancement of pain facilitatory actions at the spinal cord mediated by local $5\text{-}HT_3$ receptors (Silva et al., 2013, 2016a; Guo et al., 2014). Collectively these changes lead to increase in descending pain facilitation which may account for chronic pain installation. During the progression of pain, and due to the continuous barrage of nociceptive input from the spinal cord, a disruption of local RVM circuits occurs with the appearance of massive oxidative stress damage and hyperactivation of glial cells (Silva et al., 2016a). The subsequent neuroinflammation may lead to neurodegeneration, associated with neuronal loss, which represents a non-plastic effect of chronic pain installation at the RVM. Neuroplastic changes in key descending pain modulatory areas were shown to be crucial for the generation and maintenance of chronic pain (Woolf and Salter, 2000), the studies compiled here strongly suggest that the alterations occurring at the RVM-VLM-DRt triad during chronic pain are also fundamental to its maintenance. Additionally, our results highlight the necessity of taking into account the alterations occurring at the levels of the RF for the design of efficient therapies for the challenging treatment of chronic pain.

FUTURE DIRECTIONS IN THE STUDY OF THE RETICULAR FORMATION

Functional neuroanatomical studies rely on the coupling of neural tracing with other neuroscientific techniques such as electrophysiology and behavior. In the last few years these studies have been massively propelled by the use of novel technologies such as optogenetics and chemogenetics which allow simultaneously manipulating circuitries and linking those circuits to behavioral outputs. Optogenetics use channels that are activated by light (Copits et al., 2016), while chemogenetics uses engineered G protein-coupled receptors, that are activated by otherwise inert drug-like small molecules, to activate or silence neuronal firing (Roth, 2016). Hitherto, optogenetics have been successfully used to study brain circuits such as those involved in regulation of the sensory and affective aspects of pain, namely cortico-limbic networks (Copits et al., 2016). Chemogenetics have also been successfully used to examine spinal circuits involved in pain modulation (Bourane et al., 2015; Peirs et al., 2015; Saloman et al., 2016) and the study of

descending pain modulation is also starting to be approached by both technologies (Cai et al., 2014; Hickey et al., 2014; Jurik et al., 2015; Li et al., 2016). The RF contains areas relaying and integrating nociceptive information, from the brain and the spinal cord with other physiological functions. In the future the use of optogenetics and chemogenetics will allow a more comprehensive and precise study of the integrative role and the specificity of the intricate circuits of the RF.

At the clinical setting, the technical improvements at the brain imaging field will also allow to study the RF in more detail. By proposing that the medullary RF has a triad of pain control centers (the RVM-VLM-DRt), the technical problems of studying small components may be surpassed as the triad should be considered as a gateway from the brain to the spinal cord. The RVM-VLM-DRt triad probably represents an example of the "brain pain connectome" and should not be analyzed as to its individual components, *per se*. Future studies will analyze the RVM-VLM-DRt triad as a phylogenetically conserved structure of the RF which is vital in acute pain, namely to provide the substrate for "fight or flight responses". Since the medullary triad is a gateway from descending modulation from the brain to the spinal cord, we should study in the future how the deregulation of the RVM-VLM-DRt triad in balancing inhibition and facilitation of pain may account for chronic pain generation and/or maintenance.

AUTHOR CONTRIBUTIONS

IT was responsible for the overall organization of the manuscript and wrote the parts related to the rostroventromedial medulla and the ventrolateral medulla. IM was responsible for the items related to the role of the dorsal reticular nucleus. The overall manuscript was reviewed by both authors.

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REVIEW
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Roles of Microglial Phagocytosis and Inflammatory Mediators in the Pathophysiology of Sleep Disorders

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Sleep serves crucial learning and memory functions in both nervous and immune systems. Microglia are brain immune cells that actively maintain health through their crucial physiological roles exerted across the lifespan, including phagocytosis of cellular debris and orchestration of neuroinflammation. The past decade has witnessed an explosive growth of microglial research. Considering the recent developments in the field of microglia and sleep, we examine their possible impact on various pathological conditions associated with a gain, disruption, or loss of sleep in this focused mini-review. While there are extensive studies of microglial implication in a variety of neuropsychiatric and neurodegenerative diseases, less is known regarding their roles in sleep disorders. It is timely to stimulate new research in this emergent and rapidly growing field of investigation.

Keywords: microglia, sleep, wakefulness, disease models, animal, diseases

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INTRODUCTION

Humans spend approximately a third of their lives sleeping. Sleep is an involuntary process, required to sustain good mental and physical health. During sleep, the brain processes information, consolidates memories, and undergoes a number of maintenance processes that help its function upon waking (Graves et al., 2001; Abel et al., 2013; Tononi and Cirelli, 2014; Vorster and Born, 2015). Loss or restriction of sleep is associated with multiple detrimental consequences (Musiek and Holtzman, 2016; Pires et al., 2016).

Microglia are macrophages derived from the embryonic yolk-sac that permanently reside in the brain alongside neurons, astrocytes, and oligodendrocytes (Gomez Perdiguero et al., 2013). These cells are uniformly distributed and their ramified processes constantly survey the brain parenchyma during normal physiological conditions. Microglia respond to pathology in various manners—they enlarge their cell bodies, increase their mobility, and hyper-ramify or reduce their processes. The microglia field has recently undergone explosive growth, especially since microglial roles in the healthy brain are becoming uncovered (Tremblay et al., 2011; Sierra et al., 2014). These mononuclear phagocytes are now recognized as essential contributors to neuronal survival, synaptic pruning, and dendritic spine formation during development, as well as synaptic maintenance and plasticity, neurogenesis, learning, memory, and cognition into adulthood (Salter and Beggs, 2014; Hong and Stevens, 2016; Tay et al., 2016). As the brain's immune cells, microglia help defend against pathogens, and play pivotal roles in recovery from sickness and injury

(Kettenmann et al., 2011; Tremblay and Sierra, 2014). Microglia phagocytose cellular debris and release pro- and anti-inflammatory cytokines, trophic factors, and various other molecular mediators, processes critical for adaptation of the brain to the ever-changing environment (Delpech et al., 2015; Shemer et al., 2015; Tian et al., 2017).

Microglia detect changes in homeostasis through their recognition of exogenous or endogenous danger signals, pathogen-associated molecular patterns (PAMPs; small molecular motifs conserved within a class of microbes that are recognized by cells of the innate immune system via pathogen recognition receptors) and danger-associated molecular patterns (DAMPs; host-derived motifs that also regulate the activation of pathogen recognition receptors and can be triggered by enhanced neuronal activity and psychological stress). Microglia respond by orchestrating neuroinflammation through their interactions with peripheral immune cells which invade the brain, especially if the blood-brain-barrier is compromised (notably occurs during chronic sleep restriction, He et al., 2014), as well as vascular cells, glial cells, and neurons (Xanthos and Sandkuhler, 2014). In some contexts, microglia perform adaptive immune functions and present antigens to T cells upon binding to MHC class II expressed on their surface (Greter et al., 2015). Their responses can be homeostatic, leading to adaptation, but also dysfunctional, contributing to, or causing pathology. Anti-inflammatory mechanisms can be triggered in parallel to terminate neuroinflammmation and reduce pathological outcomes (Xanthos and Sandkuhler, 2014).

Considering that sleep and microglia share essential homeostatic functions, we have focused this review to examine the roles of microglial phagocytosis and inflammatory mediators, among various brain regions, in the pathophysiology of sleep disorders (see **Table 1** for a summary). Although the number of studies providing direct evidence is currently limited, we anticipate this field of investigation to expand in the near future, considering the exponential growth of microglial research (and increased availability of tools to study these cells specifically) in recent years. Since very interesting findings on the topic recently came out, we also hoped to stimulate further the interest toward microglia and sleep.

Infection-Induced Sleep Gain

"Sickness" is defined as the conjunction of adaptive changes that are elicited by activation of the immune system. Among those changes, infections induce stereotypic alterations in sleep activity. In rodents, sleep is divided into (1) non-rapid eye-movement (NREM) sleep [a state of deep sleep that is characterized by slow electroencephalogram (EEG) delta (0.5–4 Hz) waves] and (2) rapid eye-movement (REM) sleep (displaying electrical oscillations typical of brain activation but with inhibition of muscle tone and involuntary saccadic eye movements). During sickness, the ratio of NREM to REM sleep is increased (i.e., time in NREM sleep is increased while time in REM sleep is reduced, Lancel et al., 1995; Kapas et al., 1998; Mullington et al., 2000). A significant increase in the power of slow wave activity (SWA) is also observed (Kapas et al., 1998; Mullington et al., 2000; Majde and Krueger, 2005). SWA is commonly referred to as slow oscillation (Steriade et al., 1993a,b,c; Cowan and Wilson, 1994) and considered a physiological measure of the homeostatic drive for sleep (Borbely, 1982, 2001; Franken et al., 2001). Numerous clinical reports concur that both acute and chronic infections, as well as inflammatory diseases, are associated with sleep-related symptoms such as reduced sleep quality and increased fatigue (for review see Rohleder et al., 2012).

Many pathogens comprising gram-positive and gram-negative bacteria (Toth and Krueger, 1988, 1989; Krueger and Majde, 1994), viruses (influenza virus, rhinovirus, and human immunodeficiency virus) (Norman et al., 1988, 1990; Toth et al., 1995; Opp et al., 1996; Gemma and Opp, 1999), fungal organisms that include *Candida albicans* and the protozoan *Trypanosoma brucei brucei* (Kent et al., 1988; Toth and Krueger, 1989; Toth et al., 1994) induce a sleep response (for review, Krueger and Opp, 2016). Infectious agents do so via PAMPs that enhance the systemic and central production of pro-inflammatory cytokines, such as interleukin (IL)-1 and tumor necrosis factor-alpha (TNFα) (Imeri and Opp, 2009; Besedovsky et al., 2012; Krueger and Opp, 2016). IL-1 and TNFα are well-established sleep-regulatory substances that regulate NREM sleep duration and intensity by modulating neuronal activity in the hypothalamic preoptic area (Obal and Krueger, 2003), locus coeruleus (De Sarro et al., 1997), dorsal raphe nucleus (Manfridi et al., 2003), and cerebral cortex (Yoshida et al., 2004; Churchill et al., 2008). Hence, these cytokines act both at the circuit level and locally in the cortex and brainstem to promote sleep (Krueger, 2008). All these findings have been extensively reviewed (Krueger, 2008; Krueger et al., 2011; Zielinski et al., 2013; Krueger and Opp, 2016).

Microglia are the main producers of cytokines within the nervous system during inflammatory diseases (Renno et al., 1995; Van Dam et al., 1995; Buttini et al., 1997; Medana et al., 1997; Gregersen et al., 2000; Kettenmann et al., 2011; Delpech et al., 2015), indicating that these cells could play a crucial role in infection-induced sleep alterations. The effects of cytokines on sleep–wake behavior involve communication between neurons and microglia. As microglial processes constantly survey synaptic elements in a neuronal activity-dependent manner (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009; Tremblay et al., 2010; Hristovska and Pascual, 2015), a recent publication hypothesized that pro-inflammatory cytokines may exert their somnogenic effects by promoting microglial attraction to synapses (Karrer et al., 2015). In line with this assumption, the authors found that TNFα induces neuronal production of the chemokines (**chemo**attractant cyto**kines**) CCL2, CCL7, and CXCL10, which bind to their receptors expressed by microglia and promote microglial process extension (Karrer et al., 2015). TNFα additionally upregulates neuronal Homer1a (Karrer et al., 2015), which was shown to drive the homeostatic scaling-down of excitatory synapses during sleep (Diering et al., 2017). According to the synaptic homeostasis hypothesis, information processing and decision-making during wakefulness drive strengthening of synapses, which is counterbalanced during sleep by a global weakening (de Vivo et al., 2017). Mice with a deletion of Homer1a also show reduced wakefulness with increased NREM sleep during the dark period (Naidoo et al., 2012). While it has yet

TABLE 1 | Microglial functions and relevance to sleep disorders.

Neuroinflammation	Experimental findings	Suggested implication
	Infection-induced sleep gain	
Microglia are the main producers of cytokines in the central nervous system during inflammatory diseases.	IL-1 and TNFα are well-known to promote sleep in humans and in animal models (Krueger, 2008; Krueger et al., 2011; Zielinski et al., 2013; Krueger and Opp, 2016).	Pro-inflammatory cytokines may exert somnogenic effects by promoting microglial attraction to synapses (Karrer et al., 2015). TNFα induces the production of chemokines (CCL2, CCL7, and CXCL10) by neurons, which bind to corresponding receptors expressed by microglia and are known to promote microglial process extension (Karrer et al., 2015).
	Recreational-drug induced sleep loss	
	PET scans of chronic d-METH self-administrating individuals reveals increased binding of the radiotracer [11C](R)-PK11195 that labels "activated" microglia (McCoy et al., 2007).	
	In mice undergoing microglial depletion, the duration of daily wakefulness produced by d-METH is reduced by nearly 1 h. *Ex vivo* nitric oxide synthase (NOS) activity, and *in vivo* NOS expression are also elevated in cortical $CD11b^+$ microglia from wild-type mice upon acute d-METH exposure. Additionally, $CD11b^+$ cells are the only ones found to exhibit changes in sleep-regulatory IL-1β expression in response to d-METH (Wang et al., 2014).	The effects of d-METH on sleep and wakefulness could be mediated partly by microglia, through exacerbated oxidative stress and pro-inflammatory cytokine release (Wang et al., 2014).
	Narcolepsy	
	Increased levels of IL-6 and TNFα are measured in the sera or plasma from narcoleptic patients (Cartier et al., 2005; Eltayeb et al., 2007; Dauvilliers et al., 2014).	Decrease in CCR expression could lead to a defect in the recognition and phagocytosis of damaged cells by microglia and consequently to a delayed resolution of acute inflammation. These defects could lead to enhanced autoimmunity resulting in the loss of hypocretin neurons.
	Reduced levels of microglia/macrophage-derived CCR1 and CCR3 are measured in peripheral blood samples from narcolepsy patients (Mignot et al., 1995; Tafti et al., 1996; Partinen et al., 2014).	
Antigen presentation		
Microglia can present antigens to T cells upon their binding to MHC class II expressed on their surface.	An increased microglial expression of MHC class II is measured in the central nervous system of narcoleptic dogs (Tafti, 2009).	Local infusion of a low-dose of the endotoxin lipopolysaccharide in rats, as a model of chronic inflammation, induces the loss of hypocretin neurons and increases the number of MHC class II-positive microglia in the lateral hypothalamus. Microglia-mediated inflammation might be a trigger for the loss of hypocretin neurons during narcolepsy (Maurovich-Horvat et al., 2014).
Phagocytosis		
Microglia prune synapses in a complement-dependent manner in contexts of health and disease.	A distinctive complotype, i.e., a combination of polymorphisms defining complement activity: BfS, C4A3, and C4B1, was identified in narcopleptic patients (Savill et al., 2002).	Exacerbation of complement-dependent microglial phagocytic activity is a plausible mechanism leading to the loss of hypocretin neurons.
	Obstructive sleep apnea	
	Sleep fragmentation	
	Microglial co-localization with the marker of glutamatergic axon terminals VGLUT1 is increased after 5 days of chronic sleep deprivation in mouse prefrontal cortex (Bellesi et al., 2017). In parallel, the total length of microglial process arborization, and the proportion of the microglial population showing less ramified morphologies, are significantly reduced (Bellesi et al., 2017).	These observations suggest an exacerbated microglial pruning of synapes, which could be mediated by the classical complement pathway considering that expression of the complement protein C3 was concomitantly increased by sleep deprivation (Bellesi et al., 2017).

(Continued)

TABLE 1 | Continued

Neuroinflammation	Experimental findings	Suggested implication
Immune surveillance	***Chronic intermittent hypoxia***	
Microglia detect changes in homeostasis through their recognition of exogenous or endogenous danger signals, such as danger-associated molecular patterns (DAMPs).	Elevated levels of DAMPs are measured in blood samples from obstructive sleep apnea patients (Sapin et al., 2015), as well as in the hippocampus of rodent models of chronic intermittent hypoxia (Kiernan et al., 2016).	Microglia could respond to DAMPs induced by enhanced neuronal activity or psychological stress. The consequences on neuronal health and cognitive function are still undetermined however.

to be demonstrated *in vivo*, these data provide a provocative mechanism by which TNFα could regulate the sleep-wake cycle.

Interestingly, microglia were recently revealed also to follow a circadian rhythm for protein expression that is controlled by their intrinsic molecular clock (Hayashi et al., 2013). This notably affects their production of cytokines (Fonken et al., 2015), and could not only alter their response to infectious agents, but also their influence on sleep.

Overall, the literature suggest microglia play direct (structural interactions with synapses) as well as indirect (release of inflammatory cytokines) roles in infection-induced sleep gain.

Narcolepsy

Narcolepsy is characterized by excessive daytime sleepiness, sleep/wake fragmentation, hallucinations, sleep paralysis, and disturbed nocturnal sleep. The prevalence of this chronic sleep disorder ranges between 0.02 and 0.05% of the general population, with first symptoms appearing around 20–30 years of age, preferentially in males. After one or more years of progression, the disease stabilizes (Siegel, 1999; Longstreth et al., 2007). Narcolepsy is caused by a specific loss of hypothalamic hypocretin-producing neurons (85–95% of cells in humans), which coincides with low hypocretin levels in patients' cerebrospinal fluid (CSF) (Nishino et al., 2000; Thannickal et al., 2000; Mignot et al., 2002). Hypocretin knockout mice recapitulate human disease and are commonly used as an animal model of narcolepsy (Chemelli et al., 1999; Hara et al., 2001; Tabuchi et al., 2014). However, it remains unclear how hypocretin neurons are lost in the human pathophysiology.

Both genetic heritability and environmental triggers were found to be involved in disease progression. The cause and pathogenesis of narcolepsy are still unclear but likely involve the immune system. Human narcolepsy is closely associated (>95% of cases) with human leukocyte antigen (HLA) class II alleles in the major histocompatibility (MHC) region (Langdon et al., 1984; Matsuki et al., 1992; Mignot et al., 1994; Tafti, 2009), a classical hallmark of autoimmune diseases. Yet, its classification as an autoimmune disease is still a matter of debate (for review, see Mignot et al., 1995; Partinen et al., 2014).

Accumulating evidence allows us to consider a possible microglial implication in narcolepsy. Increased microglial expression of MHC class II (a hallmark of chronic activation) has been reported in the central nervous system (CNS) of narcoleptic dogs. Reactive microglia were predominantly located in the brainstem (including the reticular formation), basal forebrain, and amygdala (Tafti et al., 1996). A recent publication also reported reduced levels of the chemokine receptors CCR1 and CCR3 in peripheral blood samples of patients with narcolepsy (Cartier et al., 2005; Eltayeb et al., 2007; Toyoda et al., 2015). These receptors are expressed by microglia (in the brain) and other macrophages (in the bloodstream and other tissues) to ensure proper and coordinated inflammatory responses. It is hypothesized that a decrease in CCR expression leads to a defect in the recognition and phagocytosis of damaged cells by microglia and consequently to a delayed resolution of acute inflammation. These defects could lead to enhanced autoimmunity resulting in the loss of hypocretin neurons.

Clinical studies have also revealed that narcolepsy patients have subtly dysregulated cytokine levels in their serum and CSF consistent with markers of microglial reactivity (Okun et al., 2004; Dauvilliers et al., 2014; Maurovich-Horvat et al., 2014; Tanaka et al., 2014). Multiple independent studies that include different ethnic populations showed a preponderant increase in IL-6 and TNFα expression in sera or plasma from patients with narcolepsy (Okun et al., 2004; Maurovich-Horvat et al., 2014; Tanaka et al., 2014). Other studies have identified changes in IL-4 (Dauvilliers et al., 2014) or IL-8 (Tanaka et al., 2014) levels. To further assess the role of inflammation in the degeneration of hypocretin neurons, Gerashchenko et al. (Gerashchenko and Shiromani, 2004) infused a low-dose of the endotoxin lipopolysaccharide (LPS) in the lateral hypothalamus of rats as a model of local chronic inflammation. This induced a decline in the number of neurons, including hypocretin neurons, in the lateral hypothalamus. Chronic LPS infusion also increased the number of MHC class II-positive microglia in the lateral hypothalamus. These data suggest that microglia-mediated inflammation might be a trigger for the loss of hypocretin neurons during narcolepsy (Gerashchenko and Shiromani, 2004).

In addition to their important roles in inflammation and immune response, microglia are the main phagocytes of the brain (Sierra et al., 2013). Microglial phagocytosis is a pivotal mechanism for the clearance of cellular elements in the CNS, as demonstrated by their ability to engulf brain-specific cargo, such as axonal and myelin debris or apoptotic neurons. Additionally, recent data has shown that microglia can execute neuronal death by phagocytosing stressed-but-viable neurons, a process termed "phagoptosis" (Brown and Neher, 2014). Microglia are equipped with a complementary array of receptors enabling them to recognize their targets (the

so-called "eat-me" signals) including proteins of the classical complement cascade (Savill et al., 2002; Ravichandran, 2010). Interestingly, a clinical study reported a correlation between narcolepsy and complement receptors (Cuccia et al., 1991). The thirty narcoleptic patients studied had a distinctive complotype, i.e., a combination of polymorphisms defining complement activity: BfS, C4A3, and C4B1 (Cuccia et al., 1991). One could hypothesize that exacerbation of complement-dependent microglial phagocytic activity is a plausible mechanism leading to the loss of (hypocretin) neurons as also observed in other neurodegenerative conditions such as Alzheimer's disease (Hong et al., 2016).

Overall, this evidence indicates that the homeostatic function of microglia is altered in the pathogenesis of narcolepsy, leading to an increased release of inflammatory factors, and alteration of their phagocytic activity. Both events alter microglia-neuron interactions and might support the degeneration of hypocretin neurons and subsequent sleep alterations.

Obstructive Sleep Apnea: Sleep Fragmentation and Chronic Intermittent Hypoxia

Obstructive sleep apnea (OSA), often associated with obesity, is a multifactorial systemic disease affecting approximately 10–25% of the general population worldwide (Peppard et al., 2013; Heinzer et al., 2015; Senaratna et al., 2017). In OSA the upper airways are narrowed or collapsed during sleep causing repetitive pauses of breathing (apneas) and concomitant reductions of blood oxygen level. Apneas are followed by increased breathing efforts, which in turn lead to episodic increases in blood carbon dioxide concentration. These events cause repeated arousals from sleep (sleep fragmentation) and/or excessive daytime sleepiness. OSA is often accompanied by neurocognitive dysfunction leading to impairments in attention and vigilance, learning, and memory, as well as executive functions (Zhou et al., 2016). If untreated, OSA associates with several comorbidities (Vijayan, 2012) such as cardiovascular disease (Sanchez-de-la-Torre et al., 2013) and metabolic syndrome, including obesity (Shechter, 2016) and insulin resistance (Ip et al., 2002), as well as mild cognitive impairment/dementia (Yaffe et al., 2011, 2014). Recent epidemiological studies also indicated that OSA may be associated with an increased risk of cancer (Nieto et al., 2012; Palamaner Subash Shantha et al., 2015; Gozal et al., 2016).

The two major acute pathophysiological consequences of OSA are: (1) tissue oxygen shortage/hypoxia with rapid re-oxygenation (i.e., chronic intermittent hypoxia, CIH) and (2) sleep fragmentation (SF) (Young et al., 1993). In OSA, most tissues, including the brain, are repeatedly exposed to reduced oxygen levels or a total lack of oxygen followed by rapid increases in oxygen availability. This pattern of repeated hypoxia-re-oxygenation (HRO) increases tissue levels of reactive oxygen species/reactive nitrogen species (ROS/RNS) and concomitantly activates the immune response as measured by the production of pro-inflammatory cytokines (Lavie, 2003, 2015). However, these cellular responses are not uniform across all tissues and vary with the severity of OSA.

In OSA patients, the poor health outcomes are a consequence of complex interactions between CIH and SF. However, with animal models it has been possible to begin dissecting the individual pathophysiological contributions of SF and tissue oxygenation. In the next sections we review each of them separately in light of a hypothesized microglial contribution.

Chronic Intermittent Hypoxia

Because the HRO processes in OSA are similar to the ischemia-reperfusion events in a wide range of brain pathologies, data from these disease models provide valuable information regarding the cellular signaling pathways which are possibly involved. However, it should be noted that in OSA, the tissue exposures to hypoxia are cyclic, and most importantly chronic, and therefore the cellular responses can differ substantially from those of acute exposures (Almendros et al., 2014). The main trigger of the inflammatory response measured in CIH is oxidative stress (Wang et al., 2010), defined as an imbalance between pro-oxidant and anti-oxidant systems, resulting in an excessive production of ROS such as superoxide, hydrogen peroxide, and various RNS (Lavie, 2015). ROS damage critical cellular biomolecules thus leading to cell injury or death. In OSA models of CIH, signs of injured cells have been reported in various brain areas but the hippocampus and cerebral cortex appear to be most sensitive (Gozal et al., 2001; Xu et al., 2004). These lesions were proposed to contribute to the widespread neurocognitive defects encountered in OSA (Lim and Veasey, 2010; Harper et al., 2013).

Evidence of microglial phenotypic transformation such as microglia-specific inflammatory gene expression as well as morphological changes have been reported in CIH models of OSA (Smith et al., 2013; Sapin et al., 2015). A recent review (Kiernan et al., 2016) posits several putative mechanisms by which microglial physiological functions, which are now recognized to be crucial for plasticity, learning, and memory, may be affected by CIH pathophysiology:

(i) By ROS. Either directly (although this hypothesis was not yet confirmed) or indirectly, by local signals generated by ROS injured/dying neurons.
(ii) By peripheral inflammation, a condition well established in OSA (Unnikrishnan et al., 2015). Signals of inflammation could reach microglia via neural transmission of vagal afferents reacting to circulating pro-inflammatory agents or the pro-inflammatory agents themselves could cross the blood-brain barrier to directly affect microglia.
(iii) By responding to DAMPs such as HSP60, HMGB1, and MRP8/14. Elevated levels of DAMPs have been reported in blood samples from OSA patients (Wu et al., 2010), as well as in the hippocampus of rodent models of CIH (Gozal et al., 2002).

Sleep Fragmentation

There is a growing literature indicating that short or insufficient sleep is associated with low grade inflammation (Everson, 2005; Mullington et al., 2010; Aho et al., 2013) as well as cellular

stress (Naidoo, 2012; Hakim et al., 2015). Recently, findings from longitudinal follow-up studies in patients with idiopathic REM sleep behavior disorder also revealed that most patients eventually develop neurodegenerative disorder, particularly Parkinson's disease or other synucleopathies (Stokholm et al., 2017). Positron emission tomography (PET scan) studies using the radiotracer [^{11}C](*R*)-PK11195, which labels "translocator protein 18 kDa (TSPO)," mainly expressed by microglia (Banati, 2002) and considered to modulate their inflammatory activity and phagocytosis (Karlstetter et al., 2014), revealed an increased binding of the radiotracer in the *substantia nigra* of patients with idiopathic REM sleep behavior disorder, thus identifying microglial "activation" as a potential therapeutic target for halting or delaying the neurodegenerative process.

Two experimental studies in rodent models of chronic sleep deprivation have also reported microglial morphological changes that comprise the enlargement of their cell body (Hsu et al., 2003) and increased expression of pro-inflammatory cytokines (Wisor et al., 2011a). Very recently, Bellesi et al. revealed using confocal microscopy analysis that microglial homeostatic surveillance of the parenchyma is reduced after 5 days of chronic sleep deprivation in mouse prefrontal cortex. The total length of microglial process arborization and the proportion of microglial cells showing ramified morphologies were significantly reduced. These changes were accompanied by an increased microglial co-localization with the marker of glutamatergic axon terminals VGLUT1, suggesting exacerbated phagocytosis, which could be mediated by the classical complement pathway considering that expression of the complement protein C3 targeting synapses for elimination (Schafer et al., 2012) was concomitantly increased by sleep deprivation (Bellesi et al., 2017). Expression of the TAM receptor MERTK, which regulates microglial surveillance of the parenchyma and phagocytic behavior under physiological conditions (Fourgeaud et al., 2016), was also found to be increased by sleep deprivation (Bellesi et al., 2017). Interestingly, astrocytes were additionally shown in Bellesi et al., using high-resolution serial block-face scanning electron microscopy, to phagocytose synaptic elements, mainly presynaptic axon terminals, both after acute and chronic sleep deprivation (Bellesi et al., 2017). Glial cells reactivity thus appears to be implicated in the detrimental consequences of sleep loss, through neuronal circuit remodeling or loss of their essential physiological functions. Synaptic loss is considered the best pathological correlate of cognitive decline across a variety of conditions that include aging and neurodegenerative diseases (Duman and Aghajanian, 2012; Spires-Jones and Hyman, 2014).

In the case of OSA, however, the main sleep phenotype is SF (rather than sleep loss) coupled with an excessive daytime sleepiness and widespread cognitive dysfunction. In the last decade rodent models of SF have demonstrated that SF alone (without CIH) induces sleepiness and cognitive impairment (McCoy et al., 2007; McKenna et al., 2007; Tansey et al., 2007; Tartar et al., 2010; Ramesh et al., 2012), obesity (Wang et al., 2014), immune mediated metabolic dysfunction (Zhang et al., 2014), cardiovascular alterations (Carreras et al., 2014), and alterations of the gut microbiota (Poroyko et al., 2016). All of these consequences have an oxidative stress-mediated inflammatory component that could directly or indirectly affect microglial functions as proposed for CIH above. Gut microbiota can also directly influence microglial maturation, leading for instance to an attenuated production of pro-inflammatory cytokines upon immune stimulation (Erny et al., 2015). In addition, SF mediated increase in microglial surveillance of synaptic elements or remodeling of neuronal circuits could underlie some of the reported impairments in cognitive performance through synaptic loss.

Recreational-Drug Induced Sleep Loss

Recreational drugs have deleterious consequences on sleep, with dextro-methamphetamine (d-METH) exerting its sustained wake-promoting effects (for up to several days) by elevating the monoaminergic tone. It is rarely prescribed due to concerns involving neurotoxicity, notably to dopaminergic and serotoninergic neurons, in addition to its aphrodisiac and euphoriant effects, among other mind-altering properties, and strong addictiveness. PET scans of chronic d-METH self-administrating individuals using the radiotracer [^{11}C](*R*)-PK11195 revealed exacerbated microglial reactivity across several brain regions that receive dopaminergic or serotoninergic innervation, including the midbrain, striatum, thalamus, orbitofrontal, and insular cortices (Sekine et al., 2008). This microglial transformation is likely plastic and reverts to normal over longer periods of abstinence, since the binding levels of [^{11}C](*R*)-PK11195 correlated inversely with the duration of withdrawal in d-METH abusers (Sekine et al., 2008).

The effects of d-METH on sleep and wakefulness could be mediated by microglia, through exacerbated oxidative stress and pro-inflammatory cytokine release (Wisor et al., 2011b). In mice undergoing microglial depletion [through delivery of ganciclovir in transgenics expressing the suicide agent herpes thymidine kinase under control of the CD11b promoter], the duration of daily wakefulness produced by d-METH was reduced by nearly 1 h. *Ex vivo* nitric oxide synthase (NOS) activity, and *in vivo* NOS expression were also elevated in cortical CD11b$^+$ microglia from wild-type mice upon acute d-METH exposure. Additionally, CD11b$^+$ cells, which mainly comprise microglia in the brain, were the only ones among the cerebral cortex found to exhibit changes in sleep-regulatory IL-1β pro-inflammatory cytokine expression in response to d-METH (Wisor et al., 2011b). In a follow up study by the same group, it was further shown that IL-1 receptor(R) deficiency potentiates, although modestly, the wake-promoting effects of d-METH (Schmidt and Wisor, 2012). The increased time spent in NREM sleep subsequent to d-METH-induced wakefulness was abolished in IL-1R knockout mice, while the increased time spent asleep after 3 h of behaviorally enforced wakefulness was similarly prevented in the knockouts. These findings indicate that microglial IL-1β signaling through IL-1R contributes to the hypersomnolence that ensues sleep loss, whether it is triggered pharmacologically by recreational drugs or through behaviorally-induced sleep deprivation (Schmidt and Wisor, 2012).

CONCLUSION AND PERSPECTIVES

While microglial function was not a primary focus in several of the studies covered in this review, their overall findings indicate that microglial dysfunction (in the form of exacerbated phagocytic activity and neuroinflammation) may contribute to the pathophysiology of sleep disorders. These activities would be exerted in concert with other brain cells, vascular, glial, or neuronal, and the peripheral immune cells transiting to the brain. In light of these combined findings, substantial amount of evidence point toward an emerging role for neuroinflammatory processes in the pathophysiology of sleep disorders. Microglial (and astrocytic) phagocytosis of synaptic elements was also found to be exacerbated in rodent models of sleep deprivation, indicating their possible implication in its detrimental consequences on cognition. Microglia thus emerge as an important subject of investigation for future sleep-related studies, as already recognized in a variety of neuropsychiatric and neurodegenerative contexts. In addition to the diseases discussed in this review, it would be interesting to study the roles of microglia in the regulation of sleep functions: their contribution to memory consolidation and transformation (Dudai et al., 2015), synaptic homeostasis which downscales synapses during sleep to ensure learning during wake (Tononi and Cirelli, 2014), and the brain clearance from its toxic metabolites during sleep, in cooperation with astrocytes, which form a system of perivascular tunnels named the "glymphatics" (Xie et al., 2013).

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frontiers in Neuroanatomy

REVIEW
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The Effects of Amphetamine and Methamphetamine on the Release of Norepinephrine, Dopamine and Acetylcholine From the Brainstem Reticular Formation

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Amphetamine (AMPH) and methamphetamine (METH) are widely abused psychostimulants, which produce a variety of psychomotor, autonomic and neurotoxic effects. The behavioral and neurotoxic effects of both compounds (from now on defined as AMPHs) stem from a fair molecular and anatomical specificity for catecholamine-containing neurons, which are placed in the brainstem reticular formation (RF). In fact, the structural cross-affinity joined with the presence of shared molecular targets between AMPHs and catecholamine provides the basis for a quite selective recruitment of brainstem catecholamine neurons following AMPHs administration. A great amount of investigations, commentary manuscripts and books reported a pivotal role of mesencephalic dopamine (DA)-containing neurons in producing behavioral and neurotoxic effects of AMPHs. Instead, the present review article focuses on catecholamine reticular neurons of the low brainstem. In fact, these nuclei add on DA mesencephalic cells to mediate the effects of AMPHs. Among these, we also include two pontine cholinergic nuclei. Finally, we discuss the conundrum of a mixed neuronal population, which extends from the pons to the periaqueductal gray (PAG). In this way, a number of reticular nuclei beyond classic DA mesencephalic cells are considered to extend the scenario underlying the neurobiology of AMPHs abuse. The mechanistic approach

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Abbreviations: 5-HT, serotonin; 6-OHDA, 6-hydroxydopamine; A6sc, nucleus subcoeruleus; Ach, acetylcholine; AMPH(s), amphetamine(s); AP, area postrema; α1-AR(s), alpha1-adrenergic receptor(s); α1B-AR, type B alpha1-adrenergic receptor(s); β-ARs, beta-adrenergic receptor(s); Cart, cocaine- and amphetamine-regulated transcript; ChAT, choline acetyltransferase; CTZ, chemoreceptor trigger zone; DA, dopamine; DAT, DA transporter; DBH, DA beta-hydroxylase; DMV, dorsal nucleus of the vagus; DNB, dorsal NE bundle; DRD, dorsal dorsal raphe; DRL, lateral dorsal raphe; DRV, ventral dorsal raphe; DSP-4, NE neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine; E, epinephrine; ICSS, intracranial self-stimulation; LC, A6, locus coeruleus; LDTg, Ch6, laterodorsal tegmental nucleus; MAO, monoamine oxidase; METH, methamphetamine; MFB, medial forebrain bundle; NAc, nucleus accumbens; nACh-R(s), nicotinic ACh receptor(s); NE, norepinephrine; NET, NE transporter; NTS, nucleus of the solitary tract; OX1R, orexin type 1 receptors; PAG, periaqueductal gray; PB, parabrachial nucleus; PKC, protein kinase C; PPN, PPTg, Ch5, peduncolopontine nucleus or tegmentum; RF, reticular formation; RVLM, rostral ventrolateral medulla; SERT, 5-HT transporter; SNpc, Substantia Nigra pars compacta; SPNs, sympathetic pre-ganglionic neurons; VLPAG, ventrolateral PAG; VMAT-2, vesicular monoamine transporter type-2; VNB, ventral NE bundle; VTA, A10, Ventral Tegmental Area.

followed here to describe the action of AMPHs within the RF is rooted on the fine anatomy of this region of the brainstem. This is exemplified by a few medullary catecholamine neurons, which play a pivotal role compared with the bulk of peripheral sympathetic neurons in sustaining most of the cardiovascular effects induced by AMPHs.

Keywords: methamphetamine, norepinephrine, brainstem reticular formation, addiction, arousal, neurotoxicity, hypertension

INTRODUCTION

Amphetamine (AMPH) and mostly methamphetamine (METH) are widely abused psychostimulants, which possess a phenylethylamine structure. In the present review article, we focus on both compounds, which, from now on are referred to as AMPHs meant "*sensu stricto*" to rule out other amphetamine-related compounds. Both acute and chronic AMPHs intake/administration determines behavioral, purely motor, and vegetative alterations; each effect relies on a quite specific constellation of reticular nuclei, which often overlap. This calls for a constant reference to the functional anatomy of the brainstem reticular formation (RF), which stands as the seminal brain area to comprehend the neurobiology of AMPHs. Short-term effects of AMPHs include intense euphoria, increased heart rate, hypertension, hyperthermia, excitation, alertness and wakefulness (Meredith et al., 2005; Homer et al., 2008; Moratalla et al., 2017), which can be related to specific groups of brainstem nuclei. On the other hand, reiterated intake/administration of AMPHs produces long-lasting alterations, which may be the consequence of neurotoxicity or being produced by persistent epigenetic changes driving marked plastic phenomena in some brain areas (Battaglia et al., 2002; Robison and Nestler, 2011; Godino et al., 2015; Limanaqi et al., 2018). Behavioral alterations include motor and psychiatric effects such as hyper-locomotion, stereotypies, addiction, craving, aggressiveness, anorexia, psychosis, depression, cognitive impairments, and altered cortical excitability ranging from sleep alterations up to seizures (Meredith et al., 2005; Brown et al., 2011; Marshall and O'Dell, 2012; Moratalla et al., 2017). All these effects vary over time following reiterated exposure and some of them occur as the consequence of neurotoxicity or the onset of "neuronal sensitization" (Robinson and Berridge, 2000). In fact, when administered chronically and/or at high doses, AMPHs and mostly METH produce toxicity in specific brain regions or even in peripheral organs, especially those receiving dense innervation by the sympathetic nervous system (Liu and Varner, 1996; Albertson et al., 1999; Darke et al., 2008; Volkow et al., 2010; Thanos et al., 2016). Most of the effects induced by AMPHs are grounded on the powerful release of a variety of neurotransmitters, which occurs through a mechanism owning a fair anatomical specificity. The lateral column of the brainstem RF contains neuronal populations, which possess common molecular targets characterizing specific neuronal phenotypes. These very same targets are recruited quite selectively by AMPHs administration (**Figure 1**). In fact, the chemical structure of AMPHs is characterized by an aromatic ring and a nitrogen on the aryl side-chain (Biel and Bopp, 1978), which recapitulates most monoamine neurotransmitters including catecholamines (dopamine, DA, norepinephrine, NE, epinephrine, E) and indoleamines (serotonin, 5-HT, tryptamine and other trace amines; Heal et al., 2013). In the light of a cross affinity, AMPHs behave as a competitive substrate for monoamine transporters including NE transporter (NET), DA transporter (DAT) and 5-HT transporter (SERT; Rothman and Baumann, 2003; Fleckenstein et al., 2007; Sitte and Freissmuth, 2015). This is the reason why AMPHs target quite selectively those neurons, which produce monoamine as neurotransmitters. Such a simple concept explains why the RF is, at large, the target brain region for the mechanism of action of AMPHs. Likewise, we focus this review on this very same area to analyze in depth those effects not fully explained by DA neurons which are hosted in the high mesencephalic reticular nuclei. In detail, most monoamine-containing nuclei of the ponto-medullary RF are placed in a quite restricted brain region corresponding to the median and lateral column of the RF of the brainstem. The basis for both short- and long-term behavioral, motor and vegetative effects of AMPHs stems from such a selective uptake and common intracellular targets of AMPHs within these monoamine-containing neurons (Bucci et al., 2017). These neurons produce widespread innervation of a variety of limbic, motor and iso-cortical areas where ultimately the effects of AMPHs are produced. In fact, at sub-cellular levels, the targets of AMPHs are both the cell body and mostly, the axon terminal. The latter represents the primary AMPHs' target. In fact, the powerful release of monoamines relies on the effects of AMPHs on axon varicosities, where monoamines are concentrated in baseline conditions. AMPHs massively release neurotransmitters in high amount, which can be quantitatively assessed by placing microdialysis probes within all brain regions innervated by monoamine neurons. At the same time, this can be correlated with a number of systemic effects, which are mediated by each brain region under a powerful reticular monoamine innervation. In fact, following AMPHs there are dramatic changes in breathing, blood pressure, locomotor activity, muscle tone, sleep-wake cycle, mood, orienting to novelty, arousal, anxiety, reward along with innumerous vegetative and somatic phenomena occurring in the human body. A great amount of investigations, commentary manuscripts and books focused on the effects of AMPHs abuse on DA-containing neurons within the mesencephalon (Seiden et al., 1976; Wagner et al., 1979; Nielsen et al., 1983; Di Chiara and Imperato, 1988; Sonsalla et al., 1989; Cadet et al., 1994; Delle Donne and Sonsalla, 1994; Fornai et al., 1997, 2001; Battaglia et al., 2002; Ferrucci et al., 2008; Moratalla et al., 2017). Instead, the contribution of low

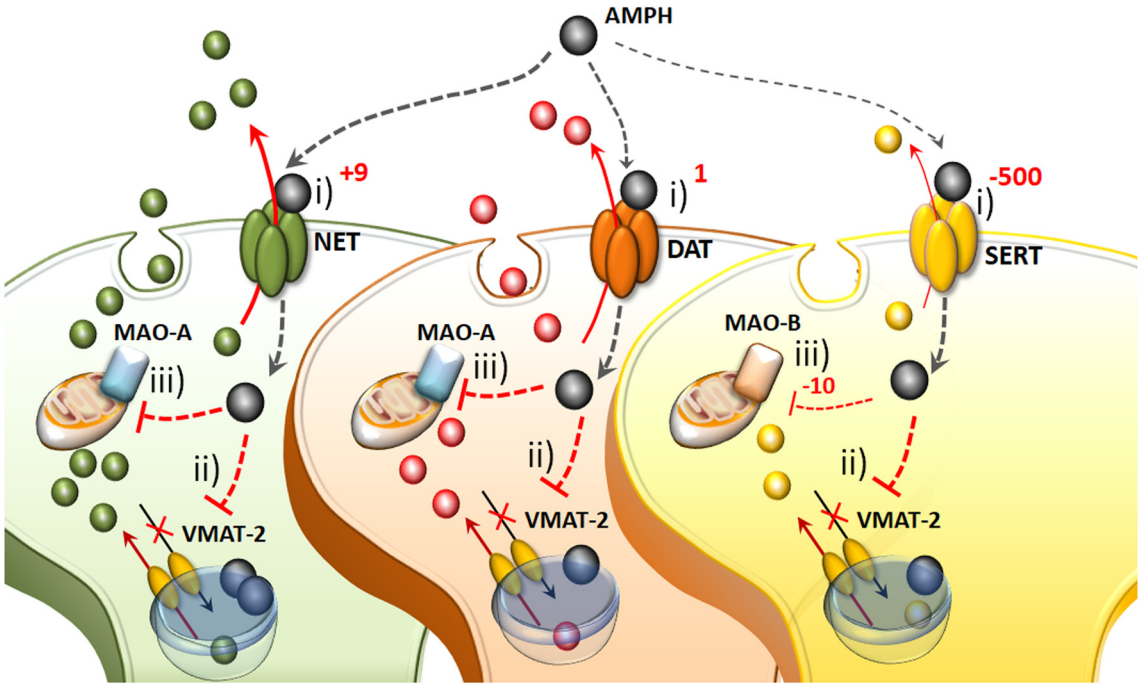

FIGURE 1 | The molecular mechanisms of amphetamine(s) (AMPHs) in monoamine-containing neurons. (i) The primary molecular target, which provides neuronal selectivity for AMPHs, consists in the plasma membrane transporter. In fact, AMPHs behave as competitive substrates for the re-uptake through the NE transporter (NET), dopamine (DA) transporter (DAT) and 5-HT transporter (SERT; Rothman and Baumann, 2003; Fleckenstein et al., 2007). These transporters normally work by taking up extracellular monoamines to the axoplasm, which is the main mechanism to terminate their activity (Iversen et al., 1965; Axelrod and Kopin, 1969; Coyle and Axelrod, 1971; Aggarwal and Mortensen, 2017). Cross-affinity between AMPHs and neurotransmitters contributes to generate the quite selective storage of AMPHs within specific neurons. Once bound to the plasma membrane transporter, AMPHs enter the axoplasm while reverting the transport direction (Sulzer et al., 1993). This occurs mostly for catecholamine neurons since AMPHs strongly discriminate between SERT, to which they bind with much lower affinity (500-fold less) compared with DAT and NET (Rothman and Baumann, 2003). In particular, AMPHs bind to the NET with five-to-nine-fold higher affinity compared with the DAT (Rothman and Baumann, 2003). This is the main reason why AMPHs release NE more potently than DA and much more than 5-HT (Rothman et al., 2001). (ii) Within monoamine axons, AMPHs encounter a second specific target called vesicular monoamine transporter type-2 (VMAT-2), which is also shared with monoamines. In this way, AMPHs enter the synaptic vesicles. At this level, AMPHs impair the acidification of the vesicle, which generates an acidic pH (Sulzer and Rayport, 1990; Sulzer et al., 1993, 1995). This acidic environment is erased by AMPHs, which rise the vesicular pH value from 4 up to 7, which corresponds to a 1,000-fold increase in the concentration of H^+ ions. Thus, monoamines, which are weak bases, are charged at low pH, while at a neutral pH lose their charge, and diffuse through the vesicle membrane, thus massively invading the axoplasm (Brown et al., 2000, 2002; Pothos et al., 2000). In this way, axonal monoamines either passively or *via* a reverted plasma membrane transporter fill extracellular space where they reach a massive concentration (Sulzer et al., 1995, 2005). (iii) The third molecular target, which is impaired by AMPHs, is the mitochondrial-bound enzyme monoamine oxidase (MAO). Both MAO-A/-B iso-enzymes oxidatively deaminate DA, NE and 5-HT. Nonetheless, MAO-A/-B isoforms differ in substrate preference, inhibitor affinity and regional distribution within either single neurons or different animal species (Robinson et al., 1977; Youdim, 1980; Sourkes, 1983; Gesi et al., 2001; Youdim et al., 2006; Bortolato et al., 2008). These differences are seminal to explain the specific effects of AMPHs within various monoamine neurons. In fact, MAO-A, are competitively inhibited by methamphetamine (METH) with a 10-fold higher affinity compared with MAO-B. MAO-A is placed within synaptic terminals of DA and NE neurons, while MAO-B are the only isoform operating within 5-HT terminals and non-catecholamine neurons. Thus, apart from rats and a few animal species, the effect of AMPHs on the amount of extracellular monoamines is remarkable concerning NE and DA, being less pronounced for 5-HT.

brainstem nuclei is much less investigated and, apart from the Locus Coeruleus (LC), only a few manuscripts investigated the recruitment of the variety of catecholamine neurons within the low brainstem during AMPHs administration. Despite not being widely investigated, this point deserves very much attention since the specific functional anatomy of these nuclei may lead to comprehension of the brainstem-related nature of a variety of AMPHs-induced alterations. Therefore, in the present review article, we avoid the analysis of DA neurons of the Substantia Nigra pars compacta (SNpc) and Ventral Tegmental Area (VTA), and focus instead on catecholamine nuclei of the low brainstem. In particular, we focus on NE nuclei located within the lateral column of the bulbo-pontine RF and a mixed neuronal population within the dorsal raphe/periaqueductal gray (PAG), which contains a subset of NE and DA neurons (Battenberg and Bloom, 1975; Saavedra et al., 1976; Steinbusch et al., 1981; Nieuwenhuys et al., 1988; Baker et al., 1990, 1991; Lu et al., 2006; Li et al., 2016; Bucci et al., 2017; Cho et al., 2017). We also overview cholinergic cells placed in the lateral column of the pontine RF, which extend up to the lateral wings of the dorsal raphe (Satoh et al., 1983; Nieuwenhuys et al., 2007; Vasudeva and Waterhouse, 2014; de Oliveira et al., 2016). In detail, the dorsal raphe contains 5-HT neurons in all nuclear regions, while catecholamine and acetylcholine cells are placed in the most rostral extent of the dorsal raphe both in rodents and humans (Saavedra et al., 1976;

Steinbusch et al., 1981; Baker et al., 1990, 1991; Nieuwenhuys et al., 2007; Mai and Paxinos, 2012; Li et al., 2016; Cho et al., 2017). As shown by pioneer studies, the amount of NE neurons in the dorsal raphe is remarkable, being about one-third of 5-HT neurons. Instead, the amount of DA cells is roughly a half compared with NE neurons, at least in the rat (Saavedra et al., 1976). While the median ventral PAG contains a few 5-HT neurons, both NE and DA neurons are placed in the ventral/ventrolateral corner of the PAG (Hökfelt et al., 1984; Dougalis et al., 2012; Mai and Paxinos, 2012). DA cells occurring within this brain region are known as A10dc (dorsal central) in order to distinguish them from the adjacent VTA DA neurons (Hökfelt et al., 1984). Again, NE neurons placed at this level represent the dorsal and rostral extent of the LC complex. This rostral extent was described in humans as nucleus epicoeruleus by Mai and Paxinos (2012). In addition to the presence of NE-containing neurons, the dorsal raphe receives a powerful NE innervation. This is supported by findings in humans, where the amount of NET in the ventral nuclei of the dorsal raphe matches the amount of NET which can be measured within the LC (Ordway et al., 1997). There is a remarkable affinity of AMPHs for NET compared with DAT and SERT. In fact, AMPHs administration produces a more powerful release of NE compared with DA (Rothman et al., 2001; Weinshenker and Schroeder, 2007; Schmidt and Weinshenker, 2014). This means that behavioral effects induced by AMPHs in various animal species including humans can be partly explained by massive NE release (Rothman et al., 2001; Weinshenker et al., 2002; Weinshenker and Schroeder, 2007). Thus, despite a DA solely-based perspective about AMPHs and behavior, NE creeps back in participating to AMPHs-induced behavioral changes. This calls for dissecting further anatomical areas within NE-containing brainstem nuclei to comprehend the effects induced by AMPHs, along with considering the anatomical connections linking the bulbo-pontine with the mesencephalic RF as a biological substrate, which may sustain a key circuitry mediating the effects of AMPHs.

THE FUNCTIONAL ANATOMY OF THE CATECHOLAMINE RETICULAR NUCLEI OF THE BRAINSTEM IN THE EFFECTS OF AMPHs

Since the present review is an attempt to relate the effects of AMPHs with specific NE nuclei of the brainstem, a preliminary synthetic overview of the neuroanatomy of these nuclei appears to be mandatory. This will make it easier to orient within the brainstem when referring to the site-specificity of the effects induced by AMPHs.

NE-Containing Reticular Nuclei

Catecholamine-containing nuclei are mainly housed within the lateral extent of the RF (**Figure 2**). A very recent original manuscript provided stereological morphometry data encompassing all brainstem reticular catecholamine nuclei at one glance (Bucci et al., 2017). These include NE neurons of the pons and medulla, which were identified by using TH immunostaining (Bucci et al., 2017). For this reason, we will refer to NE-containing nuclei and we will include the E-related sub-nuclei as a putative attribute, since most of them are believed to represent a continuum with NE areas. This is the case of nuclear complexes known as A/C nuclear groups, where the letter "A" indicates NE neurons and the letter "C" indicates E neurons (Hökfelt et al., 1974). The A1/C1 cell group is placed in the sub-pial aspect of the rostral ventrolateral medulla (RVLM). The A2/C2, also known as dorsomedial cell group appears medially on the floor of the IV ventricle. Reticular neurons of A2/C2 intermingle with neurons of the dorsal nucleus of the vagus (DMV) and nucleus of the solitary tract (NTS) to constitute an overlapped, neuromelanin pigmentated area, which is named ala cinerea. The posterior region of ala cinerea extends towards the obex to constitute the area postrema (AP), which corresponds approximately to the chemoreceptor trigger zone (CTZ; Potes et al., 2010). A3/C3 area is still poorly investigated due to species variability (Howe et al., 1980; Vincent, 1988; Paxinos et al., 1995; Menuet et al., 2014). Similarly, fragmentary information deals with the A4 nucleus once believed to occur only in primates though it was recently identified in rodents (Bucci et al., 2017). The A5 nucleus is placed ventrally in the pons, close to the roots of the facial nerve. Moving towards the dorsal and medial aspect of the pons, these neurons form a continuum with other NE neurons belonging to the A6sc (nucleus subcoeruleus) and A6 (locus coeruleus, LC) nuclei. A5 and A6 (LC) represent the primary sources of NE afferents to the VTA and A1/C1 (Bucci et al., 2017). The A7 nucleus (lateral lemniscus nucleus) is placed in the pons, immediately lateral to the rostral end of the parabrachial (PB) nucleus. A6 (LC) is the biggest NE-containing nucleus within the central nervous system (CNS) and it is located in the upper part of the floor of the IV ventricle, within the pons. NE-containing neurons of LC, together with A6sc and the scattered TH-positive cells within the medial PB form a tube-shaped continuum, which is named LC complex. The A4 area, when present, can be considered within this complex as well (Bucci et al., 2017). In humans, the LC complex also includes the nucleus epicoeruleus, which occurs in the rostral dorsal raphe, within the ventrolateral PAG (Mai and Paxinos, 2012).

Neurons belonging to the LC region profusely send their axons to the entire CNS, providing the main source of NE to the brain, and mostly, to the whole cerebral cortex (Loughlin et al., 1982). In addition, the fine neuroanatomy of NE (and catecholamine) fibers possesses typical features. In fact, apart from the marked spreading of axonal projections due to profuse collateralization, which is typical for neurons forming the isodendritic core of the RF, axon collaterals are characterized by the presence of varicosities, named "boutons en passage" (**Figure 3**). Since once released following AMPHs, NE persists in the extracellular space for a considerable amount of time until being taken back up by the NE terminal, the time persistency and the volume filled by AMPHs-induced extracellular NE are noticeable. In fact, AMPHs promote NE release and impair NE uptake. This allows NE to be released in the extracellular medium from each varicosity along the course of axon fibers to produce extra-synaptic,

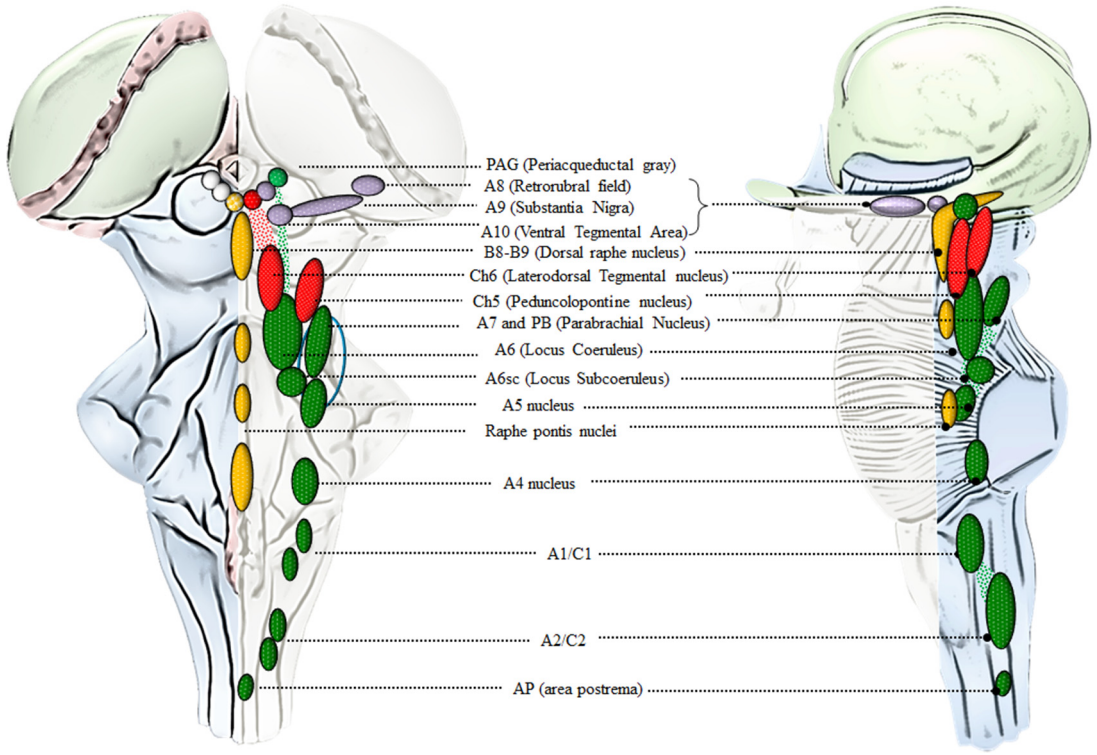

FIGURE 2 | An anatomical overview of the brainstem reticular formation (RF). This cartoon provides a synthetic overview of the neuroanatomy of brainstem RF nuclei in order to foster orientation within the brainstem when referring to the site-specificity of the effects induced by AMPHs. Monoamine-containing nuclei are placed in a quite restricted brain region corresponding to the median and lateral column of the brainstem RF. In detail, the median column hosts 5-HT-containing nuclei (yellow), which extend along the medulla and pons to reach the mesencephalon at the level of the periaqueductal gray (PAG). 5-HT containing nuclei at this level represent the rostral extent of the dorsal raphe nucleus (B8-B9). Since an analysis of 5-HT neurons in the effects of AMPHs is beyond the scope of the present review article, we focus on the dorsal raphe nucleus just concerning the PAG region, which contains catecholamine neurons. In fact, the PAG hosts a mixed population of catecholamine and acetylcholine (ACh)-containing nuclei which are placed in the lateral column of the RF. This is the case of DA (violet), NE (green) and ACh (red) nuclei which represent the rostral extent of A10 Ventral Tegmental Area (VTA), A6 (LC) and CH6, respectively. Moving downstream to the mesencephalic DA nuclei (A8, A9, A10), a constellation of nuclei appears within the lateral column of RF. These include the two pontine ACh nuclei (Ch5 and Ch6) and a number of NE nuclei placed along the ponto-medullary RF, namely A6 (LC), A6sc (subcoeruleus), A7 which intermingles with NE neurons of the rostral PB nucleus (here represented as a continuum), A5, A4, and two mixed NE/E-containing groups in the medulla, namely A1/C1 [rostral ventrolateral medulla (RVLM)] and A2/C2 (dorsomedial cell group), which extends towards the obex within the AP (area postrema). The A7 neurons together with PB-NE neurons and the A5 nucleus contribute to define the Kolliker-Fuse nucleus (blue circle, Milner et al., 1986; Byrum and Guyenet, 1987). The A6 nucleus together with the A6sc, the medial PB in the rostral pons and the epicoeruleus nucleus within the PAG, constitute the LC complex.

paracrine effects, even distant from the release site *via* a volume transmission. Altogether, these features synergize to produce extremely widespread effects following AMPHs activation of NE nuclei.

The Role of NE Nuclei in AMPHs-Induced Behavioral Effects

NE is key in mediating behavioral correlates effects of AMPHs. Specifically, NE sourcing from the RF strongly modulates AMPHs-induced behaviors and reward, which is the pre-requisite for sustaining reinforcing behavior leading to AMPHs addiction. In fact, reiteration of drug intake induces plastic changes within specific neuronal systems, which are necessary to develop craving and addiction (Pierce and Kumaresan, 2006). In this paragraph, specific reticular NE nuclei will be related to rewarding effects induced by AMPHs. From a general viewpoint, NE mediates a large amount of the rewarding effects induced by AMPHs as shown early in the '60s (Poschel and Ninteman, 1963; Stein, 1964; Sofuoglu and Sewell, 2009). This occurred in the context of the "Catecholamine theory of reward" (Hanson, 1966; Crow et al., 1972; Crow, 1972, 1973; Wise, 1978), where NE was thought to be the main neurotransmitter to produce reward following a variety of psychostimulants. In line with this, drugs reducing NE activity (by depleting NE stores, or inhibiting NE synthesis, or damaging NE axons) were shown to dampen intracranial self-stimulation (ICSS), which was used as an experimental model for reward (Fibiger and Phillips, 1974). A more detailed knowledge about the anatomy of the low brainstem RF provided the substrate to confirm the contribution of NE nuclei to the neurobiology of reward induced by psychostimulants (Wise, 1978; Ordway et al., 1997; Weinshenker and Schroeder, 2007). For instance, positive self-stimulation sites exist in the LC (A6) as well as along ascending NE pathways. These include:

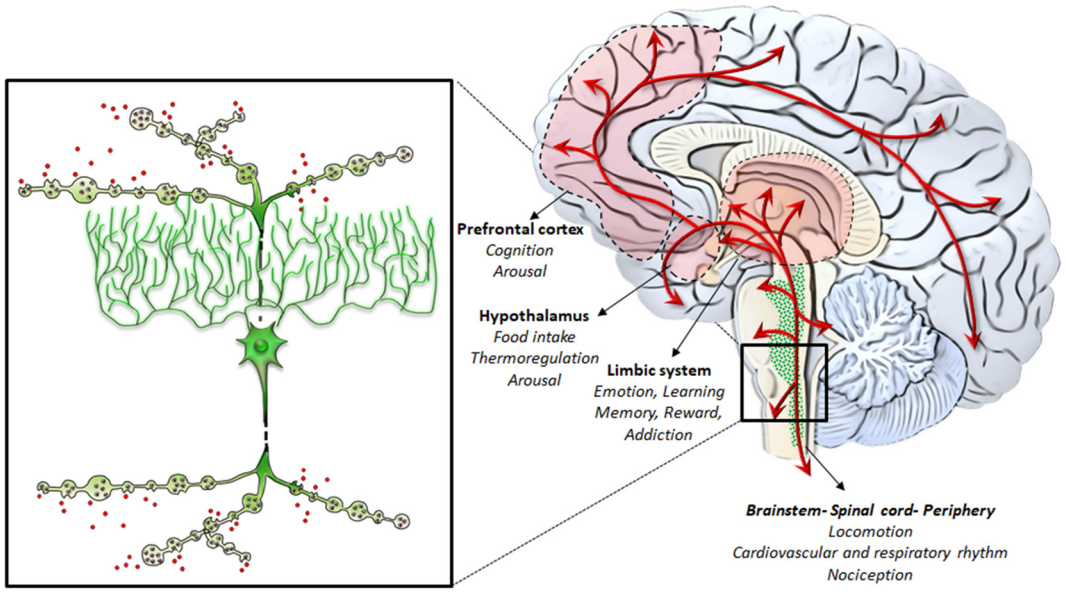

FIGURE 3 | NE-containing neurons of the RF: from gross anatomy to the fine iso-dendritic nature. NE-containing neurons of the RF profusely branch their axons to release NE throughout the whole central nervous system (CNS) and the autonomic nervous system. This is grounded on the typical fine neuroanatomy of NE fibers. In fact, apart from the marked spreading of axonal projections due to a profuse collateralization (which is typical for neurons forming the iso-dendritic core of the RF), axon collaterals are gifted with varicosities, also named "boutons en passage." This is key in the case of AMPHs, which promote massive NE release while impairing NE uptake. This allows NE to be released in the extracellular medium from each varicosity along the course of axon fibers to produce extra-synaptic, paracrine effects, even remotely from the release site *via* volume transmission. Altogether, these features synergize to produce widespread extra-synaptic effects following AMPHs-induced NE release in each brain area. This explains why AMPHs activate wide brain areas leading at the same time to different behavioral, motor and vegetative effects. These include the limbic system, the hypothalamus, the prefrontal cortex, as well as the whole brainstem, spinal cord and peripheral organs receiving NE sympathetic innervation.

(i) the ventral NE bundle (VNB, which originates from the LC) as well as; (ii) the dorsal NE bundle (DNB); and (iii) the medial forebrain bundle (MFB), which originate from a number of NE nuclei within the pons and medulla, including LC, PB, A1 and A2 (Dresse, 1966; Stein and Wise, 1969; Crow et al., 1972; Wise et al., 1973; Ritter and Stein, 1974; Carlezon and Chartoff, 2007). In fact, apart from LC, caudal NE nuclei such as the A1/C1 and A2/C2 cell groups project profusely to the forebrain and hypothalamus (Nieuwenhuys et al., 1982; Mai et al., 2008; Berridge et al., 2012). As shown by retrograde tracing studies, the LC provides nearly 50% of NE input to the forebrain and hypothalamus, while A1/C1 and A2/C2 groups contribute from 25% to 40% (España and Berridge, 2006). This partly explains why AMPHs promote arousal (Berridge et al., 1999; Berridge and Stalnaker, 2002; Berridge, 2006). AMPHs-induced cortical arousal *via* NE release from these nuclei is associated with drug-seeking behavior and relapse (España et al., 2016). This may occur also *via* NE acting on hypothalamic perifornical orexin neurons. Orexins are hypothalamic neuropeptides implicated in a variety of behaviors including sleep/wakefulness, feeding and reward (Sakurai et al., 1998; Sakurai, 2007; Sakurai and Mieda, 2011). Recent studies suggest that orexins play a role in drug-induced sensitization and drug-seeking motivation, which occurs through a fair neuro-anatomical specificity (Sharf et al., 2010; Mahler et al., 2012). The effects of orexin-containing nuclei are grounded on their strong connection with reticular NE nuclei (**Figure 4**). In fact, NE nuclei of the brainstem represent the most densely orexin-innervated neuronal population (Date et al., 1999; Nambu et al., 1999; Peyron et al., 2000; Marcus et al., 2001). This occurs mostly within NE LC neurons, where orexin type 1 receptors (OX1R) are densely expressed to promote arousal and locomotor activity (Hagan et al., 1999; Marcus et al., 2001). A reciprocal modulation occurs between LC-NE and hypothalamic perifornical orexin-containing neurons (van den Pol et al., 2002; Bayer et al., 2005; Gompf and Aston-Jones, 2008). This is key in the case of METH, which increases orexin levels in METH abusers (Chen et al., 2016). Likewise, METH administration strongly activates orexin-producing neurons, as shown by the increase in c-Fos expression (Estabrooke et al., 2001; Cornish et al., 2012). A considerable amount of orexin receptors is present also in lower reticular NE groups including A1/C1 and A2/C2 (Marcus et al., 2001), which explains functional studies addressing the role of such a connection in food intake and AMPHs-induced anorexia (McCabe and Leibowitz, 1984; Li et al., 2015; Ritter, 2017). Apart from providing molecular and anatomical specificity, NE neurons are also involved in genetic susceptibility to AMPHs-induced behavioral effects. In fact, a genetic polymorphism affecting the limiting enzyme for NE synthesis, DA beta-hydroxylase (DBH), is involved in substance abuse disorders (Kalayasiri et al., 2014). Such a hypothesis

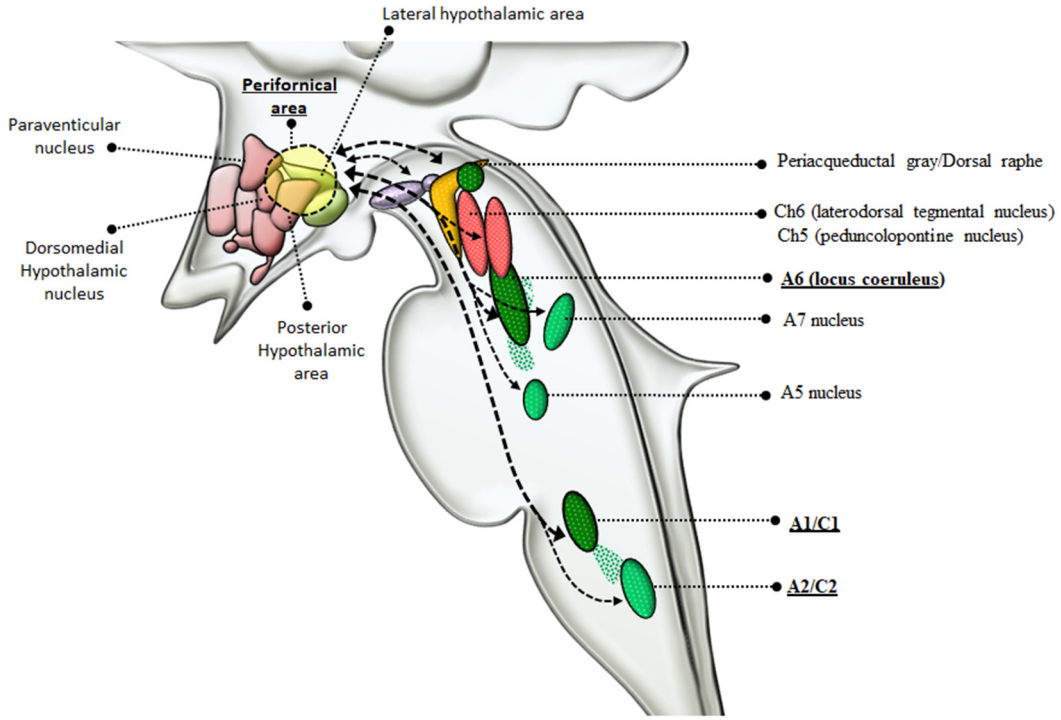

FIGURE 4 | Connections between reticular nuclei and orexin-containing perifornical neurons of the hypothalamus. Orexin-containing neurons are placed within the dorsolateral hypothalamus corresponding to the perifornical area. Orexin perifornical neurons are profusely connected with a number of brainstem RF nuclei including the PAG/dorsal raphe, VTA, Ch6, Ch5, A6, A5, A7, A1/C1, A2/C2 (Marcus et al., 2001; Sharf et al., 2010; Mahler et al., 2012). However, among these neuronal groups, orexins display the highest neuro-anatomical and pharmacological specificity with NE-containing neurons, as witnessed by the strong reciprocal connections with LC and A1/C1 nuclei (thick dashed connectors) and the abundance of orexin receptors in these nuclei (McCabe and Leibowitz, 1984; van den Pol et al., 2002; Bayer et al., 2005; Gompf and Aston-Jones, 2008; Li et al., 2015; Ritter, 2017). In the light of such connections with NE neurons, orexin-containing neurons are dragged in a variety of functions including, arousal, locomotion, feeding, reward, sensitization, motivation (Sakurai et al., 1998; Sakurai, 2007; Sakurai and Mieda, 2011). Since AMPHs are powerful NE releasers and they strongly activate orexin-producing neurons as well, it is likely that the strong connections between orexin and NE-neurons of the LC and A1/C1 are implicated in the effects induced by AMPHs.

was tested specifically in DBH KO mice, where AMPH was much more effective in producing DA-release and behavioral sensitization (Weinshenker et al., 2008). It is remarkable that such a potentiation of DA-release and behavioral sensitization is reminiscent of what occurs in LC-damaged mice following METH administration.

Molecular Mechanisms Bridging Reticular NE Nuclei to AMPHs-Induced Behavioral Effects

This paragraph adds on the well established evidence that AMPHs-induced DA release is key to produce locomotor stimulation, sensitization and neurotoxicity to encompass the synergistic role of NE reticular nuclei in sustaining these effects. In fact, the powerful NE release contributes to AMPHs-induced hyper-locomotion and stereotypies. Stereotypies, which occur upon reiterated AMPHs administration increase progressively following the same dose of AMPHs as a typical expression of AMPHs-induced behavioral sensitization. This is achieved by multiple mechanisms, which include the stimulation of alpha1-adrenergic receptors (α1-ARs), as demonstrated since the early '80s (Dickinson et al., 1988; Drouin et al., 2002a,b; Vanderschuren et al., 2003). Consistently, reduced hyper-locomotion and a loss of sensitization occur after α1B-AR inhibition or in mice genetically lacking this receptor subtype (Auclair et al., 2002, 2004). Consistently we found that α1B-AR-KO mice are also protected from METH-induced toxicity (Battaglia et al., 2003). This confirms data from Zuscik et al. (2000) who showed that overexpression of α1B-ARs in mice leads to an extended degeneration, which appears reminiscent of METH-induced neuronal damage (Fornai et al., 2004) and multiple system atrophy (Zuscik et al., 2000). These mice also develop spontaneous seizures, which are a typical effect observed upon METH-induced sensitization. Remarkably, when we used α1B-AR-KO mice, seizures were prevented (Pizzanelli et al., 2009). Again, activation of α1B ARs accounts for the increase in burst firing of midbrain DA neurons induced by AMPHs (Pan et al., 1996; Paladini et al., 2001), while α1B ARs antagonists suppress DA-related behaviors stimulated by AMPHs (Poncelet et al., 1983; Snoddy and Tessel, 1985; Tessel and Barrett, 1986; Dickinson et al., 1988; Mavridis et al., 1991; Blanc et al., 1994). Thus, α1B-ARs play a strong role in the deleterious effects induced by METH in the brain. In contrast, the peripheral effects

induced by METH on NE neurons appear to rely on α1A-ARs since no effects are determined by α1B-ARs (Kikuchi-Utsumi et al., 2013).

Consistently, the selective inhibition of α1-AR within the nucleus accumbens (NAc) or prefrontal cortex abolishes hyperlocomotion induced by AMPHs (Blanc et al., 1994; Darracq et al., 1998). Again, amplification of AMPHs-induced locomotor activity occurs by increasing extracellular levels of NE *via* NET inhibition or by enhancing NE release *via* blockade of inhibitory pre-synaptic α2-ARs (Dickinson et al., 1988; Xu et al., 2000; Juhila et al., 2005). Data concerning the effects of the pharmacological modulation of NE receptors on AMPHs-induced behavior are mostly related to NE released from LC neurons. Thus, LC-NE activity appears to be crucial for sensitizing AMPHs-induced behavior and toxicity, although other nuclei need to be investigated more extensively. For instance, catecholamine neurons of the AP are relevant, since a damage to this area increases locomotor activity while facilitating stereotypies (Costall et al., 1981). In addition, A1/C1 and, to a lesser extent, A2/C2 neurons, which are connected with orexin-containing perifornical neurons of the hypothalamus (**Figure 4**), modulate food intake and contribute to anorexia induced by AMPHs (McCabe and Leibowitz, 1984; Li et al., 2015; Ritter, 2017). The A5 nucleus sends descending axons to the spinal cord down to the lumbosacral tract (Westlund et al., 1981). A5- together with A7-neurons are involved in anti-nociceptive effects, and they are likely to mediate AMPHs-dependent analgesia (Proudfit, 1988; Miller and Proudfit, 1990). AMPHs also target the PB, a critical integrative site within the brainstem being involved in pain, satiety, taste, arousal, breathing and blood pressure (Hajnal et al., 2009; Martelli et al., 2013; Davern, 2014). The involvement of PB nucleus was investigated independently of NE release only in the context of conditioned taste aversion induced by AMPHs (Krivanek, 1997). In fact, within PB, AMPHs increase protein kinase C (PKC) activity, placed downstream to METH-induced signaling and toxicity (Lin et al., 2012).

The Brainstem NE Nuclei and AMPHs-Induced Autonomic Effects

A1/C1 neurons are anatomically organized in a roughly viscerotopic manner, in order to allow specific subsets of cells to control different visceral functions, encompassing circulation, breathing, glycemia, inflammation (Guyenet et al., 2013). A1/C1 neurons are mostly involved in regulating blood pressure (Reis et al., 1984). In fact, apart from activating pre-ganglionic vasomotor neurons, A1/C1 neurons control vasopressin release and sodium/water balance (Blessing and Willoughby, 1985; Guyenet, 2006). Thus, due to a powerful NE-release by AMPHs, it is expected that all regions being innervated by the A1/C1 complex will be activated during AMPHs administration. This may explain why AMPHs induce a severe increase in blood pressure (Liu and Varner, 1996), which was once believed to be solely due to peripheral NE. Thus, quite selective effects of AMPHs on a discrete neuron number of the medulla are supposed to provide a generalized increase in blood pressure (**Figure 5**). This key catecholamine nucleus adds on and surpasses the whole peripheral NE system in mediating AMPHs-induced hypertension. Thus, hypertension produced by AMPHs largely depends on a few central neurons, which regulate the vascular tone. Similarly to behavioral effects, the visceral responses to AMPHs are characterized by sensitization. Remarkably, even a single dose of METH may induce a sensitized response in blood pressure, which is accompanied by increased c-Fos immunoreactivity within TH-positive neurons of A1/C1 area and LC (Marchese et al., 2017). These findings confirm an overlap between behavioral and vegetative effects induced by AMPHs. In sharp contrast with a severe increase in blood pressure, chronic METH may lead to sudden and severe hypotension with bradycardia, which may lead to a lethal cardiovascular collapse (Chan et al., 1994; Ago et al., 2006; Miyashita et al., 2007). This is also related to a direct effect of METH within A1/C1 area, which mediates METH-induced increase in heart rate and arterial pressure (Liu and Varner, 1996), while at high and/or reiterated doses, METH may produce a selective neuronal death of A1/C1 (Li et al., 2012). This neuronal loss abolishes the descending activation of sympathetic pre-ganglionic neurons (SPNs), which are no longer able to stimulate the heart and produce contraction of smooth muscle within the blood vessels. This causes a sudden fall in blood pressure leading to METH-induced cardiovascular collapse (Li et al., 2012). These data are relevant to understand the key role of the reticular nuclei in regulating blood pressure while disclosing a previously overlooked neurotoxicity of METH on central NE neurons. In fact, these data demonstrate that NE neurons, apart from modulating METH toxicity to DA cells (Fornai et al., 1995, 1996a,b, 1997, 1998, 1999; Weinshenker et al., 2008), may also represent a primary target of METH toxicity. In fact, the A1/C1 nuclei innervate the SNPs being the pivot to provide direct excitatory input to the thoraco-lumbar sympathetic column of the cord (Ross et al., 1984). These neurons represent a critical link between the central respiratory rhythm generator and the vasomotor outflow (Guyenet et al., 1990). A multi-faceted signaling mechanism between A1/C1 cell groups and SPNs in the spinal cord is witnessed by several neuropeptides (such as prolactin, substance P, and cocaine- and amphetamine-regulated transcript, Cart, peptide), which are co-released within target areas (Chen et al., 1999; Dun et al., 2002). It is remarkable that METH persistently increases the expression of Cart peptide *via* epigenetic mechanisms (Jayanthi et al., 2017), which suggest that in addition to NE itself, neuropeptides produced by reticular NE neurons play a role in AMPHs-induced autonomic alterations. This is not surprising since NE nuclei are widely involved in neural circuitries, which regulate both behavioral and autonomic effects induced by AMPHs. For instance, A1/C1 sends visceral information to LC (Aston-Jones et al., 1986, 1991; Guyenet, 1991), which in turn, projects to midbrain DA neurons (Kirouac and Ciriello, 1997; Mejías-Aponte et al., 2009). In this way, DA neurons are recruited by these neurons, which mediate visceral effects produced by AMPHs. Such an integrated scenario indicates that behavioral and vegetative effects produced by AMPHs, despite being primarily processed within different nuclei, then converge in a common circuitry, which encompasses most

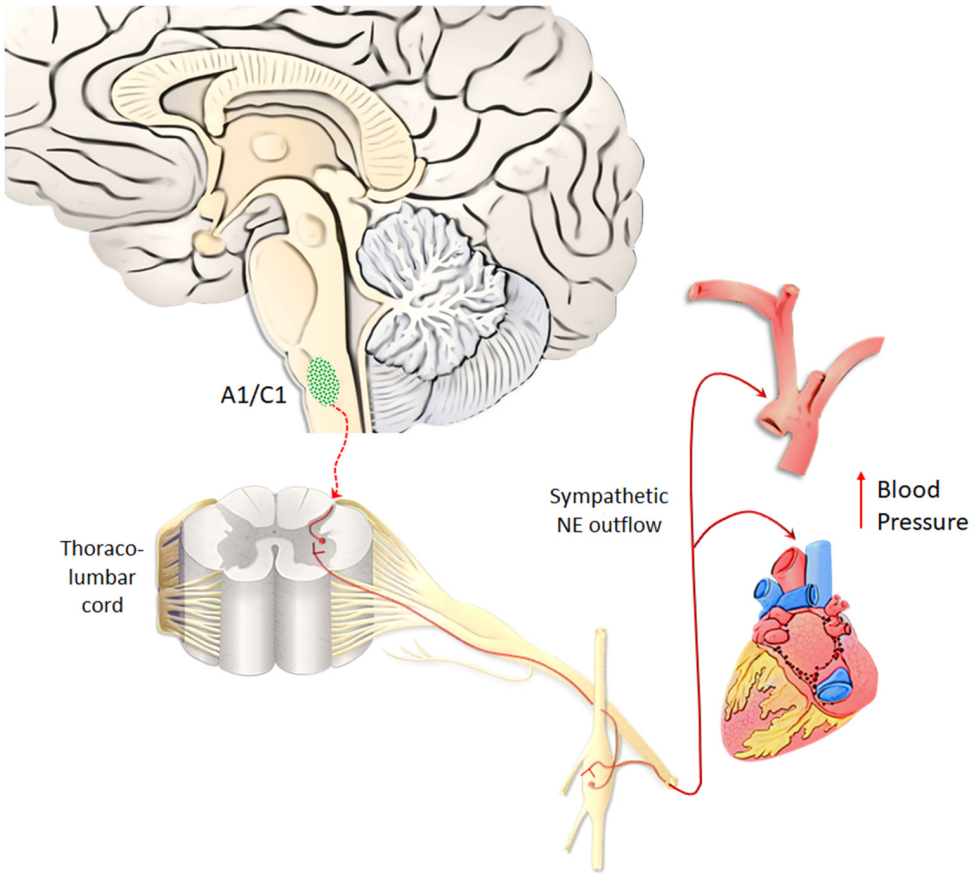

FIGURE 5 | A1/C1 neurons as a key center to control blood pressure. Hypertension produced by AMPHs largely depends on A1/C1 medullary neurons, which regulate the vascular tone, heart rate and blood pressure. NE released from A1/C1 neurons regulates blood pressure by directly activating sympathetic pre-ganglionic neurons (SPNs), which in turn stimulate the heart and produce contraction of smooth muscle within blood vessels. Due to a powerful NE-release by AMPHs, these peripheral targets innervated by the A1/C1 complex are strongly activated during AMPHs administration (Liu and Varner, 1996). This response occurs in a sensitized manner following repeated dosing of AMPHs (Marchese et al., 2017).

catecholamine containing nuclei of the brainstem RF. In this way, depending on which nucleus we focus on, different effects produced by AMPHs can be mechanistically explained by the specific neuro-anatomical connections of this very same nucleus. This is not surprising at all when considering the simultaneous activation of the peripheral NE sympathetic nervous system when a demanding environmental task is activating the NE ascending reticular nuclei to produce arousal or during rewarding stimuli.

THE INTERPLAY BETWEEN NE AND DA IN AMPHs-INDUCED BEHAVIOR

Within a context of NE-dependent reward, a balanced dual perspective indicates that AMPHs need to converge on both DA and NE cells in order to be fully effective in producing reward. This was already hypothesized in a pioneer manuscript by Fibiger and Phillips (1974). In fact, caudal nuclei of the RF strongly connects with midbrain reticular DA nuclei. This circuitry originated during phylogeny, as biochemical, anatomical and physiological features strongly witness for an evolutionary continuum between mesencephalic DA neurons and more caudal NE cell groups (Bucci et al., 2017). In fact, profuse and reciprocal connections establish between NE/E nuclei within the pons and medulla and midbrain DA nuclei (Simon et al., 1979; Deutch et al., 1986; Grenhoff and Svensson, 1993; Grenhoff et al., 1993; Liprando et al., 2004; Mejías-Aponte et al., 2009). For instance, DA neurons of the Retrorubral Field (RRF, A8) and VTA (A10) receive abundant NE innervation, which is provided mainly from the nuclei A1, A2, A5 and A6 (Mejías-Aponte et al., 2009). Thus, DA and NE systems do not represent separate compartments within the CNS but rather an interconnected system, which share key neurobiological features making it as the endogenous circuitry where AMPHs electively impinge to produce a number of systemic effects. The strong anatomical connections between NE and DA systems are conserved at molecular level. This is best represented by the phylogeny of NET and DAT, which indeed represent the evolutionary divergence of an archaic single catecholamine transporter (meNET), which was isolated and characterized already in the brain of the teleost fish medaka

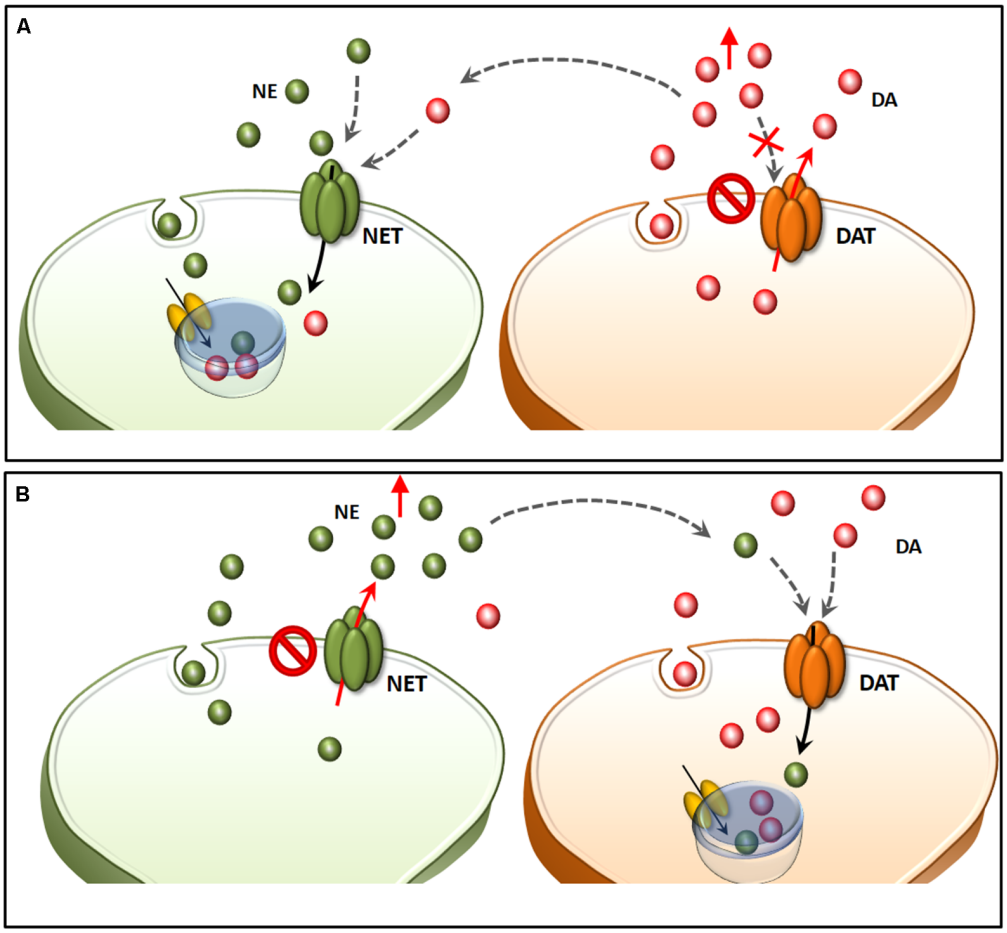

FIGURE 6 | Structural similarities between NET and DAT confound neuron-specific NE and DA compartmentalization. Both NET and DAT represent the evolutionary divergence of a single catecholamine transporter (meNET), which was isolated and characterized already in the brain of the teleost fish medaka (Roubert et al., 2001). In the light of a strong structural similarity, both NET and DAT take up extracellular DA with a similar potency **(A)**. In fact, in the presence of an excess of extracellular DA (due to either a selective blockade of DAT or a reverted direction of DA transport), this may compete effectively with NE, thus being inappropriately stored within NE terminals. This explains why NE axons may internalize DA in the absence of DAT (Rocha et al., 1998). This same phenomenon also explains why in some instances selective NET inhibitors may paradoxically increase extracellular DA **(B)**. In fact, when a powerful NE release occurs in a densely DA-innervated area, it is very likely that extracellular NE is taken up mostly by fraudulent DA axons instead of authentic NE terminals.

(Roubert et al., 2001). This ancestral carrier is very similar to both the human NET and DAT, showing 70% and 64% amino acid homology, respectively. In fact, NET responds to AMPHs similarly to DAT and it represents the main gateway for AMPHs to invade NE terminals and to reach specific sub-cellular and molecular targets (Seidel et al., 2005). For instance, following AMPHs administration there is a down-regulation of NET and DAT, which are both stored in endosomes (Annamalai et al., 2010; Hong and Amara, 2013). Following AMPHs both DAT and NET, which remain on the plasma membrane, revert their transport of catecholamine (Sulzer et al., 1993; Robertson et al., 2009). Moreover, both transporters tend to be internalized within the terminals once bound to AMPHs. As mentioned above there is a strong similarity between NET and DAT. In fact, they are able to take up extracellular DA with a similar potency (Rothman et al., 2001). This affinity may confound the neuron-specific compartmentalization (**Figure 6**). In the presence of an excess of extracellular DA, this may compete effectively with NE, thus being inappropriately stored within NE terminals (Amara and Kuhar, 1993; Ramamoorthy et al., 2011; Borgkvist et al., 2012). This explains why in some instances selective NET inhibitors may paradoxically increase extracellular DA (Reith et al., 1997), while NE axons may internalize DA in the absence of DAT (Rocha et al., 1998). Again, when a powerful NE release occurs in a densely DA-innervated area, it is very likely that extracellular NE is taken up mostly by fraudulent DA axons instead of authentic NE terminals (**Figure 6**). This needs to be taken into account when considering the effects of AMPHs, since the fine structure of a given brain region may switch considerably the ratio of a combined mechanism of action upon both DA and NE systems. This fully applies to the different brain areas which sustain the reinforcing and rewarding effects of AMPHs (the richly DA-innervated NAc compared with densely NE-enriched allo-cortical regions).

THE MOLECULAR MECHANISMS OF BRAINSTEM DA-NE INTERPLAY IN THE BEHAVIORAL EFFECTS INDUCED BY AMPHs

NE sourced by the reticular nuclei of the low brainstem is key for AMPHs-induced behavior (Rothman et al., 2001; Weinshenker and Schroeder, 2007; Weinshenker et al., 2008). This occurs also *via* amplification of DA-related rewarding and reinforcing properties. This is not surprising given the profuse reciprocal connections between NE and DA nuclei. In particular, LC innervates almost all brain areas, which receive DA innervation throughout the mesolimbic and mesocortical systems, including the ventral striatum and the prefrontal cortex (Nicola and Malenka, 1998). In fact, NE axons from LC neurons regulate DA release in the prefrontal cortex (Gresch et al., 1995; Devoto et al., 2005), while a damage to LC projections with DSP-4 alters baseline or stimulated DA release in the NAc (Lategan et al., 1992). This is consistent with studies showing that depletion of NE in the prefrontal cortex potentiates AMPHs-induced behavioral sensitization through striatal DA release (Ventura et al., 2003). In addition to α1B ARs involvement in DA-related behaviors and toxicity induced by AMPHs (Poncelet et al., 1983; Snoddy and Tessel, 1985; Tessel and Barrett, 1986; Dickinson et al., 1988; Mavridis et al., 1991; Blanc et al., 1994), the role of β-ARs has been investigated as well. In fact, Albers and Sonsalla (1955) showed that a β-AR blocker prevents AMPHs-induced DA toxicity, and a subsequent study confirmed these data showing that β-AR blockers prevent AMPHs-induced DA sensitization (Colussi-Mas et al., 2005). These data confirm that DA neurotoxicity, just like autonomic, motor and behavioral effects undergoes sensitization. This is expected since AMPHs-induced sensitization up-regulates those molecular cascades, which are the common pathway to produce all AMPHs-induced alterations.

Similarly, marked alterations in AMPHs-induced DA release within the dorsal striatum occur following a damage to LC *via* DSP-4 and following either genetic or pharmacological blockade of NE synthesis (Weinshenker et al., 2008). Such a neurochemical effect mediated by NE loss enhances the behavioral response induced by METH while potentiating nigrostriatal METH toxicity (Weinshenker et al., 2000, 2008). In addition, a reduction of TH within LC neurons (*via* RNA interference) potentiates D1 receptor-dependent AMPHs-induced sensitization in the ventral striatum, to an extent, which is not replicated by DAT inhibition (Smith and Greene, 2012). In line with this, Harro et al. (2000) found that a loss of NE axons increases AMPHs-induced locomotor activity while up-regulating striatal D2 receptors. Again, a damage to LC neurons enhances nigrostriatal METH toxicity in both mice and rats (Fornai et al., 1995, 1996a,b), an effect, which is related to increased DA sensitivity to METH rather than to METH pharmacokinetics (Fornai et al., 1997, 1998, 1999). Such a potentiation is suddenly evident by observing METH-induced behavioral changes when dramatic stereotypies occur in LC-damaged mice. It is likely that NE operates at some level within DA neurons to alter synaptic plasticity. In fact, the abnormal synaptic plasticity, which happens following pulsatile DA stimulation in a parkinsonian striatum, is worsened in LC-damaged mice, which develop severe abnormal involuntary movements following low doses of L-DOPA (Fulceri et al., 2007). These effects appear to rely more on LC compared with other NE nuclei. In fact, they can be reproduced by a bilateral stereotactic injection of the neurotoxin 6-OHDA within both LC nuclei (Fulceri et al., 2007).

RETICULAR NUCLEI WITHIN THE DORSAL RAPHE/PAG AS A PARADIGM TO DECIPHER AMPH-INDUCED BEHAVIOR

Pioneer studies carried out in both animal models and humans uncovered a highly heterogeneous nature of the dorsal raphe neurons, thus providing a seminal contribution in implementing the original description by Dahlstrom and Fuxe (1964). Such a heterogeneity holds true for either cyto-architectural, neurochemical or topographic differences characterizing subsets of neuronal populations within the dorsal raphe. In detail, the dorsal raphe nucleus (B8-B9), which extends from the rostral pons up to the midbrain within and around the ventromedial and ventrolateral PAG, can be subdivided into five sub-regions, namely caudal, dorsal, ventral, ventrolateral and interfascicular (Steinbusch et al., 1981; Baker et al., 1990, 1991). In the present paragraph, we focus on the ventromedial and ventrolateral PAG, where a number of catecholamine nuclei, targeted by AMPHs, are placed. This is the case of the NE nucleus epicoeruleus, DA neurons of the A10dc nucleus, as well as cholinergic neurons of the laterodorsal tegmental nucleus (LDTg, Ch6), which intermingle in the ventral and ventrolateral PAG with their rostral extent (Hökfelt et al., 1984; Mai and Paxinos, 2012; Vasudeva and Waterhouse, 2014; **Figure 7**).

AMPHs and Catecholamine Neurons of the PAG

Despite being poorly investigated in the specific case of AMPHs compared with low brainstem reticular nuclei, catecholamine neurons of the PAG represent an important neuro-anatomical substrate for the behavioral changes induced by AMPHs (Tasman and Simon, 1983; Sobieraj et al., 2016). This is not surprising given the plethora of functions, which are regulated by the PAG, such as pain, anxiety, arousal and escape, as well as heart rate, thermogenesis, mean arterial blood pressure and breathing (Bandler et al., 1985; Bandler and Carrive, 1988; Brandao et al., 1990; Carrive, 1991; Lovick, 1993; Coimbra and Brandão, 1997; Hayward et al., 2003). In addition, catecholamine PAG neurons are profusely connected with a variety of cortical and subcortical brain regions. These include, for instance, the thalamus, the medial prefrontal cortex, the basal forebrain cholinergic neurons, the hypothalamic orexin cells, the pontine LDTg, most of the NE bulbo-pontine nuclei, and the VTA (Li et al., 1990; Reichling and Basbaum, 1991; Bajic et al., 2001, 2012; Lu et al., 2006; Rathner and Morrison, 2006). Such a region becomes the prototype for confounding outcomes when trying to decipher the specific effects produced by each monoamine-containing nucleus in AMPHs-induced behavior, which appear

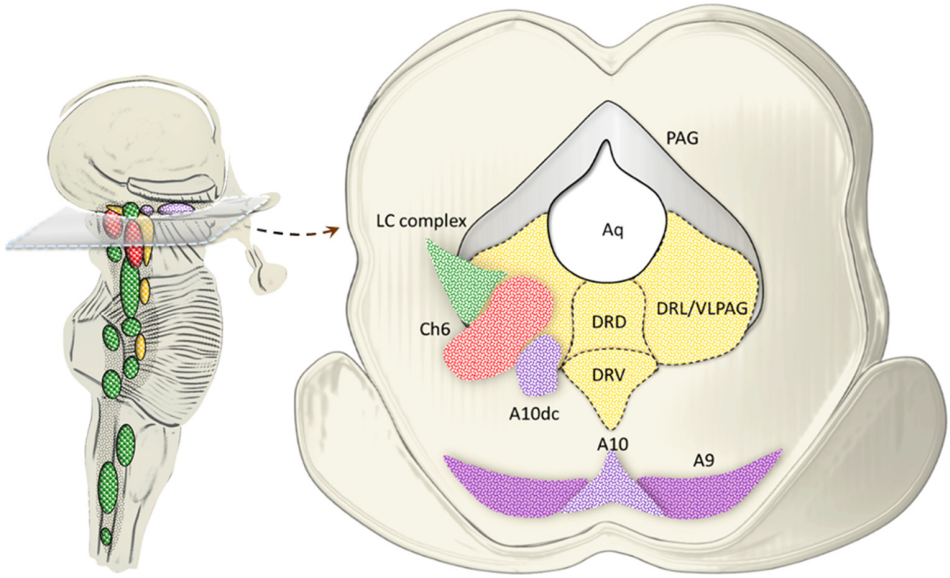

FIGURE 7 | Catecholamine and cholinergic nuclei of the dorsal raphe/PAG. The dorsal raphe nucleus extends from the rostral pons up to the midbrain within and around the ventromedial and ventrolateral PAG. In fact, at this level, three sub-regions of the dorsal raphe intermingling with PAG are identified, namely, dorsal (DRD), ventral (DRV) and lateral (DRL; Steinbusch et al., 1981; Baker et al., 1990, 1991). While 5-HT neurons are scattered throughout all the dorsal raphe, the ventromedial and mostly the ventrolateral PAG (VLPAG) hosts a mixed neuronal population, among which catecholamine and cholinergic neurons prevail at large. This is the case of the NE nucleus epicoeruleus corresponding to the rostral and dorsal extent of the LC complex. Similarly, DA neurons of the A10dc nucleus, as well as cholinergic neurons of the Ch6 (LDTg) intermingle in the DRL/VLPAG with their rostral extent.

largely related to the profuse isodendritic connections occurring within the PAG. For instance, the stimulation of dorsal raphe nucleus, besides enhancing extracellular 5-HT levels in both the forebrain and the LC, also increases extracellular NE (Hajós-Korcsok and Sharp, 2002). Such a response is not altered by a damage to 5-HT neurons of the dorsal raphe. The plethora of connections linking PAG-dorsal raphe neurons with lower NE brainstem nuclei appears critical in AMPHs-induced behavior. In fact, profuse and reciprocal connections occur between PAG and reticular NE nuclei targeted by AMPHs, including LC, A5, A7, PB, and A1/C1 group. For instance, the LC provides a major stimulatory drive to the dorsal raphe nucleus while the A1/C1 provides an inhibitory tone (Peyron et al., 1996; Kim et al., 2004; Cao et al., 2010). In the light of these projections, the PAG becomes an interface in behavioral control concerning the regulation of sleep-wake cycle and arousal, pain modulation and cardiovascular responses (Benarroch, 2012). NE connections with DA-containing nuclei of the PAG are important as well. In fact, following stimulation of LC, α1-AR-dependent NE transmission in the PAG promotes arousal *via* modulation of PAG DA neurons activity (Porter-Stransky et al., 2019). It is remarkable that besides the SNpc, METH targets DA neurons of the PAG, as shown in autopsy brains from METH abusers (Quan et al., 2005). Beyond neurotoxicity, reiterated METH administration induces plastic effects in PAG DA neurons, which associate with drug-induced reward and addiction (Sobieraj et al., 2016). An excitatory effect of TH-positive PAG neurons on the adjacent VTA DA cells (Lu et al., 2006) is likely to participate to AMPHs-induced activation of opioid receptors in the PAG, which associates with analgesia, hyperthermia, and hedonic reward reinforcement (Berridge et al., 2009; Cristina-Silva et al., 2017).

Acetylcholine-Containing Reticular Nuclei in AMPHs-Induced Behavior

A few studies demonstrated that beyond monoamines, acetylcholine (ACh) is involved in the behavioral effects of AMPHs. In line with this, METH releases ACh in adult mice (Dobbs and Mark, 2008) and alters striatal choline acetyltransferase (ChAT), the enzyme responsible for synthesizing ACh, in humans (Kish et al., 1999; Siegal et al., 2004). Given the critical role of ACh systems in cognition (van Hest et al., 1990; Muir et al., 1994; Lin et al., 1998; Mirza and Stolerman, 2000), alterations in ACh levels and receptors are suggested to contribute to the cognitive impairments observed following METH exposure. Only a few studies investigated the role of ACh produced specifically by reticular brainstem nuclei in AMPH's-induced effects. The main source of ACh in the brainstem RF is represented by two ACh pontine nuclei, which correspond to the peduncolopontine nucleus (PPN) or peduncolopontine tegmentum (PPTg) and the laterodorsal tegmental nucleus (LDTg). These nuclei are also referred to as Ch5 and Ch6, respectively. Rostrally, the Ch5 is included between two DA nuclei of the RF: ventrally, it contacts the dorsomedial aspect of the A9, while dorsally it is bordered by the A8. Caudally, the Ch5 adjoins the LC. Ch5 neuronal population is heterogeneous regarding its spatial distribution and neurochemistry. In fact, the dorsolateral portion of

Ch5, which is called pars compacta, contains densely packed cholinergic neurons forming a continuum with Ch6 cholinergic neurons. Ch6 neurons contour the Ch5 nucleus and rostrally they extend into the PAG and medial longitudinal fasciculus. Caudally to the Ch5 nucleus, Ch6 neurons are intermingled with NE neurons belonging to the epicoeruleus nucleus (Mesulam et al., 1989). Ch6 neurons are slightly smaller than those of Ch5 pars compacta. As components of the RF, Ch5 Ch6 neurons share the typical isodendritic conformation. It is well-established that Ch5 and Ch6 neurons are targeted by the basal ganglia efferent fibers. Projections from these neurons are directed to the thalamus and other nuclei of the brainstem RF (Mesulam et al., 1989). Remarkably, excitatory cholinergic fibers have been described, which project mainly from these two nuclei to the VTA, promoting burst firing in DA neurons and thus enhancing DA release, which is pivotal for AMPHs-induced behavior (Woolf, 1991; Yeomans and Baptista, 1997; Yeomans et al., 2001; Omelchenko and Sesack, 2005, 2006). In particular, Ch6 targets VTA DA neurons, while Ch5 preferentially targets SNpc DA neurons (Oakman et al., 1995). Recent studies suggest that ACh in addition to DA is primarily involved in the initiation and maintenance of hyper-locomotion induced by METH. In detail, Dobbs and Mark (2008) demonstrated that, systemic but not intra-VTA perfusions of METH in mice induce a prolonged ACh release within VTA. In particular, extracellular ACh levels persist above baseline levels for 2–3 h post-injection and it takes 180 and 300 min post-injection to return to baseline values, after a low and high dose of METH respectively. Remarkably, ACh but not DA release, within VTA, correlates dose-dependently with METH-induced locomotor activity. These data suggest that METH acts in the VTA to induce a robust, though short-lived, increase in extracellular DA release while producing a prolonged increase in ACh release, which correlates with hyper-locomotion. In the light of these findings, the same authors investigated the contribution of Ch6 and Ch5 nuclei in METH-induced locomotor activity. Inhibition of ACh release by intra-Ch6 infusion with a muscarinic receptor agonist, which binds to M2 inhibitory auto-receptors, attenuates METH-induced locomotor activity (Dobbs and Mark, 2012). As assessed by brain dialysis, the inhibition of Ch6 neuronal activity blunts METH-induced increase in ACh release within the VTA dose-dependently, while it has no effect on DA release within the NAc.

Ch6 ACh neurons are not involved in METH-induced drug-seeking behavior (Dobbs and Cunningham, 2014), but are important for METH-induced locomotor activity (Dobbs and Mark, 2012). On the other hand, inhibition of ACh release from Ch5 does not produce any effects either on ACh or DA release within VTA and NAc, respectively. Therefore, it is likely that Ch6, rather than Ch5, is involved in locomotor behavior induced by systemic METH, which is mediated by ACh release in the VTA. Previous studies suggest that a damage to both Ch5 and Ch6 blunts hyper-locomotion but enhances stereotypies induced by systemic or intra-ventrolateral striatal injections of AMPHs (Inglis et al., 1994; Allen and Winn, 1995; Forster et al., 2002; Miller et al., 2002). This is due to an increase in DA outflow specifically in the dorsal but not ventral striatum, which suggests that AMPHs-induced hyper-locomotion and stereotypies largely depend on the specific effects of Ch5 and/or Ch6 upon the mesostriatal or mesoaccumbens DA systems. These findings warrant further studies elucidating the site-specificity for ACh in mediating METH-induced behavioral alterations.

Although molecular targets responsible for AMPHs-induced monoamine release are well known, the molecular mechanisms through which AMPHs release ACh are not fully established yet. A stream of interpretation indicates a close functional relationship between DA system and ACh release. This stems from evidence showing that administration of D1 and D2 receptor antagonists blunts AMPHs-induced ACh release within the striatum, hippocampus and frontal cortex (Ajima et al., 1990; Damsma et al., 1990, 1991; Imperato et al., 1993; DeBoer and Abercrombie, 1996; Keys and Mark, 1998). Nonetheless, contradictory results are obtained when a damage to the nigrostriatal DA system is induced by 6-OHDA. In fact, 6-OHDA injections produce only a slight decrease in extracellular ACh levels induced by systemic AMPHs (Mandel et al., 1994; Taguchi et al., 1998). These results led to hypothesize that AMPHs-induced ACh release may be due to connections between cholinergic and catecholamine, rather than solely DA systems. This is supported by evidence indicating that combined administration of the TH inhibitor α-methyl-*p*-tyrosine with the VMAT inhibitor reserpine, completely blocks AMPHs-induced ACh release both *in vivo* and in striatal slices (Cantrill et al., 1983; Taguchi et al., 1998). In any case, once released by AMPHs, ACh provides an important excitatory input to those neurons expressing nicotinic ACh receptors (nACh-Rs). This was mainly investigated on DA neurons, where activation of nACh-Rs leads to intracellular Ca^{2+} accumulation, which in turn facilitates DA exocytosis (MacDermott et al., 1999; Engelman and MacDermott, 2004; Lester et al., 2010). In this way, AMPHs produce DA-related effects also *via* ACh release (Drew et al., 2000; Camarasa et al., 2008; Chipana et al., 2008; Hondebrink et al., 2012). Recently, nACh-Rs-mediated Ca^{2+} increase and subsequent nitric oxide-synthase activation have been linked to AMPHs-induced neurotoxicity (Pubill et al., 2011). In line with this, the pharmacological blockade of α7 nACh-Rs attenuates METH-induced oxidative damage and nigrostriatal neurotoxicity both *in vivo* and striatal synaptosomes. Studies focusing on the effects of AMPHs-induced ACh release on NE system are missing so far, which warrants additional studies to test ACh-NE interplay following AMPHs.

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MF wrote the article. FL contributed to important intellectual content. FB, CB and LR contributed to the literature review and made artwork. FF was the coordinator of the article. He participated in drafting the article and also in critically revising the article.

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frontiers in Neuroanatomy

MINI REVIEW
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The Neuroanatomy of the Reticular Nucleus Locus Coeruleus in Alzheimer's Disease

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Alzheimer's Disease (AD) features the accumulation of β-amyloid and Tau aggregates, which deposit as extracellular plaques and intracellular neurofibrillary tangles (NFTs), respectively. Neuronal Tau aggregates may appear early in life, in the absence of clinical symptoms. This occurs in the brainstem reticular formation and mostly within Locus Coeruleus (LC), which is consistently affected during AD. LC is the main source of forebrain norepinephrine (NE) and it modulates a variety of functions including sleep-waking cycle, alertness, synaptic plasticity, and memory. The iso-dendritic nature of LC neurons allows their axons to spread NE throughout the whole forebrain. Likewise, a prion-like hypothesis suggests that Tau aggregates may travel along LC axons to reach out cortical neurons. Despite this timing is compatible with cross-sectional studies, there is no actual evidence for a causal relationship between these events. In the present mini-review, we dedicate special emphasis to those various mechanisms that may link degeneration of LC neurons to the onset of AD pathology. This includes the hypothesis that a damage to LC neurons contributes to the onset of dementia due to a loss of neuroprotective effects or, even the chance that, LC degenerates independently from cortical pathology. At the same time, since LC neurons are lost in a variety of neuropsychiatric disorders we considered which molecular mechanism may render these brainstem neurons so vulnerable.

Keywords: neurofibrillary tangles, basal forebrain nuclei, phospho-Tau, amyloid, mild cognitive impairment, pre-clinical AD

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INTRODUCTION

Alzheimer's Disease (AD) affects more than 45 million of people worldwide (Alzheimer's Disease International, 2015). Despite research efforts, key molecular mechanisms of disease remain uncertain. Milestones in AD pathology consist in alterations of the cytoskeleton-associated Tau protein along with abnormal β-amyloid (Aβ) depositions. A long-lasting time interval exists since the onset of early pathological alterations until the appearance of cognitive deterioration. In fact, a sub-clinical phase, in which cortical AD pathology occurs in the absence of a frank cognitive impairment is documented. Such a stage, in which a cognitive intact subject already bears AD markers is defined "pre-clinical asymptomatic at-risk for AD" (Dubois et al., 2014).

We wish to emphasize that, despite being a fascinating hypothesis, the causal relationship between Tau pre-clinical pathology and later cognitive deterioration remains to be established. Cortical pathology consists of: (i) argyrophilic structures formed by abnormal intracellular aggregates of phospho-Tau (P-Tau) fibrils, known as neurofibrillary tangles (NFT); and (ii) extracellular aggregates of Aβ known as amyloid plaques (Hyman et al., 2012; Montine et al., 2012).

An updated hypothesis considers six stages of NFT pathology. At stage I, a low amount of Gallyas-positive NFT is present, mainly within the trans-entorhinal region, which corresponds to the peripheral entorhinal cortex (Braak and Braak, 1991 or the parahippocampal gyrus facing the fusiform gyrus with the collateral sulcus interposed (Taylor and Probst, 2008). At stage I scattered NFT can be detected also within entorhinal mesocortex, CA1, dorsomedial thalamus and basal forebrain nuclei (Braak and Braak, 1991). At stage II the trans-entorhinal cortex is more affected, while the entorhinal mesocortex is consistently affected along with CA1 and prosubiculum; dorsomedial thalamus and basal forebrain nuclei appear as at stage I. At stage III, NFT densely cluster in the superficial layer of both trans-entorhinal and entorhinal cortex, while hippocampal involvement extends towards subiculum. Isocortex (neocortex) is spared apart from a few NFT within associative ventral areas. At stage IV all layers of trans-entorhinal and entorhinal cortex are filled with NFT, while the CA1 region features ghost tangles, and the basal frontal and insular isocortex are involved. At this stage also the amygdala and endopiriform nucleus feature NFT. At stage V, the parasubiculum and the whole hippocampal formation are involved massively in a continuum with trans-entorhinal and entorhinal cortex. Remarkably, CA2 is mostly spared along with dentate gyrus (Dudek et al., 2016). This is reminiscent of hippocampal damage following seizures and ischemia (Giorgi et al., 2008). At this stage the associative isocortex is widely affected. Finally, at stage VI, primary sensory and motor areas are affected. In most patients stage I and II are rather asymptomatic, while during stage III memory impairment may appear.

Aβ aggregates follow an opposite spreading direction (Thal et al., 2002) since they first appear in the isocortex (phase 1) and later on within allocortex (phase 2), then downstream to diencephalon (phase 3) and furtherly towards brainstem nuclei including substantia nigra (phase 4). In the last step Aβ extends to caudal reticular nuclei including locus coeruleus (LC; phase 5).

The spreading of NFT through interconnected brain regions may occur trans-synaptically via a prion-like transmission of Tau (Mohamed et al., 2013; Goedert, 2015). These synaptic mechanisms of degeneration are supposed to start caudally within the iso-dendritic core of the reticular formation. In fact, LC and other reticular nuclei feature an impressive collateralization allowing a single axon to innervate multiple brain regions making these cells ideal spreading vectors. A variety of brainstem reticular nuclei features NFT. Similarly, when considering cortical neurons long-projecting pyramidal cells of layer V or hippocampal pyramidal cells appears more vulnerable to degeneration (Ovsepian et al., 2016; Schaeffer et al., 2017). The present review emphasizes the role of LC as a powerful brainstem nucleus, which projects mono-synaptically to all cortical regions (Nagai et al., 1981). Nonetheless, while writing this article we already feel the limits of such a LC-centered hypothesis, since other nuclei such as the rostral dorsal raphe, the parabrachial nuclei and the pedunculopontine nucleus are important as well. Thus, the LC should be regarded more as a paradigm, rather than the unique anatomical entity, which connects NFT pathology from the brainstem to the cortex.

The trans-synaptic spreading of Tau from LC to other brain regions may occur "a rebour" (i.e., in opposite directions) by hippocampal injections of Tau fibrils, which produce a frank pathology down to the LC (Iba et al., 2015).

TAU PATHOLOGY OCCURS EARLY WITHIN BRAINSTEM, CORTICAL-PROJECTING NUCLEI

Recent studies indicate early impairment of LC with potential outcomes on preclinical staging and neurobiology of disease (Braak et al., 2011). In detail, stereology consistently evidenced that the number of LC neurons negatively correlates with AD pathology. Pioneer studies by Tomlinson et al. (1981), Bondareff et al. (1982) and Mann et al. (1982, 1984) described a reduction of LC neurons in AD patients. A few years later Chan-Palay and Asan (1989) proposed a rostro-caudal gradient of neuronal loss within LC of AD patients, differently from PD patients where LC is uniformly affected. In a large series of non-selected brains ($N > 2300$), Braak et al. (2011) by using P-Tau antibodies detected "pre-tangle material" (negative for Gallyas reaction) within LC of almost all the brains in the absence of Tau-related pathology in the trans-entorhinal region. This emphasizes the occurrence of subcortical deposition of Tau in the absence of NFT in any cortical area (less than stage I). Depending on the severity of brainstem involvement, a progressive staging ("a", "b", "c") up to early cortical recruitment (stages "1a" and "1b") is described (**Table 1**). These stages are age-related, being stages "a-c" present solely at age 20–30, while stages > I–II being detected only after the age of 40. These findings lead to the following statements: (i) LC features abnormal Tau deposits before a frank neuronal loss; (ii) Tau deposits in LC occur decades before the average onset of cortical AD pathology; (iii) P-Tau accumulation is likely to be key in the process of NFT formation; and (iv) Tau alterations anticipate dementia. The latter observation challenges quite directly the classic amyloid cascade hypothesis, according to which, an impairment in Aβ-pathway may trigger Tau pathology (Hardy and Selkoe, 2002; Jack et al., 2010). In contrast, one might hypothesize that early Tau pathology predisposes to Aβ accumulation, which in turn exacerbates Tau pathology. Very recently, Theofilas et al. (2017) applied up-to-date stereology to brains at various disease stages and showed a two-fold increase in P-Tau positive LC neurons from pre-stage 1 to stage I, with a positive correlation between the number of P-Tau positive cells in the LC and disease stage. They also found that volume in the rostral part of LC decreases already from pre-stage 1 to

TABLE 1 | Staging of neurofibrillary tangles (NFT-related) pathology in Locus coeruleus (LC).

Pretangle Stages	Subcortical pretangles stages	a	P-Tau accumulation within the axon hillock of brainstem reticular neurons, mostly LC neurons.
		b	P-Tau accumulation extended further into LC cell bodies.
		c	Involvement of other reticular (or reticular-related) ascending nuclei (e.g., dorsal raphe nucleus, nuclei of the basal forebrain).
	Cortical pretangles stages	1a	Pre-tangle material in LC axons within the trans-entorhinal and entorhinal regions.
		1b	Pre-tangle inclusions within pyramidal cells of the trans-entorhinal region connected with NFT positive axons.

stage I. The number of LC neurons decreases form stage II onward. In another study in asymptomatic patients, Andrés-Benito et al. (2017) confirmed these data. On the other hand, these studies are grounded on static descriptions, where a dynamic mechanism remains at hypothetical level. The cross-sectional evidence reported in these studies does not represent a proof of principle for an actual spreading of pathology. Thus, casualty cannot be ruled out and a supreme vulnerability of LC neurons represents a sort of background noise occurring in a number of neurological disorders encompassing AD, PD, seizures, multiple system atrophy, Down syndrome and many others (Fornai, 2007; Phillips et al., 2016). In fact, the loss of brain norepinephrine (NE) levels characterizes a number of neurological patients and involves multiple brain areas (Gesi et al., 2000; Giorgi et al., 2003, 2004; Marien et al., 2004; Fornai et al., 2011; Ruffoli et al., 2011; Pifl et al., 2012, 2013) and various experimental neurological disorders (Fornai et al., 1995a,b, 1997a,b, 1998, 1999, 2007; Siciliano et al., 1999; Soldani and Fornai, 1999; Ferrucci et al., 2002, 2013; Fulceri et al., 2004; Giorgi et al., 2006; Weinshenker et al., 2008).

THE NEUROANATOMY OF LC IN AD

LC is a tube-like shaped group of NE neurons placed in the upper part of the pons; it is composed of medium-sized neurons (Brodal, 1981). LC extends rostrocaudally for approximately 16 mm (German et al., 1988; Baker et al., 1989; Halliday, 2004). These neurons send their branched axons to innervate the entire cerebral cortex (Nagai et al., 1981). Thus, by releasing NE through "bouton en passage" (or synaptic varicosities), LC modulates the activity of several cortical areas. In fact, LC modulates sleep/wake cycle, learning and memory, early gene expression and neuroprotection (Fornai et al., 1990; Foote et al., 1991; Cirelli et al., 1996; Cirelli and Tononi, 2000; Giorgi et al., 2003, 2004; Aston-Jones, 2005; Aston-Jones et al., 2007; Fornai, 2007; Giorgi et al., 2008; Weinshenker et al., 2008; Sara, 2009; Fornai et al., 2011; Counts and Mufson, 2012; Ferrucci et al., 2013). LC mostly innervates limbic cortex compared with neocortex (here defined as isocortex, Jones and Yang, 1985; Loughlin et al., 1986; Giorgi et al., 2003). The ventral part of caudal LC sends projections even to lower medulla and spinal cord, and regulates autonomic functions (Ward and Gunn, 1976), while neurons which innervate the allocortex are placed in the rostral part of the nucleus (Ward and Gunn, 1976; Loughlin et al., 1986).

The projection of LC neurons to the cortex may occur monosynaptically (Fallon et al., 1978; Nagai et al., 1981; Harley, 1987), via the allothalamus (Krout et al., 2002; Garcia-Rill et al., 2013), or through basal cholinergic nuclei. These are severely involved in AD (Davies and Maloney, 1976; Coyle et al., 1983; Sassin et al., 2000). In particular, degeneration of Ch1 and Ch2 neurons is responsible for the loss of septo-hippocampal connections leading to memory impairment (Gertz et al., 1987; Brayda-Bruno et al., 2013). The Ch4 (nucleus basalis of Meynert) nuclear complex, which is divided into various subfields (Mesulam, 2013) and receives dense NE projections from LC degenerates as well (Mesulam et al., 1983a,b, 1984; Mesulam and Geula, 1988; Smiley and Mesulam, 1999; Haghdoost-Yazdi et al., 2009). The Ch4 sends a strong cholinergic input to allocortical and mesocortical areas (Mesulam et al., 1986). In line with this, the joint involvement of cholinergic and NE nuclei encoding motivational salience in spatial attention explains the loss of motivational information and spatial attention that is critically lost in dementia (Mohanty et al., 2008; Sara, 2015; Chandler, 2016). Another limbic region innervated by LC is the amygdala (Asan, 1998) which is involved in AD (Knafo, 2012). The entorhinal and trans-entorhinal cortex as well as the hippocampal formation are monosynaptically innervated by LC (Fallon et al., 1978; Room and Groenewegen, 1986; Harley, 1987). Entorhinal cortex is involved in memory consolidation and retrieval (Dolcos et al., 2005; Bott et al., 2016; Cho et al., 2016; Fayed et al., 2017). Within hippocampus, LC axons innervate the stratum radiatum of CA1 and stratum lucidum of CA3 (Melander et al., 1986; Moudy et al., 1993). Despite a preferential limbic innervation which is in line with AD pathology, a fine anatomical analysis of the cytotectonic pattern of LC innervation within allocortex does not provide any site-specificity which may justify a preferential damage to layer V long-projecting neurons which occurs in AD. In fact, the pattern of catecholamine innervation is rather uniform in the various layers of the allocortex (apart from the scarce density in layer I; Gaspar et al., 1989). This rules out a site-specific, disease-related synaptic contact with those cortical cells (pyramidal) which are mostly affected in AD. Thus, there is no anatomical overlapping between the fine pattern of NE innervation and the fine pattern of neuropathology within various cortical layers. Such a discrepancy also applies to NE innervation of isocortical (neocortical regions; Gaspar et al., 1989). Remarkably, albeit primary sensorimotor regions are affected only at latest AD stages, they possess the highest NE innervation among isocortical regions (Gaspar et al., 1989). Thus, we have to rule out a

point-by-point matching between NE axons and degenerating cortical neurons, which in turn rules out a trans-synaptic disease spreading between NE fibers and cortical neurons. This calls for considering alternative mechanisms, which may link a damage to LC axons to cortical degeneration. In fact, when analyzing the pattern of NE release rather than a point-by-point connection between pre- and post-synaptic sites a massive extra-synaptic neurotransmitter diffusion takes place. In fact, NE release allows the amine to affect close glial cells and brain vessels (Stone and Ariano, 1989; Toussay et al., 2013).

THE MECHANISMS WHICH MAY LINK LC DEGENERATION TO AD

In early studies LC neurons were quantified without stereology. This explains a great variability in LC cell counts even within control subjects.

Modern stereology confirms LC neuronal loss (Grudzien et al., 2007; Counts and Mufson, 2010; Kelly et al., 2017). P-Tau positive LC neurons are associated with a dramatic reduction of synaptophysin-positive perineural dots, which leads to a loss of synaptic connectivity, a sort of anatomical de-afferentiation of these neurons from activating inputs (Andrés-Benito et al., 2017). These authors found over-expression of α2-adrenergic receptors at stage I, while these receptors were reduced at stage IV in the hippocampus (Andrés-Benito et al., 2017).

Altogether these pieces of information suggest that P-Tau accumulates within LC very early, producing subtle, yet potentially relevant, functional alterations affecting NE release in target areas.

P-Tau pre-tangles accumulate within pyramidal cortical limbic cells after NE axon terminals are filled with pre-tangles themselves (pre-tangle stage 1b). Later on, the occurrence of NFT inclusions in trans-enthorinal cortical neurons parallels the increase of P-Tau inclusions within LC axons, and spreads to other cortical structures (Braak et al., 2011). At stage III, when prodromal form of AD may appear, P-Tau burdens in LC neurons are severe enough to cause neuronal death. Early pre-tangle impairment of LC leads to an altered pattern of NE release within target regions. In baseline conditions NE release is finely tuned, and it is regulated by strong afferents from different sensory inputs (Samuels and Szabadi, 2008) which are altered early in the course of disease (Andrés-Benito et al., 2017), making surviving LC cells less effective. The specific anatomy of LC neurons makes them ideal vectors throughout the CNS. In fact, Tau aggregates may pass trans-synaptically from LC axons to cortical neurons and from one cortical region to another and back again (Mohamed et al., 2013; Goedert, 2015).

At advanced stages, the loss of NE neurons enhances the accumulation of Aβ in cortical regions: this in line with experimental evidence. In fact, the neurotoxin DSP-4 (which causes selective degeneration of LC axons, Fornai et al., 1996) enhances Aβ plaque accumulation in the brain (Heneka et al., 2006). Thus, the loss of NE appears to directly foster Aβ plaque accumulation (Kalinin et al., 2007) and LC

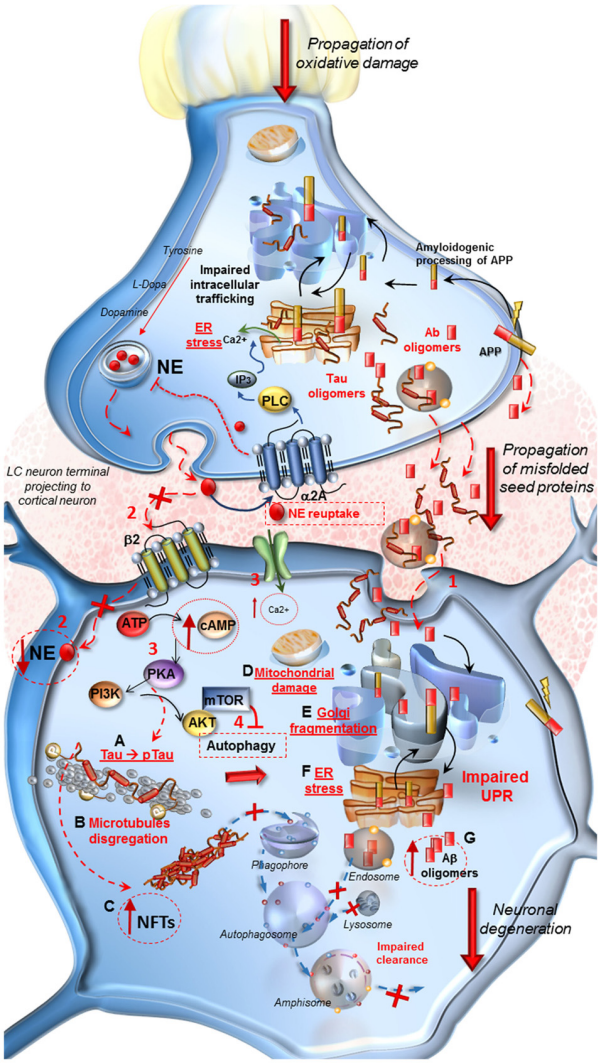

FIGURE 1 | Molecular events occurring in Alzheimer's Disease (AD) following locus coeruleus (LC) norepinephrine (NE) loss and autophagy impairment. *At LC pre-synaptic terminal*. Decreased expression of β2-adrenergic receptors in the pre-synaptic terminal leads to autophagy suppression. Oxidative stress, NE decrease and IP3 all contribute to endoplasmic reticulum stress and impairment of proteins processing, folding and trafficking in the trans-Golgi network. Aβ oligomers seeds, deriving from an amyloidogenic cleavage of APP, as well as Tau protein, are engulfed into vesicles and released into the axo-somatic synaptic cleft. *At post-synaptic cortical neuron*. **(1)** Aβ and Tau seeds are spread into the synaptic cleft and internalized into the cell body of the cortical neuron. **(2)** The loss of β2-adrenergic receptors (β2R) stimulation decreases autophagy. Thus the lack of NE, cannot exert its protective effect in the cell, it drives a cascade of detrimental intracellular effects instead. **(3,4)** Ca^{2+} entry into the cell, increase of cAMP levels, activation of PKA and PI3K/AKT/mTOR pathway lead to a further inhibition of autophagy. **(A–G)** PKA hyper-phosphorylates Tau protein **(A)** which leads to microtubules disgregation **(B)** and to the formation of neurofibrillary tangles (NFTs). **(C)** These effects translate into mitochondrial damage **(D)**, Golgi fragmentation due to the inactivation of associated binding proteins **(E)** and ER stress **(F)**. These events disrupt the Unfolded Protein Response (UPR) which does not provide for a proper intracellular trafficking, processing and sorting of misfolded Aβ, leading to further increase of harmful oligomers into the cell **(G)**. Misfolded proteins are internalized into the endosomal compartment but autophagy impairment does not allow their removal and fosters trans-synaptic propagations.

degeneration alters microglial activity, which may indirectly induce amyloid mismetabolism (Heneka et al., 2010). These changes correlate with cognitive impairment (Jardanhazi-Kurutz et al., 2011).

Again, a damage to LC produces a lack of neuroprotection. In fact β2-receptor stimulation is key in activating autophagy (Aránguiz-Urroz et al., 2011; Farah et al., 2014; Wauson et al., 2014) which removes P-Tau from the trans-Golgi network.

A recent study demonstrates an accumulation of P-Tau pyramidal cell bodies from AD patients correlates with alterations of the Golgi apparatus (Antón-Fernández et al., 2017).

A lack of β2-mediated autophagy stimulation both on pre-synaptic LC axons and post-synaptic cortical neurons might be key in triggering NFT (**Figure 1**). Similarly, the loss of β-receptors stimulation leads to a decrease in growth factors expression (Follesa and Mocchetti, 1993) and permanently alters immediate-early-genes expression (Cirelli and Tononi, 2000). Most directly, NE activation of neurotrophic pathways was shown to protect against neuronal amyloid toxicity (Counts and Mufson, 2010). Again, NE innervations, which occur following a volume transmission, may alter a variety of anatomical structures including astrocytes, glial cells and blood vessels which can be reached through a paracrine diffusion of NE (Stone and Ariano, 1989; Stone and John, 1991; Gesi et al., 2000; Marien et al., 2004). In this way, the neurovascular unit can be affected at multiple levels by a lack of NE innervation (Lecrux and Hamel, 2016). For instance, LC activation increases brain perfusion which triggers over-activity of multiple cortical cells (Toussay et al., 2013). This may contribute to explain why in vascular dementia Tau and amyloid accumulation occur as well (Mendel et al., 2010; Day et al., 2015; Saito et al., 2015). On the other hand, one should consider the chance that co-transmitters released in concomitancy with NE may participate to neuroprotective effects. This is the case of adenosine, which is known to be neuroprotective, as well as galanine, and others (Crawley, 1993, 1996; Stone et al., 2009; Huang et al., 2011). Just like NE these co-transmitters act by increasing autophagy, which removes both Tau and Aβ, while inducing a higher perfusion rate in the neurovascular unit and by modulating Ach release.

The time window, lasting years, from the onset of P-Tau accumulation in the LC to the onset of LC neuronal loss, might be the best timing for trying to halt AD onset and progression (Mather and Harley, 2016; Ehrenberg et al., 2017). Thus, molecular mechanisms and sub-cellular sites, which first accumulate P-Tau under the effects of NE loss, need to be investigated. The high vulnerability of the isodendritic cells of the LC reticular neurons relies on the archaic nature of these cells which are preserved to transmit multimodal activities and do not develop effective protective mechanisms (Theofilas et al., 2015; Gambardella et al., 2017). In fact, these neurons are sensitive to a wide spectrum of stressful stimuli. This implies the convergency of a number of excitatory pathways making these cells a paradigm for critical overstimulation within the brain. A balanced stimulation of the autophagy machinery, which removes both Tau and Aβ could be an important step in relenting the neurobiology of disease.

AUTHOR CONTRIBUTIONS

FSG wrote the article. LR contributed to the writing of the article and made artwork. RR provided a critical review of the literature. FB contributed to the artwork. FL contributed to the writing of the article and conceptualization. MF contributed the writing of the article. CLB contributed to the review of the literature. UB provided a review of the literature. FF wrote the article and provided a critical review of the whole manuscript.

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MINI REVIEW
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The Monoamine Brainstem Reticular Formation as a Paradigm for Re-Defining Various Phenotypes of Parkinson's Disease Owing Genetic and Anatomical Specificity

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The functional anatomy of the reticular formation (RF) encompasses a constellation of brain regions which are reciprocally connected to sub-serve a variety of functions. Recent evidence indicates that neuronal degeneration within one of these regions spreads synaptically along brainstem circuitries. This is exemplified by the recruitment of various brainstem reticular nuclei in specific Parkinson's disease (PD) phenotypes, and by retrospective analysis of lethargic post-encephalitic parkinsonism. In fact, the spreading to various monoamine reticular nuclei can be associated with occurrence of specific motor and non-motor symptoms (NMS). This led to re-consider PD as a brainstem monoamine disorder (BMD). This definition surpasses the anatomy of meso-striatal motor control to include a variety of non-motor domains. This concept clearly emerges from the quite specific clinical-anatomical correlation which can be drawn in specific paradigms of PD genotypes. Therefore, this review article focuses on the genetics and neuroanatomy of three PD genotypes/phenotypes which can be selected as prototype paradigms for a differential recruitment of the RF leading to differential occurrence of NMS: (i) Parkin-PD, where NMS are rarely reported; (ii) LRRK2-PD and slight SNC point mutations, where the prevalence of NMS resembles idiopathic PD; (iii) Severe *SNCA* point mutations and multiplications, where NMS are highly represented.

Keywords: Parkinson's disease, non-motor symptoms, pyramidal syndrome, genetic Parkinsonism, genotype-phenotype correlation

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INTRODUCTION

In the process of re-defining Parkinson's disease (PD) a task force is working on various symptoms which, despite being unrelated to the extra-pyramidal motor system, are now considered as fundamental features of PD (Poewe, 2008; Fornai and Ruggieri, 2013; Marras and Chaudhuri, 2016; Wei et al., 2016). Although most cardinal symptoms of PD involve the extra-pyramidal motor system, an in depth knowledge of PD patients led to describe a variety of non-motor alterations

as well as pyramidal motor dysfunctions which are presently under intense scrutiny (Li et al., 2010; Fornai and Ruggieri, 2013; Fornai et al., 2013; Natale et al., 2013; Xu et al., 2015; Zou et al., 2016). In fact, the occurrence of these symptoms may be helpful to discern between various PD genotypes/phenotypes, while it provides new vistas on the variety of brainstem reticular nuclei, which may or may not, be recruited during the disease course (Chao et al., 2015). Despite being missed out for a long time or being considered as an unexpected complication of a pure extra-pyramidal motor disorder, non-motor symptoms (NMS) are now a fundamental feature of PD. At the same time NMS provides an unusual perspective to elucidate the anatomical network sub-serving a brainstem monoamine disorder (BMD) which is the anatomical core of PD. In PD, NMS are often present during the disease course (Chaudhuri et al., 2006; Li et al., 2010; Bonnet et al., 2012; Chege and McColl, 2014; Bastide et al., 2015; Gao et al., 2015). In fact, brain areas, which control extra-pyramidal motor systems concomitantly, affect other neurological and psychiatric domains. This is evident when examining several reticular brainstem nuclei, whose functions affect both extra-pyramidal motor circuitries and a variety of non-motor domains during the neuropathology of sporadic PD (S-PD; Fornai and Ruggieri, 2013). Within these sub-cortical regions a variety of activities beyond extra-pyramidal motor control take place, which explains why non-motor alterations should be expected to occur rather than being unusual in most PD patients. In a recent article, we emphasized such a concept focusing on the involvement of a number of brainstem monoamine nuclei (Fornai and Ruggieri, 2013). In this analysis, we proposed the definition of BMD as more balanced to define the neuroanatomy of PD. In fact, a constellation of nuclei belonging to the brainstem reticular formation (BRF), are affected at various disease/stage severity. There is an appreciable site-specificity, which connects neuroanatomy with the onset of NMS and this works as a general model to build a clinical anatomical correlation within various PD syndromes. Within this context, progress in neurogenetics provided a powerful tool to improve PD nosography since specific gene/protein alterations connect quite specifically with clusters of both motor and NMS, which in turn, are related to a damage in quite selective brain areas. This configures PD as a spectrum of brainstem disorders which ranges between the occurrence of solely extra-pyramidal motor symptoms, up to the coexistence of pyramidal, extra-pyramidal symptoms along with NMS. Remarkably, a deep insight in the genetics of PD, along with the progressive awareness of NMS in PD, provided two key elements, which fostered the need to re-define PD itself. This is listed in the form of three paradigms shown in **Figure 1** as PD due to mutations in three different loci (*Parkin; LRRK2; SNCA*). Similarly, **Figure 2** shows the anatomy of the BRF and highlights those reticular nuclei which are often recruited in each paradigm of PD (Parkin; LRRK2; SNCA). When combining **Figures 1**, **2**, one may get a general correlation between PD

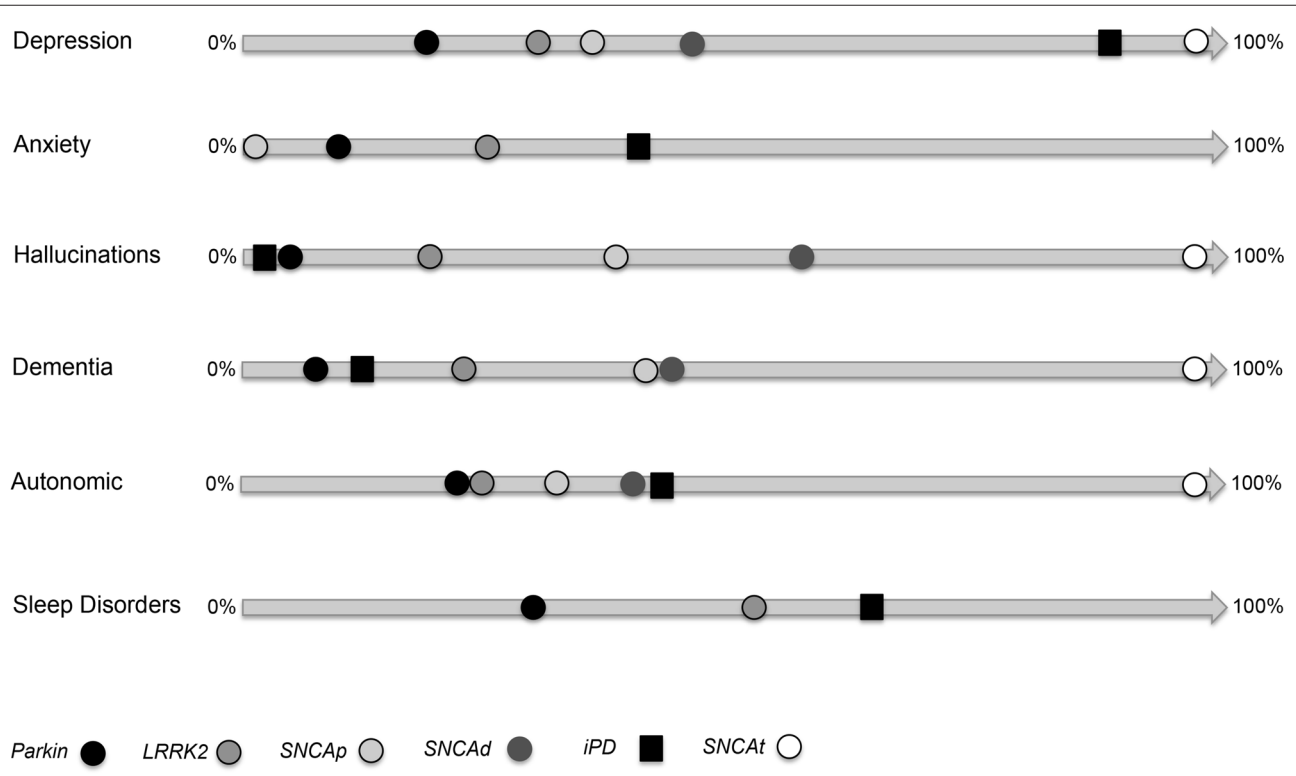

FIGURE 1 | Non-motor symptoms (NMS) and paradigms of genetic Parkinson's disease (PD). This image shows the frequencies of each NMS (depression, anxiety, hallucination, dementia, autonomic dysfunction, sleep disorders) for Parkinsonism related to *Parkin*, *LRRK2*, *SNCA* point mutation (SNCAp), *SNCA* duplication (SNCAd), *SNCA* triplication or homozygote duplication (SNCAt). Image adapted from tables reported in Chaudhuri et al. (2015).

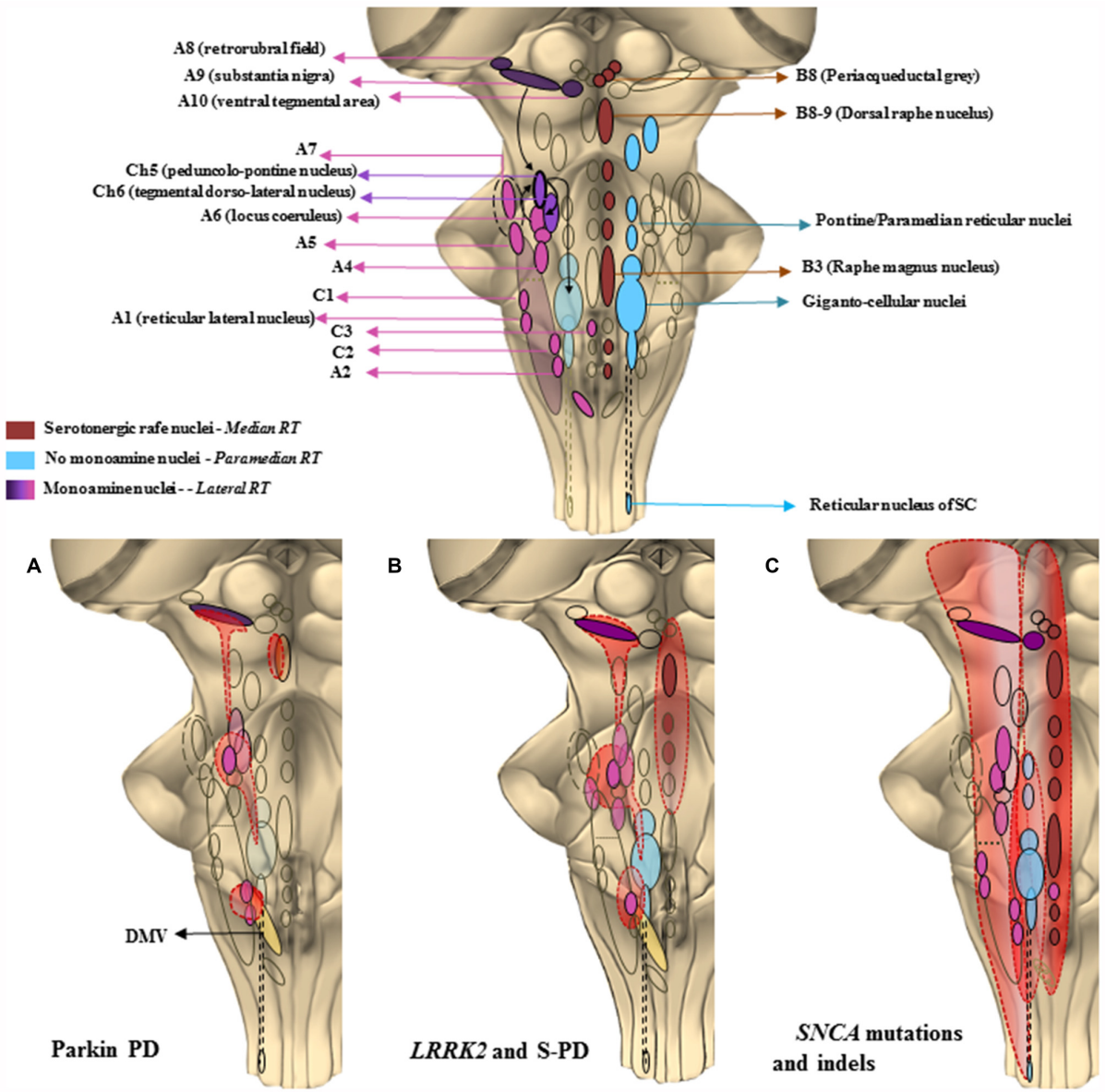

FIGURE 2 | The chemical neuroanatomy of the brainstem reticular formation (BRF) and its involvement in specific PD paradigms. This cartoon offers a schematic description of those brainstem areas properly belonging to the reticular formation (RF), which may be the key in PD pathology, as well as their selective recruitment in the three PD paradigms taken into account. In the upper part is shown the constellation of the RF nuclei following a neurotransmitter chemical classification. The isodendritic morphology of the neurons composing the RF nuclei, configures them as crucial stations of both afferent and efferent projections descending and projecting up to the cortex and spinal cord (SC). This network of overlapping connections is involved in a plenty of either extrapyramidal motor and non-motor functions. The major monoamine containing areas, mainly localized in the lateral RF except from C3, are the noradrenergic (A1–A7) adrenergic (C1–C3) dopaminergic (A8–A10) and cholinergic (Ch5–Ch6) nuclei. These are crucial for respiratory activity and for regulating blood pressure and heart rate, micturition, sweat, sleep-wakink cycle as well as descending motor control. Serotonergic nuclei are found in the median RF raphe nuclei, mainly in the B3, B8 and B9 areas. They control vegetative functions such as mood, sleep and sexual behavior, depression and pain. The medial RF, found between the median and the lateral column, is a region lacking monoamine nuclei, but whose giganto-cellular and paramedianpontine nuclei act as a station for fibers connecting with monoamine regions such as A6 (LC) and Ch6. They are involved in voluntary movement regulation, as well as in optical, acoustic and olfactory control due to their connections respectively, to the spinal cord and to the main cranial nerves' nuclei. In the second part of the figure, it is shown how a progressive and selective anatomic recruitment of such nuclei may be phenotypically and genotypically related to specific PD subtypes. **(A)** Parkin PD—Impairment of ventral SNpc (A9), mild impairment of LC (A6) leading to solely motor symptoms. A minimal alteration of the dorsal raphe nucleus (B8–9) may lead to apathia or anhedonia. DMV is affected as well, which may lead to a partial impairment of the overlapping C2/A2 area controlling the parasympathetic outflow. **(B)** *LRRK2* and slighter *SNCA* point mutations—Featuring the typical PD motor symptoms, along with the presence of sleep disorders, depression and dementia which are here related with a more extended involvement of monoamine nuclei of the lateral RF as well as of the median raphe nuclei. **(C)** Severe *SNCA* point mutations and large gene rearrangements—They present as a predominance of non-motor autonomic, psychotic and cognitive symptoms relying on the massive involvement of rostral and caudal areas of the RF extending above and beyond the brainstem.

genotype, the occurrence of specific NMS, and the recruitment of specific brainstem reticular nuclei. For instance, Parkin disease represents an almost pure extra-pyramidal motor disorder. This is well described by Doherty et al. (2013) showing the paucity of symptoms (**Figure 1**) and affected brain areas (**Figure 2**), limited to the substantia nigra pars compacta (SNpc) dorsal tier, locus coeruleus (LC) and dorsal motor nucleus of the vagus. Conversely, the specific point mutation p.G51D in the *SNCA* gene (Lesage et al., 2013) or multiplications of *SNCA* gene itself leads to a plethora of non-motor alterations (**Figure 1**). In this latter SNCA-dependent syndrome, motor alterations surpass the extra-pyramidal circuitry featuring also a pyramidal disorder with fatal prognosis (Mutez et al., 2011; Chen et al., 2015; Kiely et al., 2015). The p.G51D mutation additionally shifts the disease towards multiple system atrophy (MSA). The great amount of NMS shown in **Figure 1** is consistent with massive recruitment of brainstem nuclei shown in **Figure 2**. Roughly, in the middle of these extremes we describe most *LRRK2*-associated PD, which features extra-pyramidal motor symptoms along with some NMS (Zimprich et al., 2004). This resembles most *SNCA* point mutations but p.G51D, as well as S-PD patients (**Figure 1**).

THE CASE OF PARKIN DISEASE

The *Parkin* gene (OMIM 602544), codes for a protein called Parkin, a E3 ubiquitin-ligase, which tags altered proteins by ubiquitin chains (Hristova et al., 2009; Yoshii et al., 2011). The loss of function of parkin activity leads to a restricted pathology of the mesencephalic RF (SNpc; Takahashi et al., 1994), which is affected only in the ventral tier (Doherty et al., 2013). This is quite odd in PD since we are now aware that in most PD cases the pontine nucleus of LC (A6) is massively affected and represents a hallmark for pathological diagnosis (Braak et al., 2000; Dickson, 2012). Mutations in *Parkin* produce autosomic recessive (AR) parkinsonism with early onset. Alterations are spread over the entire gene and include deletions and duplications of one or more exons in more than 50% of cases (Matsumine et al., 1997; Kitada et al., 1998; Abbas et al., 1999; Labandeira-Garcia et al., 2011). Motor symptoms of PD patients with two *Parkin* mutations present with classic parkinsonism, slow disease progression and more symmetrical onset, with fewer NMS than classic PD (Lesage and Brice, 2009; Lohmann et al., 2009; Kasten et al., 2010), supporting the concept of limited pathology (Kägi et al., 2010). In line with this, the frequency of dementia in *Parkin* mutations matches the frequency of the general population above the age of 65 (Khan et al., 2003; Macedo et al., 2009; Xu et al., 2012). Even psychiatric symptoms are almost absent (Lohmann et al., 2009; Alcalay et al., 2014). These observations may be unexpected since the occurrence of psychiatric disorders PD patients is connected with dopamine (DA)-induced motor fluctuations. Indeed, these patients undergo DA-dependent motor fluctuations early in their life, nonetheless these motor fluctuations do not contaminate the psychiatric domain. This evidence is critical to understand the neurobiology of DA-dependent psychiatric disorders. The occurrence of fluctuations in response to DA replacement therapy is concomitant with a massive loss of DA axons (Lohmann et al., 2009). Thus, the DA replacement therapy generates peaks and valleys of extracellular DA concentrations, which trigger a non-canonical transduction pathway within post-synaptic neurons (Gerfen, 2006; Biagioni et al., 2009). When the DA axons are present, the DA substitution therapy does not generate fluctuations. In fact, the excess of DA concentrations is quickly buffered by the powerful uptake from surrounding DA terminals via the DA transporter (DAT). In PARK2 patients, the loss of DA selectively occurs in the dorsal striatum which produces motor fluctuations only consistent with the loss of ventral tier of the SNpc. In contrast, the meso-limbic DA rising from the reticular nucleus of ventral tegmental area (VTA) pathways is spared preserving DA fluctuations in the ventral striatum and brain areas, which are key for psychopathology. Other psychiatric symptoms such as depression, panic, and anxiety are absent as well, which is likely to depend on sparing 5-hydroxytryptamine (5-HT) and the NA neurons of the RF (**Figure 2**; Fornai et al., 2013). In fact, depressive symptoms in PD as a whole syndrome, or a few items such as apathy or anhedonia, are likely to rely on degeneration of reticular 5-HT dorsal raphe nucleus and/or NA LC nucleus (Politis and Loane, 2011). Interestingly, although *PINK1* mutation carriers are clinically indistinguishable from *Parkin* mutation carriers, frequency of depression is higher in *PINK1* carriers, calling for more detailed anatomical comparisons between these two diseases (Schneider and Klein, 2010; Ricciardi et al., 2014; Chaudhuri et al., 2015). Autonomic dysfunction often relies on medullary A1, A2 and C1 and C2 noradrenergic and adrenergic neurons, respectively, which provide the descending fibers to preganglionic sympathetic neurons (**Figure 2**). The absence of disease spreading caudally may explain the preservation of blood pressure, micturition, sweating and other autonomic functions in PARK2 patients (Palma and Kaufmann, 2014). This is further explained by the lack of involvement of post-ganglionic ortho-sympathetic neurons, which otherwise degenerate in most PD cases. The lack of upstream disease progression and preservation of cholinergic and noradrenergic ascending reticular pathways as shown in **Figure 2** may explain why cognitive dysfunction and sleep disorders are absent in these patients. As reported for *PINK1* and *DJ-1*, these PD patients show a largely preserved sense of smell, adding evidence to the observations that autosomal dominant forms of monogenic parkinsonism exhibit more severe olfactory impairment than the recessive ones (Yoritaka et al., 2011). Only in a few cases slight psychiatric symptoms may occur in Parkin PD patients (Cooney and Stacy, 2016; Lynch and Fujioka, 2016).

LRRK2 MUTATIONS

Mutations of the *LRRK2* gene (leucine-rich repeat kinase 2, OMIM 609007) is considered the most common genetic cause of late-onset, autosomal-dominant (AD) familial PD (PARK8; Paisán-Ruíz et al., 2004). The gene codes for a member of the leucine-rich repeat kinase family, widely expressed in isocortex, striatum, cerebellum, BRF and hippocampus (Sweet et al., 2015).

Several *LRRK2* mutations have been reported so far. These genotypes differently affect the amount of catecholamine nuclei of the BRF, and produce various NMS patterns (Vitte et al., 2010). *LRRK2* overlaps with alpha-synuclein pathology in the BRF, where deposition of LRRK2 appears to anticipate alpha-synuclein pathology (Alegre-Abarrategui et al., 2008). This makes the involvement of brainstem reticular nuclei in LRRK2 PD closely resembling point *SNCA* mutations and idiopathic PD.

In fact, clinical data support that in LRKK2 patients the prevalence of NMS is similar to idiopatic PD (i-PD; Estanga et al., 2014; Gaig et al., 2014).

Depression is quite common and can affect up to 40% of individuals with *LRRK2* mutations (Chaudhuri et al., 2006; Langston, 2006; Schrag and Schott, 2006; Shanker et al., 2011; Gaig et al., 2014; Pont-Sunyer et al., 2015). Similarly, REM sleep behavioral disorder (RBD) is quite frequent (Trinh et al., 2014). This suggests a recruitment of ascending reticular nuclei extending beyond the SNpc. In fact, just like idiopathic PD, LRRK2 involves the substantia nigra and LC (Vitte et al., 2010).

The occurrence of dementia in 17% of LRKK2 patients is lower than i-PD (Aarsland and Kurz, 2010). This appears to rely on altered synaptic activity within hippocampus. Supporting this concept, transgenic mice expressing p.G2019S *LRRK2* mutations show altered long-term depression (LTD) with potential impairment of learning and memory as shown in PD patients carrying *LRRK2* mutations (Shanker et al., 2011; Sweet et al., 2015). Olfactory functions is preserved in PD patients carrying the *LRRK2* mutations which is confirmed in preclinical models where mice expressing p.R1441C *LRKK2* mutations exhibit normal olfactory function (Tsika et al., 2014). These latter findings may be in contradiction with the classic Braak staging of PD. Nonetheless, the brainstem progression involves monoamine containing nuclei of the RF and starts from the dorsal nucleus of the vagus and nucleus solitarius (Kingsbury et al., 2010). According to **Figure 2** this area corresponds roughly to the A2/C2 catecholamine region of the medulla. The cortical pathology is independent from the recruitment of the brainstem and so would be the olfactory allocortex including the olfactory bulb.

SLIGHT AND SEVERE SNCA POINT MUTATIONS AND MULTIPLICATIONS

The *SNCA* gene (alpha-synuclein, OMIM 163890) was the first gene to be associated with familial parkinsonism (Houlden and Singleton, 2012). This gene codes for the protein alpha-synuclein which is altered in both familial PD (F-PD) and S-PD (Oczkowska et al., 2013). Both point mutations (PARK1) and gene multiplications (PARK4), have been reported to produce PD with different age at onset, penetrance and clinical motor and non-motor features (Singleton et al., 2003; Puschmann et al., 2009; Kiely et al., 2013).

SNCA Multiplications

Triplication carriers have disease onset about 10 years earlier and a more rapid disease course than duplication carriers, who overall closely resemble i-PD patients, while higher clinical variability has been reported for different point mutations (Miller et al., 2004; Ferese et al., 2015). NMS are constantly present in all SNCA patients. Remarkably, those patients carrying four copies of *SNCA* undergo a severe phenotype of PD that leads to death in a few years from diagnosis. These patients possess early onset sleep disorders, autonomic dysfunction, and psychotic episodes, with massive involvement of the BRF (**Figure 2**). This is evidenced by a massive deposition of alpha- synuclein oligomers in the BRF (Roberts et al., 2015). These oligomers are made up of prion-like partially proteinase-K resistant alpha-synuclein just like oligomers in the brainstem of PD patients. These patients show rapid cognitive and motor deterioration which surpasses the extrapyramidal circuitry affecting the corticospinal pathway as evidenced by a pyramidal syndrome which adds on the extrapyramidal movement disorder which is the main cause of death in these patients (Singleton et al., 2003; Ferese et al., 2015). These latter syndromes indicate that neuronal degeneration recruits brain areas above the brainstem level extending well beyond the classic neuropathology described by Tretiakoff in 1919 (Parent and Parent, 2010).

SNCA Duplication

SNCA duplications lead to various NMS (dysautonomia, RBD, hallucinations, depression; Konno et al., 2016) albeit with lower prevalence compared with carriers of four *SNCA* copies, but still higher compared with i-PD. *SNCA* duplication shows an incomplete penetrance in some carriers and, interestingly, NMS are reported in both healthy people and PD patients carrying this kind of mutation. Such carriers are reported to show an impairment of reward learning, suggesting that a copy number variation of the *SNCA* gene may be associated with NMS (especially selective learning impairment) more than producing classic motor parkinsonism (Kéri et al., 2010). In contrast, olfactory dysfunction and RBD are observed only in symptomatic carriers (Nishioka et al., 2009).

SNCA Point Mutations

High clinical variability has been reported in *SNCA* point mutations, where NMS are less frequent if compared with *SNCA* multiplications, and dementia is also less frequent when compared with i-PD patients (Aarsland et al., 2005; Aarsland and Kurz, 2010). NMS features are variably associated with different point mutations. p.A53T and p.A30P are reported to correlate with dementia (Puschmann et al., 2009), p.E46K with dementia and visual hallucinations (Zarranz et al., 2004), and p.G209A with olfactory dysfunction and RBD. In G209A carriers, prominent motor decline and deterioration of autonomic and cognitive function occur.

Remarkably, the point mutation p.G51D leads to a rapidly evolving syndrome. In this case, despite no gene multiplication occurs, there is a widespread neuropathology that, similarly to patients carrying four *SNCA* copies, leads to the involvement of the pyramidal tract. Even in this case, the cortico-spinal degeneration is the main cause of death with a clinical course and neuropathology which surpasses PD and features MSA, where a plethora of NMS and massive areas in the brainstem are affected

to extend rostrally to the prosencephalon and caudally to the spinal cord (Lesage et al., 2013; Kiely et al., 2015).

CONCLUSIONS

The occurrence of both motor NMS in most cases of PD is now well established. This is related to the engagement of a constellation of brain areas mostly located in the core of the BRF. The severity and variety of motor symptoms and mostly the various occurrence of NMS produce different PD syndromes, which appear more and more as a variety of diseases. This is substantiated by the number of genetic alterations, which may produce PD, and it is confirmed by the variety brain nuclei which may be involved. The present manuscript suggests a clinical anatomical correlation based on different genetic alterations which produce PD. These genetic conditions were used as paradigms for a clear-cut separation of different PD syndromes. The various involvement of brainstem reticular nuclei in these three paradigms was discussed.

Evidence was provided that the onset of an almost pure extrapyramidal syndrome due to a *Parkin* mutation was related to a quite unusual pathology mostly confined to the ventral tier of the SNpc. On the other hand, the multiplications of the *SNCA* genes and the p.G51D *SNCA* point mutation were related to the most severe phenotype in which a plethora of NMS (psychotic and mood disorder, sleep disorder, cognitive alterations, autonomic dysfunctions) were associated with a massive involvement of several nuclei of the BRF. Remarkably the most severe condition (point mutation p.G51D is definitely considered as MSA rather than PD). In the middle of these paradigms, we described the clinical anatomical correlation in the case of most *LRRK2* mutation and *SNCA* point mutation, which resembles S-PD. An interesting correlation was evident between specific NMS and specific brainstem reticular nuclei. This is quite remarkable for anxiety, mood and sleep disorders which associate with serotonergic neurons and lateral reticular noradrenergic nuclei. Similarly, the occurrence of autonomic dysfunctions witnesses for the involvement of the bulbar noradrenergic and adrenergic areas (A1/A2 and C1/C2, respectively). The impairment of oculo-motor activities suggests the impairment in the paramedianpontine RF, while the presence of DA-dependent psychotic symptoms relates with the involvement of the mesencephalic VTA of the RF which projects to limbic and iso-cortical regions. In the most severe phenotypes, the disease surpasses the definition of BMD and configures as a MSA where the early involvement of the brainstem is expected to spread quickly to distant brain regions also including the motor cortex and spinal cord. The spreading of symptoms in the disease course is likely to rely on the prion-like properties of key proteins such as alpha-synuclein. The variety of brain areas is not totally unexpected in the light of the remarkable branching and collateralization of the iso-dendritic neurons forming the core of the BRF which represents a powerful stream to drive physiological and altered synaptic activity.

AUTHOR CONTRIBUTIONS

SG: coordination and writing the review. RF: pubmed research and state of the art about genetic of parkin. FB: pubmed research and state of the art about genetic of *LRRK2*. CLB: pubmed research and state of the art about genetic of *SNCA*. RC: participated in critically revising the article. AMPG: participated in drafting the article. FL: pubmed research and state of the art about genetic of *SNCA* duplication and triplication. GN: participated in critically revising the article for important intellectual content. MS: participated in drafting the article. FF: coordinating and writing the article. Participated in drafting the article. Participated in critically revising the article for important intellectual content.

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frontiers in Cellular Neuroscience

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Neuronal Release of Cytokine IL-3 Triggered by Mechanosensitive Autostimulation of the P2X7 Receptor Is Neuroprotective

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Mechanical strain due to increased pressure or swelling activates inflammatory responses in many neural systems. As cytokines and chemokine messengers lead to both pro-inflammatory and neuroprotective actions, understanding the signaling patterns triggered by mechanical stress may help improve overall outcomes. While cytokine signaling in neural systems is often associated with glial cells like astrocytes and microglia, the contribution of neurons themselves to the cytokine response is underappreciated and has bearing on any balanced response. Mechanical stretch of isolated neurons was previously shown to trigger ATP release through pannexin hemichannels and autostimulation of P2X7 receptors (P2X7Rs) on the neural membrane. Given that P2X7Rs are linked to cytokine activation in other cells, this study investigates the link between neuronal stretch and cytokine release through a P2X7-dependent pathway. Cytokine assays showed application of a 4% strain to isolated rat retinal ganglion cells (RGCs) released multiple cytokines. The P2X7R agonist BzATP also released multiple cytokines; Interleukin 3 (IL-3), TNF-α, CXCL9, VEGF, L-selectin, IL-4, GM-CSF, IL-10, IL-1Rα, MIP and CCL20 were released by both stimuli, with the release of IL-3 greatest with either stimuli. Stretch-dependent IL-3 release was confirmed with ELISA and blocked by P2X7R antagonists A438079 and Brilliant Blue G (BBG), implicating autostimulation of the P2X7R in stretch-dependent IL-3 release. Neuronal IL-3 release triggered by BzATP required extracellular calcium. The IL-3Rα receptor was expressed on RGCs but not astrocytes, and both IL-3Rα and IL-3 itself were predominantly expressed in the retinal ganglion cell layer of adult retinal sections, implying autostimulation of receptors by released IL-3. While the number of surviving ganglion cells decreased with time in culture, the addition of IL-3 protected against this loss of neurons. Expression of mRNA for *IL-3* and *IL-3Rα* increased in rat retinas stretched with moderate intraocular pressure (IOP) elevation; BBG blocked the rise in *IL-3*, implicating a role for the P2X7R in transcriptional regulation *in vivo*. In summary, mechanical stretch triggers release of cytokines from neurons that can convey

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neuroprotection. The enhancement of these signals *in vivo* implicates P2X7R-mediated IL-3 signaling as an endogenous pathway that could minimize damage following neuronal exposure to chronic mechanical strain.

Keywords: P2X7, cytokine release, mechanosensitivity, IL-3, neuroprotection, ATP release, glaucoma, pannexin hemichannels

INTRODUCTION

Mechanical strain can trigger neuronal loss, and inflammatory signals are increasingly recognized as contributing to the response. Cytokines and chemokines have been implicated in traumatic brain injury (TBI), tumor-related swelling, increased intraocular pressure (IOP) and other pathological conditions linked to mechanical strain (Ziebell and Morganti-Kossmann, 2010; Freedman and Iserovich, 2013; Wei et al., 2014; Gyoneva and Ransohoff, 2015). Given that cytokines and chemokines can initiate both pro-inflammatory and protective responses, understanding the pathways linking mechanical strain to cytokine signaling has broad relevance for neural trauma.

Although cytokines are traditionally associated with release from cell types of the inflammatory system, they are now recognized as general signaling molecules (Iwasaki and Medzhitov, 2010; Lacy and Stow, 2011; Arango Duque and Descoteaux, 2014). Within neural tissues, microglial cells are a major source of cytokines (Hanisch, 2002), but other glial cells like astrocytes can also signal with cytokines in health and disease (Domanska et al., 2011; Kan et al., 2012; Choi et al., 2014). Neurons are typically examined as a target for cytokine signaling, with various cytokine receptors expressed on neural membranes (Bajetto et al., 2002; Gougeon et al., 2013). However, neurons are also a source of releasable cytokines; while this has been known for some time, the signaling pathways that link neural stimulation to cytokine release remain unclear (Freidin et al., 1992; Yamamoto et al., 2011).

Purinergic signaling pathways provide a likely route to connect mechanical strain with neuronal cytokine release. Throughout the body, mechanical strain leads to ATP release, with pannexin hemichannels implicated as a conduit for mechanosentitive ATP release in many cell types (Bao et al., 2004; Beckel et al., 2015; Furlow et al., 2015). Isolated neurons also respond to mechanical strain with the release of ATP through pannexin hemichannels (Xia et al., 2012). This released ATP autostimulates P2X7 receptors (P2X7Rs) on the neural membrane to elevate cytoplasmic calcium levels. Given that cytoplasmic calcium can contribute to the vesicular release of cytokines (Stow et al., 2009; Stanley and Lacy, 2010), and the stimulation of P2X7Rs is associated with cytokine release from multiple cell types (Pizzirani et al., 2007; Mingam et al., 2008; Clark et al., 2010), this study asked whether neurons could release cytokines in response to mechanical strain, whether this involved P2X7Rs, and probed the consequences of cytokine release for neuronal survival.

MATERIALS AND METHODS

Purification of Retinal Ganglion Cells

Isolation of retinal ganglion cells (RGCs) was performed using the immunopanning procedure of Barres (Barres and Chun, 1993) as described in detail (Zhang et al., 2006). All animals were used according to protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee. In brief, retinas of Long-Evans rat pups PD 3–7 of both genders were dissected from each eye globe and digested for 30 min at 37°C in Hank's balanced salt solution (HBSS; Gibco, Inc. Invitrogen Corp., Carlsbad, CA, USA) containing 15 U/mL papain, 0.2 mg/mL DL-cysteine and 0.004% DNase I. The retinas were washed and triturated in HBSS with 1.5 mg/mL ovomucoid, 1.5 mg/mL bovine serum albumin (BSA) and 0.004% DNase I, incubated with rabbit anti-rat macrophage antibody (10 min, 1:75, Accurate Chemical, Westbury, NY, USA) and then centrifuged at 1000 rpm for 10 min, washed and spun again. Cells were re-suspended in phosphate-buffered saline (PBS) containing 0.2 mg/mL BSA and 5 μg/mL insulin, and incubated for 15 min in a 100 mm Petri-dish coated with goat anti-rabbit IgG antibody (1:400, Jackson ImmunoResearch Inc, West Grove, PA, USA). Non-adherent cells were transferred to a second Petri-dish coated with goat anti-mouse IgM antibody (1:300, Jackson ImmunoResearch) and anti-Thy 1.1 antibody (from hybridoma T11D7e2; American Type Culture Collection, Rockville, MD, USA). After 30 min, non-adherent cells were washed off and the adherent ganglion cells were released with 0.125% trypsin for 8 min at 37°C. Enzymatic activity was neutralized using 30% fetal bovine serum in Neurobasal-A medium and the purified RGCs were collected in a centrifuge tube. The basic growth medium consisted of Neurobasal-A medium with 0.033mL/mL B-27 supplement, 3.3% rat serum, 50 ng/mL BDNF, 10 ng/mL CNTF, 50 ng/mL FGF-basic, 5 μg/mL insulin and 5 μM forskolin. Isolated RGCs were seeded onto 0.1% poly-L-lysine (Peptides International) and 1 μg/mL laminin coated coverslips or elastic silicone sheeting in stretch chambers and cultured at 37°C with 5% CO_2.

Cell Stretch Chamber

A specially designed stretch chamber was used to apply moderate stretch to isolated neurons as described for neuronal ATP release (Xia et al., 2012). A hole drilled in the top enclosure of the stretch chamber allowed the entry of a needle attached to a 3-way valve connected to both a 20 ml syringe, which allowed pressure elevation by injection of air, and simultaneously to a pressure transducer to monitor the pressure inside the chamber.

To prevent pressure leakage, silicone grease was used to cover the hole after the needle was inserted and a layer of Teflon tape was used to screw on the top enclosure. Each stretch chamber was fitted with an elastic silicone sheet and application of 20 mmHg of pressure was calculated to result in a 4.1% deformation strain. To promote cell adhesion, the sheets were coated with 0.1% poly-L-lysine for 24 h and 1 μg/mL laminin for 2 h prior to seeding. Isolated RGCs were seeded onto silicone sheets in the stretch chamber and maintained in the basic growth medium at 37°C for 16 h. Cells were washed with low-Mg^{2+} isotonic solution containing (in mM) 105 NaCl, 5 KCl, 4 Na Hepes, 6 Hepes acid, 5 $NaCO_3$, 60 mannitol, 5 glucose and 1.3 $CaCl_2$ (pH 7.4). To begin the experiment, 750 μL of isotonic solution with or without P2X7R antagonists Brilliant Blue G (BBG, Sigma Chemical #B-0770 (Jiang et al., 2000)) or A438079 (Tocris, #2972 (Donnelly-Roberts and Jarvis, 2007)) were added to the stretch chambers and 30 min stabilization time was allowed prior to taking a 250 μL baseline sample. Pressure inside the stretch chamber was increased to 20 mmHg for 4 min, returned to 0 mmHg for 1 min and the cycle repeated three times for a total duration of 15 min. Immediately following stretch, a 250 μL sample of the extracellular solution was collected from the center of the stretch chamber. Samples were frozen at −20°C and used for cytokine array or Interleukin 3 (IL-3) measurement as described below.

P2X7 Receptor Activation

Isolated RGCs were cultured for 18 h on coverslips coated with poly-L-lysine and laminin. RGCs were washed thrice with low-Mg^{2+} isotonic solution and then incubated with 1.0 mL of isotonic solution for 30 min. A 500 μL sample was collected and replaced with 500 μL of 100 μM P2X7R agonist BzATP (Young et al., 2007). After 30 min incubation in 50 μM BzATP, a 500 μL sample was collected. In some experiments 50 μM BzATP was prepared in Ca^{2+}-free isotonic solution or with 10 μM P2X7R antagonist A438079 with 30 min pre-incubation in A438079. After 30 min, a sample of the extracellular solution was collected and stored at −80°C until ready for measurement.

Cytokine Array

Samples collected before and after BzATP stimulation or samples collected from the previously detailed stretch chamber experiments were assayed for levels of cytokines using a rat antibody cytokine array (#ARY008, R&D Systems). Briefly, the experimental samples were first incubated with biotinylated detection antibodies and then incubated on nitrocellulose membranes spotted with 29 different anti-cytokine antibodies in duplicate. Cytokines present in the sample were bound to its cognate capture antibody on the membrane and the amount of attached cytokines was detected by incubating the membrane in Streptavidin-Horseradish Peroxidase (HRP) followed by chemiluminescent detection reagents (GE Healthcare). The production of light corresponding to levels of bound cytokine was determined with ImageQuant LAS4000 and the intensity of each spot was measured using ImageQuant TL analysis software (all GE Healthcare).

Measurement of IL-3

IL-3 concentrations were determined using the Rat IL-3 ELISA kit (#CSB-E07436r, Cusabio Biotech Co., College Park, MD, USA) by following the manufacturer's instructions. The ELISA plate provided in the kit had been coated with an IL-3 antibody, which binds IL-3 proteins present in the sample. After incubation with biotin-conjugated antibody specific for IL-3 and Avidin conjugated to HRP, the addition of a TMB (3,3′,5,5′-tetramethylbenzidine) substrate solution produces a change in color. A sulfuric acid solution was added to stop the enzyme-substrate reaction and the plate was read using a plate reader at wavelength of 450 nm and a correction wavelength of 570 nm. IL-3 standard curves with and without drugs used in the experiment were prepared to convert absorbance values of the samples to IL-3 concentration.

Immunoblots

Isolated cells or tissue were lysed in RIPA buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% Na-Deoxycholate, 0.1% SDS, 50 mM Tris, protease inhibitor cocktail, pH 8.0). Lysates were pulse sonicated, centrifuged at 13,000 g for 10 min at 4°C and the supernatant collected and boiled with 4× sample buffer. Controls included spleen dissected from the rat pups used for ganglion cell isolation, macrophages/microglial cells as identified in the first stage of the immunopanning procedure, and rat optic nerve head (ONH) astrocytes isolated as described (Beckel et al., 2014). Protein concentration was determined by the bicinchoninic acid (BCA) assay and equal amounts of protein sample (15 μg) were separated by 10% SDS-PAGE, followed by transfer to PVDF membrane. Non-specific sites were blocked with 5% non-fat milk and incubated overnight at 4°C with mouse anti-IL-3Rα (sc-74522, 1:100, Santa Cruz Biotechnology Inc. Dallas, TX, USA). Membranes were incubated with HRP-conjugated secondary antibody (1:3000, Santa Cruz) for 1 h at room temperature (RT). Blots were developed using a chemiluminescent detection kit (Amersham) and captured using an ImageQuant LAS 4000 as described (Lu et al., 2015).

Immunohistochemistry

Frozen retina sections (10 μm) from adult Long-Evans rats were fixed in 4% paraformaldehyde for 10 min at RT. Sections were incubated with Superblock blocking buffer (ThermoFisher Scientific# 37515) containing 10% donkey serum for 1 h at RT. The primary antibodies for IL-3 receptor alpha (1:50, mouse anti-IL-3Rα, sc-74522, Santa Cruz Biotech.) and IL-3 (1:50, goat anti-IL-3, sc-34807, Santa Cruz Biotech.) were incubated at 4°C for overnight. The secondary antibodies were conjugated to AlexaFluor 555 (1:1000, ThermoFisher Scientific) and incubated for 1 h at RT. Samples were protected from light from this point onwards. Nuclei were counterstained with 4′,6-diamidino-2-phenyindole dilactate (DAPI; Invitrogen) for 10 min at RT prior to mounting in Slow Fade Gold Antifade immunofluorescence mounting medium. Negative controls were conducted by omitting the primary antibodies. Images were visualized using a fluorescence microscope (Nikon Eclipse E600) with excitation/emission filters of 360/ >515 nm (DAPI) and

530–560/575–640 nm (AlexaFluor 555) and captured with a Retiga 2000 camera (QImaging, Surrey, BC, Canada). ImageJ[1] (Schindelin et al., 2015) was used to subtract background, modulate intensity and combine pseudocolored images, with parallel processing for all images.

Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) activity released into the extracellular solution was measured as an indicator of cell membrane integrity as described previously (Guha et al., 2013), based on a coupled two-step reaction where tetrazolium salt was reduced to the colored product formazan (Cytotoxicity Detection Kit (LDH), Roche Applied Science, Indianapolis, IN, USA). Briefly, 100 μL of extracellular solution collected from cells subject to stretch or BzATP stimulation was added to a 96-well plate. A reaction mixture consisting of catalyst and dye solution was prepared and 100 μl was added to each well as per the manufacturer's instructions. After incubation for 15–30 min at RT, the dye absorbance was measured at 490 nm using a microplate reader. Absorbance values were converted to LDH concentration by preparing LDH standards (L-LDH; from rabbit muscle, Roche) for each experiment.

Cell Viability Assay

Cell viability was determined as previously described (Zhang et al., 2010). In brief, Long Evans rat pups (PD 3–7) were anesthetized with an intraperitoneal injection of 50/5 mg/kg ketamine/xylazine, followed by the injection of aminostilbamidine into the superior colliculus to allow for retrograde labeling of the RGCs. After allowing 2–3 days for dye transport to the ganglion cell somas in the retina, animals were sacrificed and the retinas dissected in Hank's buffered saline solution (HBSS). The tissue was dissociated in 15 U/mL papain in HBSS at 37°C for 15 min, followed by washing in HBSS and trituration in culture medium to obtain a mixed retinal cell suspension. Cells were seeded onto coverslips coated with 0.1% poly-L-lysine and 1 μg/mL laminin. Cells were maintained at 37°C with 5% CO_2 in culture medium with or without 10 ng/mL IL-3 (R&D Systems, Minneapolis, MN # 2524-RL) or 20 μM AG490 (Calbiochem/EMD Billerica, MA #658411). After 24 h, the cells were fixed with 4% paraformaldehyde for 15 min and the coverslips mounted onto slides with SlowFade Gold anti-fade mounting media (Invitrogen). The number of surviving ganglion cells was quantified by counting the fluorescently labeled cells in 80–120 fields observed with a 20× objective in a masked fashion.

In Vivo Elevation of Intraocular Pressure

Transient non-ischemic elevation of IOP was performed based on the protocols developed by Morrison and Crowston (Morrison et al., 2014; Crowston et al., 2015). Sprague-Dawley rats (8–12 weeks) were anesthetized by intraperitoneal injection of ketamine/xylazine (100/10 mg/kg). One eye was cannulated with a 27-gauge needle (Becton Dickinson, NJ, USA) inserted into the anterior chamber and connected to a 20 ml syringe filled with sterile PBS. IOP was elevated to 50 mmHg by positioning the syringe at the appropriate height (68 cm H_2O), while the contralateral eye without cannulation served as the normotensive control. IOP was checked with a TonoLab tonometer (Colonial Medical Supply, Franconia, NH, USA) at the beginning and end of the elevation of the reservoir. IOP was found to be remarkably consistent both throughout the 4 h of elevation and between animals. After 4 h IOP elevation, pressure was returned to normal, the needle was removed and antibiotic ointment was applied. Rats were sacrificed 24 h later and the retina dissected and processed for molecular analysis.

Intravitreous Injection

Injections were performed under a microscope with a micropipette connected to a microsyringe (Drummond Scientific Co., Broomall, PA, USA) as described (Hu et al., 2010). P2X7R antagonist BBG (0.8%) was dissolved in sterile saline and injected 5–7 days before IOP elevation. The glass pipette filled with drug was passed through the sclera at a point approximately 1 mm from the limbus into the vitreous cavity. The total volume injected was 5 μl over a 30 s time period.

Quantitative PCR

After enucleation, the dissected retina was immediately homogenized in 1 mL of Trizol reagent (Invitrogen Inc., Carlsbad, CA, USA). Chloroform was added, followed by centrifugation at 12,000 g for 10 min. The aqueous layer was collected and the total RNA purified using the RNeasy Mini kit (Qiagen). The High Capacity RNA-to-cDNA kit (Applied Biosystems #4387406) was used to reverse-transcribed 1 μg of total RNA to cDNA. Quantitative PCR (qPCR) was performed using a Power SYBR Green master mix (Applied Biosystems) and the quantitative assessment of gene levels was performed using a 7300 Real-Time PCR System (Applied Biosystems) as described (Reigada et al., 2005). For wells with IL-3 primers, 0.75 uL of cDNA was added per well, due to low gene expression of IL-3 in the retina. For all other wells, 0.5 uL of cDNA was added per well. Primer pair sequences used for qPCR are IL-3 F: AGTGACGACAAAGCCAATCTGAGG R: TTGTAGACACCT GGCAACACAGAGT; IL-3Rα F: AGGGAACACTGAGAGCA GGA R: TGACATCGCCTCGAACATAG; IL-3Rβ F: GGGA GGACAGCAAGACAGAG R: GGTGAGGATGAGGAAGACCA; GAPDH F: ATGACTCTACCCACGGCAAG R: TACTCAGCA CCAGCATCACC. Primers were designed using Primer Blast (NCBI[2]) or Primer 3. All data were analyzed using the delta-delta Ct approach. Statistical analysis for the qPCR results was performed by comparing the change in expression of relevant genes to GAPDH, and these responses in pressurized eyes with and without antagonist BBG using a Student's *t* test.

Data Analysis

All data are expressed as mean ± standard error of the mean, with significance defined as $p < 0.05$. Statistical analysis for cytokine

[1]https://imagej.nih.gov/ij/

[2]http://www.ncbi.nlm.nih.gov/tools/primer-blast/

array experiments was determined using a paired *t*-test, while other data was analyzed using a one-way ANOVA followed by appropriate *post hoc* test unless otherwise noted. Analysis was performed using SigmaStat software (Systat Software, Inc., San Jose, CA, USA). % block of IL-3 expression was calculated as $100 \times ([RQ_c\text{-}RQ_b]/RQ_c)$ where RQ_c is expression in control and RQ_b is in BBG.

RESULTS

Neurons Release Cytokines Following Mechanical Stretch

The identification of neuron-specific cytokine release required the isolation of a pure population of neurons. As the "two-step" immunopanning procedure enables the production of RGC populations of >98% purity (Zhang et al., 2006) this preparation was used to investigate neuron-specific release. The application of 20 mmHg pressure into the stretch chamber was calculated to produce a 4.1% strain on the silicone sheet (Xia et al., 2012), leading to a moderate stretch of adherent neurons. Cells were stretched for 4 min with a rest for 1 min, with three cycles applied over 15 min. This moderate stretch elevated the levels of numerous cytokines in the extracellular bath surrounding the neurons (**Figure 1A**). Cytokines with levels significantly higher in the bath after stretch included IL-3, TNF-α, VEGF and CXCL9. While the overall magnitude of increase was modest, this was likely due to the small number of cells secreting cytokines into a relatively large extracellular volume. Critically, extracellular LDH levels did not change following stretch (**Figure 1B**). This implied that the release of cytokines was a physiological response to stretch and not attributable to neuronal lysis or non-specific leakage.

As these neurons were previously shown to release ATP in response to stretch, and this released ATP was capable of autostimulating their P2X7Rs (Xia et al., 2012), the cytokine response to P2X7R stimulation was examined directly. The P2X7R agonist, BzATP (50 μM) led to a similar release of cytokines into the extracellular bath (**Figure 1C**). Cytokines with levels significantly higher in the bath after stretch included IL-3, TNF-α, VEGF and L-Selectin. Extracellular LDH levels were not elevated after stimulation with 50 μM BzATP (**Figure 1D**).

Many of the cytokines that were significantly increased by cell stretch were also elevated by BzATP stimulation. Plotting the changes in cytokine release after stretch vs. BzATP incubation illustrated a correlation between the extent of release by the two stimuli (**Figure 1E**). While the magnitude of release triggered by stretch was generally greater than the increase by BzATP, there was considerable similarity in the overall pattern of release for both experiments. This suggested a common mechanism of cytokine release induced by stretch and P2X7R activation from the neurons.

Stretch-Induced Release of IL-3 Involves the P2X7 Receptor

While the patterns of cytokine release triggered by stretch and P2X7R activation suggested a common mechanism, additional experiments examined whether the P2X7R was involved in the stretch-mediated release. Experiments focused upon IL-3 given the relative robustness of its release from either stimuli. Baseline levels of extracellular IL-3 were 8.5 ± 0.3 pg/mL; levels rose to 13.8 ± 1.4 pg/mL after neuronal stretch, corresponding to a 1.62-fold increase in IL-3 concentration (**Figure 2A**). The stretch-triggered release of IL-3 was inhibited by 63.2 ± 7.1% and 68.2 ± 10.7%, respectively, by P2X7R antagonists A438079 (10 μM) or BBG (1 μM). Given that these antagonists target different sites, and that neuronal stretch released sufficient ATP to autostimulate the P2X7R on these cells (Xia et al., 2012), this strongly implicates the P2X7R in the stretch-dependent release of IL-3.

Additional evidence for the involvement of the P2X7R in the release of IL-3 was confirmed with agonist BzATP. Exposure to BzATP raised the IL-3 concentration surrounding the neurons from 7.4 ± 0.4 pg/mL to 10.9 ± 1.0 pg/mL (**Figure 2B**). The absence of calcium in the bath solution significantly reduced the release of IL-3 by BzATP to 8.3 ± 0.4 pg/mL. The P2X7R antagonist A438079 (10 μM) also attenuated the BzATP-triggered IL-3. This strongly suggested that BzATP was acting at P2X7Rs and not other receptor types.

IL-3 Stimulation is Neuroprotective

While the results above suggest the mechanosensitive release of IL-3 depended upon a P2X7R-dependent pathway, additional experiments probed the possible targets and consequences of IL-3. Western blot analysis revealed that the ligand-binding alpha subunit of the IL-3 receptor (IL-3Rα) was expressed by RGCs, but not by ONH astrocytes (**Figure 3A**). Immunohistochemistry identified RGCs as the primary of location for both IL-3Rα (**Figure 3B**) and IL-3 (**Figure 3C**) staining in the posterior eye of adult rats. Together, this implied the IL-3 released by ganglion cells autostimulated their own receptors.

Given reports of IL-3 as neuroprotective (Zambrano et al., 2007; Luo et al., 2012), the effect of IL-3 on neuronal survival was investigated. When grown in standard medium, the number of RGCs reduced with time (**Figure 3D**). However, incubation with IL-3 increased the number of surviving cells over 24 h (**Figure 3E**). As IL-3 activation can involve the JAK/STAT signaling pathway (Reddy et al., 2000), the effect of the JAK2 inhibitor AG490 was examined. The number of surviving neurons in the presence of IL-3 + 20 μM AG490 was reduced. When numbers were quantified across multiple trials, IL-3 increased the number of surviving neurons after 24 h in culture by 24% (**Figure 3F**), while AG490 reduced the protection by 54%. Incubating the cells with AG490 alone did not affect the neuronal survival.

Pressure-Dependent Rise in IL-3 *In Vivo* Needs P2X7R

To determine whether IL-3 signaling was involved in the response to mechanical strain *in vivo*, the effect of a non-ischemic elevation of IOP was examined. Pressure was raised to 50 mm Hg for 4 h. There was a significant rise in mRNA for *IL-3* (**Figure 4A**) and the receptor *IL-3Rα* in retinas exposed to

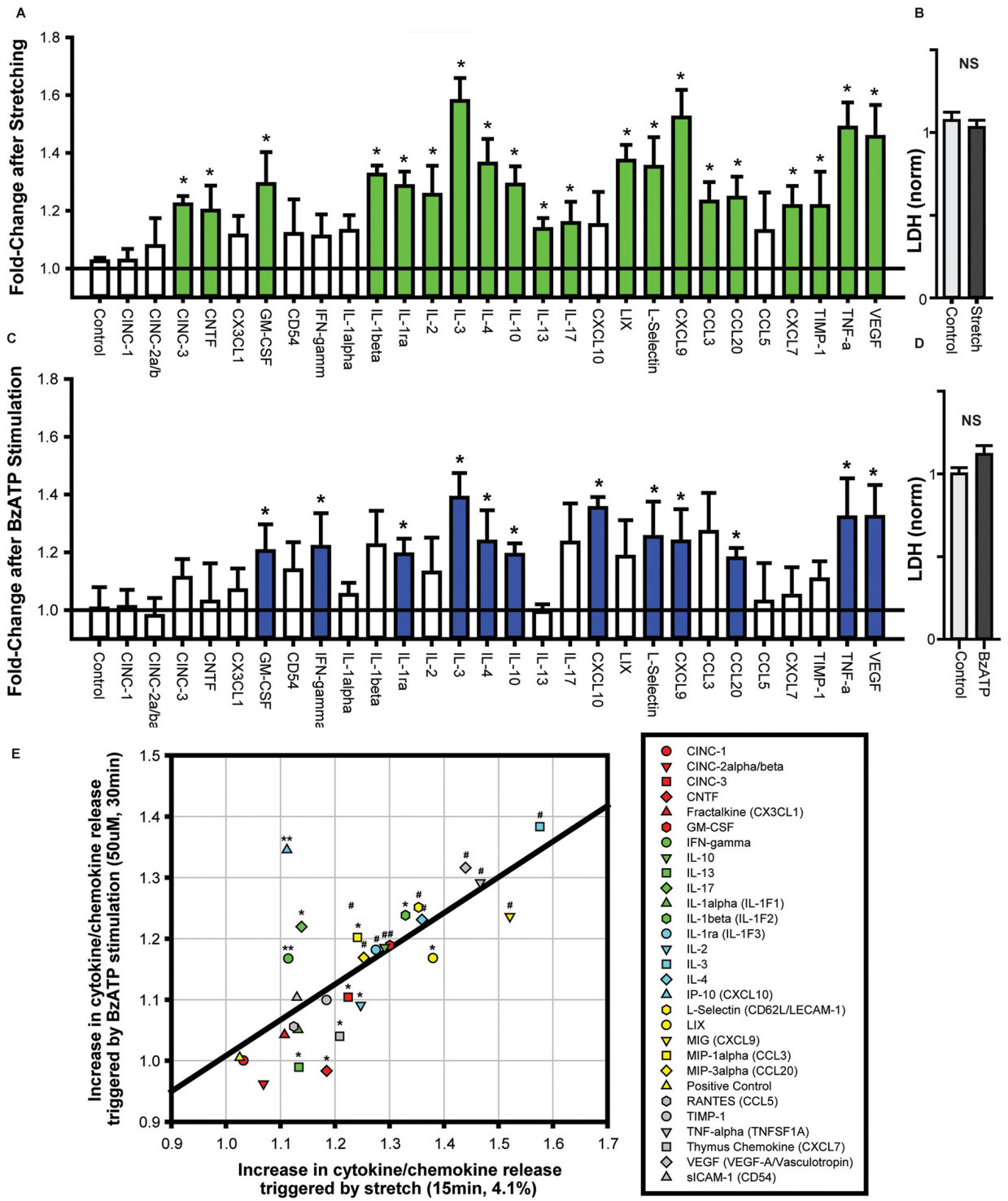

FIGURE 1 | Cytokines are released from neurons challenged with cell stretch or P2X7R stimulation. (A) Neuronal stretching with a 4.1% strain triggered the release of numerous cytokines into the extracellular bath. Cytokines that were increased significantly are shown in green ($n = 4$, $^*p < 0.05$, paired t-test). **(B)** Neuronal stretch did not increase lactate dehydrogenase (LDH) levels in the bath (NS, Not significant; $p = 0.55$, $n = 4$). **(C)** Exposure of neurons to P2X7R agonist BzATP (50 μM) for 30 min increased the level of extracellular cytokines. Cytokines that were increased significantly are shown in blue ($n = 4$, $^*p < 0.05$, paired t-test). **(D)** BzATP did not increase LDH levels in the bath ($p = 0.12$, $n = 4$). **(E)** Comparison of the increase in cytokine secretion triggered by cell stretch to the increase after BzATP stimulation (For panel **E**, $^*p < 0.05$ Stretch, $^{**}p < 0.05$ BzATP, $^{\#}p < 0.05$). The increase in cytokine release induced by stretch and BzATP displayed a relatively linear relationship, $r^2 = 0.52$.

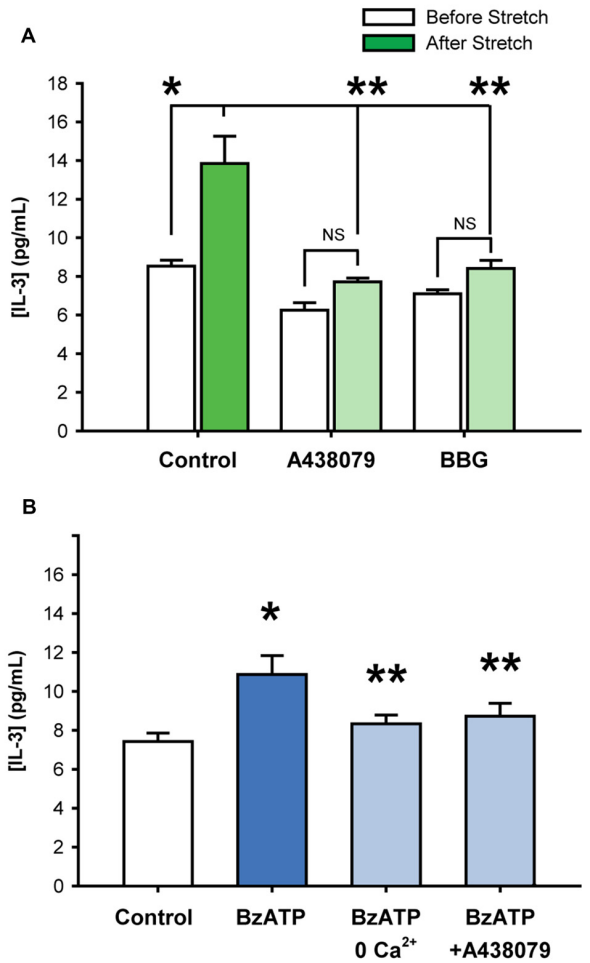

FIGURE 2 | Stretch-induced Interleukin 3 (IL-3) release involves the P2X7R. (A) Stretching isolated neurons with 20 mmHg pressure increased levels of extracellular IL-3 (*p = 0.01, n = 4). The addition of P2X7R antagonists A438079 (10 μM, **p = 0.005 vs. stretched) or Brilliant Blue G (BBG, 1 μM, **p = 0.01 vs. stretched) significantly suppressed the stretch-activated release of IL-3 by 63.2 ± 7.1% and 68.2 ± 10.7%, respectively. Note that the slight inhibition of baseline IL-3 levels by A438079 and BBG were determined to be statistically NS. **(B)** Incubation of isolated neurons with 50 μM BzATP for 30 min led to a significant increase in extracellular IL-3 concentrations, compared to isotonic control. The BzATP-induced release of IL-3 was attenuated by the removal of calcium from the extracellular solution or by the presence of P2X7 receptor antagonist A438079 (10 μM). (n = 6, *p < 0.05 vs. Control, **p < 0.05 vs. BzATP).

elevated IOP (**Figure 4B**). The mRNA for the *IL-3Rβ* isoform increased 9.6 ± 4.6 fold but this was not significant (NS; **Figure 4C**). Intravitreal injection of P2X7R antagonist BBG significantly reduced the rise in IL-3 expression, blocking the levels by 72.8 ± 7.8% (N = 5). BBG had no discernable effect on levels of IL-3Rα or IL-3Rβ.

DISCUSSION

This study demonstrates that moderate stretch triggers the release of multiple cytokines from isolated neurons. A parallel cytokine release is produced by exposing the cells to P2X7R agonist BzATP. The correlation between the release profiles triggered by stretch and P2X7R stimulation suggested that the cytokine release pathways were linked. IL-3 showed the largest proportional release by either stimuli, and the ability of two different P2X7R antagonists to inhibit the stretch-dependent IL-3 release strongly suggested the release required P2X7R stimulation. We have previously demonstrated that stretch initiates a release of ATP through pannexin hemichannels from these neurons capable of autostimulating their P2X7R; the ability of apyrase to prevent swelling activated currents through the P2X7R, to reduce the regulatory volume decrease accompanying swelling, and to block the rise in intracellular calcium that followed swelling confirmed the central role of released extracellular ATP in the stimulation of this P2X7 receptor (Xia et al., 2012). The stretch-dependent release of IL-3 is consistent with this release of ATP and autostimulation of the P2X7R (**Figure 5**).

This study identified several cytokines significantly released by both moderate stretch and BzATP including IL-3, TNF-alpha, CXCL9, VEGF, L-selectin, IL-4, GM-CSF, IL-10, IL1Ra, MIP and CCL20. Mechanistic evaluation was focused on IL-3 because it showed the most robust release with both stimuli. IL-3 is expressed in neuronal populations (Konishi et al., 1994) and its activity is mediated through cell surface receptors composed of a ligand-binding IL-3Rα subunit and transmembrane IL-3Rβ subunit (Appel et al., 1995). IL-3 exhibits predominantly neuroprotective actions; it protects cortical neurons from amyloid peptide-induced death (Zambrano et al., 2007) and protects neuroprogenitor and adult neurons cells (Luo et al., 2012). It promotes the survival of sensory neurons and triggers the formation of a neural net (Moroni and Rossi, 1995) and rescues hippocampal neurons from ischemic injury (Wen et al., 1998). Results from the present study suggest IL-3 can also protect RGCs from death. As the Jak/Stat pathway was previously shown to be activated following stimulation of the IL-3 receptor (Reddy et al., 2000) and is associated with increased protection of neurons in general, and of RGCs in particular (Peterson et al., 2000; Wang et al., 2015), involvement of this pathway is a reasonable hypothesis, although this awaits further confirmation.

The cytokine release measurements in this study were performed on isolated neurons from neonatal rats as they survive manipulation more robustly than adult cells. However, experiments from cells in adult rats exposed to mechanical stretch following a moderate elevation of IOP suggest IL-3 signaling may be relevant in adults too. The expression of IL-3 and IL-3Rα was largely restricted to the ganglion cell layer in adult retina. The rise in mRNA expression for both the cytokine *IL-3* and the receptor subunit *IL-3Rα* upon exposure to elevated pressure suggests the recruitment of the signaling system upon mechanical strain. The ability of P2X7R antagonist BBG to prevent upregulation of *IL-3* suggests P2X7R involvement in both the transcription and release of IL-3. Our preliminary results have identified a similar role for the P2X7 receptor in the upregulation IL-6 dependent on NKκB (Lu et al., 2013). Stimulation of the P2X7R leads to the nuclear translocation of NKκB in osteoclasts (Korcok et al., 2004),

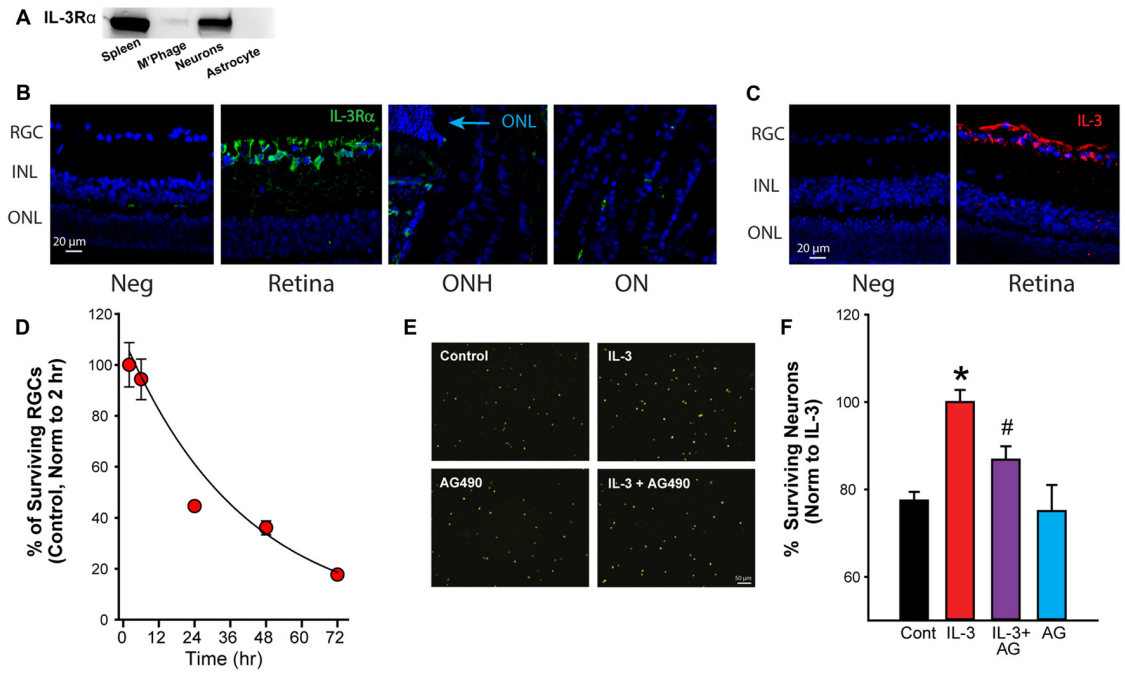

FIGURE 3 | Activation of the IL-3 receptor is neuroprotective. (A) Immunoblot showing presence of IL-3 receptor α (IL-3Rα) in immunopanned ganglion cells, but not astrocytes. Bands for IL-3Rα were also detected on material from spleen but not macrophages/microglia (M'Phage). **(B)** Staining for IL-3Rα (green) was found primarily in the RGC layer in adult retinas. Blue—4′,6-diamidino-2-phenyindole dilactate (DAPI). Neg- no primary antibody, RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; ONH, optic nerve head; ON, optic nerve. **(C)** Staining for cytokine IL-3 (red) was also found predominantly in the RGC layer of the retina. **(D)** Percentage of ganglion cells decreases with time in culture. Numbers normalized to levels 2 h after plating ($n = 3$). **(E)** Images of fluorescently labeled neurons (ex 360 nm). Neurons in mixed culture had been incubated with growth medium (Control), IL-3 (10 ng/ml), IL-3 + AG490 (20 μM) or AG490 alone for 24 h to determine the effects of IL-3 on neuronal survival. **(F)** Quantification of effect of IL-3 on cell survival. IL-3 increased the number of surviving RGCs (labeled with fluorescent marker fluorogold) after 24 h in culture ($^{*}p = 0.003$ IL-3 vs. control; $^{\#}p < 0.05$); the number of surviving cells decreases over this period in untreated conditions. The addition of JAK2 inhibitor AG490 reduced the neuroprotective effects of IL-3 ($p = 0.027$ IL-3 vs. AG490 + IL-3, $n = 5$–11, normalized to levels in IL-3).

while co-immunopreciptation indicates the C-terminal of the P2X7 receptor physically contacts MyD88 upstream of NKκB (Liu et al., 2011). The mechanisms linking P2X7R activation to priming of IL-3 in neurons remain to be determined, but these reports confirm the ability of the P2X7R to activate key components in the pathway.

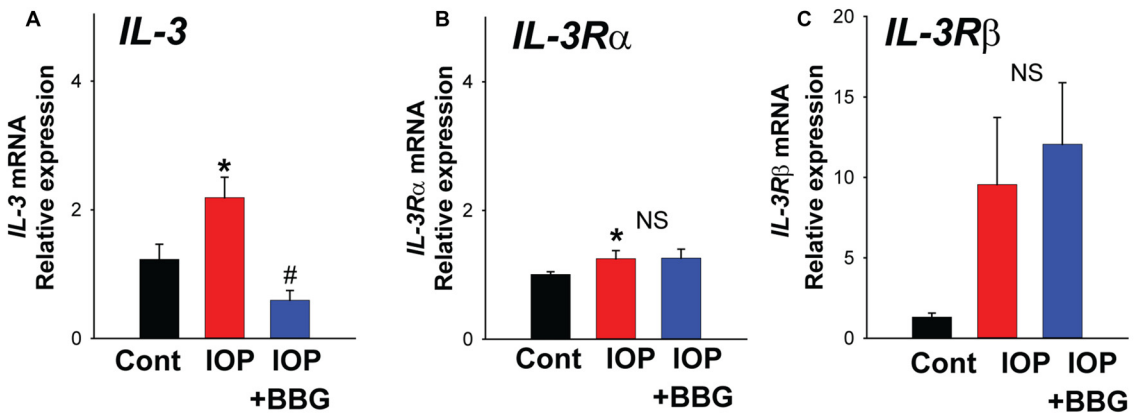

FIGURE 4 | Change in IL-3 expression *in vivo*. (A) Quantitative PCR (qPCR) for *IL-3* indicating increased expression in rat retina following non-ischemic elevation in intraocular pressure (IOP) to 50 mmHg for 4 h (IOP). Intravitreal injection of P2X7R antagonist BBG (0.8%) 5–7 days before the elevation of IOP (IOP + BBG) prevented this rise. $^{*}p = 0.010$ vs. normal IOP. $^{\#}p = 0.002$ IOP vs. IOP + BBG. **(B)** Expression of *IL-3Rα* receptor was increased following increased IOP ($p = 0.019$) but BBG did not change expression. **(C)** Levels of receptor *IL-3Rβ* mRNA rose but this was NS. $N = 5$ each for pressure vs. control and BBG + pressure vs. control.

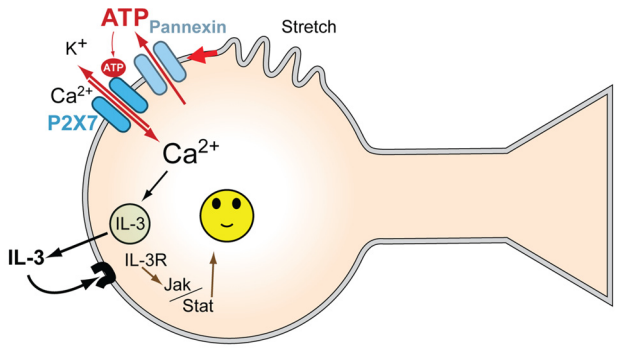

FIGURE 5 | Model for mechanosensitive P2X7/IL-3 signaling in neurons. Mechanical strain of the neurons leads to ATP release through pannexin hemichannels; this released ATP autostimulates P2X7Rs, leading to an influx of Ca^{2+}. P2X7R activation leads to the release of multiple cytokines, including IL-3, dependent upon this Ca^{2+} influx. The released IL-3 autostimulates IL-3Rα/IL-3Rβ receptors on the neurons, providing neuroprotection via JAK2 signaling pathways. Together, this provides a signaling pathway to protect neurons from moderate mechanical strain and stretch.

While the absolute levels of IL-3 released from the isolated ganglion cells into the bath are low, previous experience with ATP release suggests the levels sampled in the bath represent a 1000-fold dilution over that close to the cells. The mechanosensitive release of ATP from these neurons makes it critical to ensure the sampling probe does not touch the cells, necessitating the use of a considerable extracellular volume (Xia et al., 2012). Although the absolute concentration of ATP measured in the bath after cell stretch using this system was 10 nM, we demonstrated that the released ATP was capable of autostimulating the P2X7 receptor with measurements from patch clamp recordings, cytoplasmic calcium imaging and regulatory volume decrease analysis (Xia et al., 2012). Of note, the EC_{50} of ATP at the rat P2X7R is approximately 100 μM (Bianchi et al., 1999), i.e., four orders of magnitude greater than the levels of ATP measured in the bath. Direct measurement of released ATP levels on the cell surface with attached luciferase show a similar dilution (Beigi et al., 1999). It is thus not unreasonable to expect IL-3 detected in the bath at 10 pg/ml to correspond to 10 ng/ml close to the cell membrane. As this is the level of IL-3 capable of protecting the neurons, the concentrations required for autostimulation are reasonable. It is worth pointing out that the lack of detectable LDH was not affected by this bath dilution, as our studies have demonstrated cell lysis leads to a substantial and readily detectable LDH signal in the bath, while stretch or swelling does not (Beckel et al., 2014).

Physiological Implications

While the loss of RGCs is associated with sustained IOP elevation in glaucoma, the neurons can survive for a considerable time before their eventual death. In addition, certain conditions, such as advancing age, can make ganglion cells more susceptible to death when exposed to the same pressure rise in some models (Kim et al., 2004; Steinhart et al., 2014). Understanding the endogenous mechanisms that minimize the pathological effects of increased mechanical strain on the neurons is a key target in preventing their loss in disease. Although aberrant immune signaling has been implicated in the pathogenesis of glaucoma (Tezel and Wax, 2004; Grus et al., 2008; Wax and Tezel, 2009; Križaj et al., 2014), certain cytokines have also been recognized for their ability to protect ganglion cells (Sholl-Franco et al., 2001; Sappington et al., 2006). It is tempting to propose that the increased expression of mRNA for *IL-3* and *IL-3Rα* in eyes exposed to moderate IOP elevation may be part of an endogenous protective response, although this requires further confirmation.

Linking Mechanical Stain with Cytokine Release Through Pannexin/P2X7R Interactions

The potential neuroprotective actions of the P2X7R are at odds with its traditional identification as a "death receptor", but reflect a growing acknowledgment that expression of the receptor in stable adult neurons can actually be beneficial. Pannexin hemichannels frequently act as a conduit for the mechanosensitive release of ATP (Bao et al., 2004) and thus provide a mechanism to activate P2 signaling after swelling or stretch (Wicki-Stordeur and Swayne, 2014). Mechanical stimulation of the neurons in the present study activated ATP release through pannexins that autostimulates adjacent P2X7Rs. While pannexin-mediated stimulation of P2X7Rs can activate inflammatory pathways such as the NLRP3 inflammasome and lead to neuronal death (Gulbransen et al., 2012), the autostimulation of P2 receptors following pannexin activation can prevent apoptosis linked to mechanical deformation (Furlow et al., 2015). Clearly the consequences of pannexin/P2 signaling are context dependent and may differ in the varied purinergic signaling pathways throughout the retina and brain (Burnstock, 2013; Sanderson et al., 2014).

Finally, it should be emphasized that while the current study pertains most directly to the loss of RGCs following the rise in pressure accompanying glaucoma, the findings also have general relevance to the inflammatory responses in neural tissues after TBI. Of particular relevance may be the upregulation of genes linked to survival in astrocytes after increased cranial pressure included IL-3 (Vandevord et al., 2008). Whether this is part of a pannexin/P2X7 signaling complex, as it seems to be in the retina, remains to be determined.

AUTHOR CONTRIBUTIONS

JCL helped design the study, carried out most of the *in vitro* experiments, performed analysis of molecular and protein data and immunohistochemistry. WL performed experiments associated with the elevation of IOP and immunohistochemical staining on rats and analyzing associated data. JMB contributed to the experimental design and interpretation of data. CHM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors were involved in critical revisions of the work. All authors also

approve of the final version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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