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BRAINSTEM STRUCTURES RESPONSIBLE FOR PARADOXICAL SLEEP ONSET AND MAINTENANCE

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FOREWORDS

This paper is dedicated to our mentor, Michel Jouvet who inspired our career and transmitted to us his passion for the study of the mechanisms responsible for paradoxical sleep genesis and also that of its still mysterious functions. We expose in the following the progresses in the knowledge in this field brought during 40 years by Michel Jouvet and his team and more recently by the members of a new CNRS laboratory in which we aim to pursue in the path opened by Michel Jouvet.

THE DISCOVERY OF PARADOXICAL SLEEP

In 1959, Michel Jouvet and François Michel discovered in cats a sleep phase characterized by a complete disappearance of the muscle tone (2, 20, 41) paradoxically associated with a cortical activation and rapid eye movements (REM). In view of its singularity, they proposed to call this state paradoxical sleep (PS). It corresponds to the stage of sleep named REM sleep in 1953 by Aserinsky and Kleitman and shown to correlate in Humans with dream activity (2, 19). The discovery that a complete muscle atonia occur during this stage of sleep leaded Jouvet to propose that PS was a third state of vigilance independent of slow wave sleep and waking.

THE SEARCH OF THE "CENTER" OF PARADOXICAL SLEEP

During the forty years after the discovery of PS, Jouvet pursued with the researchers from his laboratory the study of PS. Supporting his theory of a duality of the sleep stages, he showed that PS is present in mammals and birds but absent in amphibians and reptiles in contrast to slow wave sleep. He also demonstrated that the stage of PS is initiated and maintained by structures different from those regulating SWS and W. He first showed that PS persists following decortication, cere-

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bellar ablation or brain stem transections rostral to the pons. In contrast, transection at the posterior limit of the pons suppressed PS (44). He also demonstrated that a state resembling PS is still visible in the"pontine cat", a preparation in which all the structures rostral to the pons have been removed (44). These results indicated that brainstem structures are necessary and sufficient to trigger and maintain the state of PS, a concept still valid today. Jouvet and others than showed that electrolytic and chemical lesions of the dorsal part of the pontis oralis (PnO) and caudalis (PnC) nuclei specifically suppress PS (9, 43, 45, 83, 95) indicating that these nuclei contain the neurons responsible for PS onset and maintenance.

THE RECIPROCAL ROLE OF THE MONOAMINERGIC PS-OFF AND CHOLINERGIC PS-ON NEURONS

In the sixties and seventies, following the introduction of histochemical methods to localize the cholinergic and monoaminergic neurons, that of drugs specifically increasing or decreasing the action of their neurotransmitters and the development of electrophysiological methods allowing the recordings of the single unit activity of neurons, Jouvet and his colleagues in parallel with several teams in the world reached the conclusion that the onset of PS is due to a reciprocal interaction between monoaminergic and cholinergic neurons (46, 48). Jouvet and Michel (42) were the first to demonstrate that cholinergic mechanisms play a major role in PS generation since peripheral atropine administration suppressed PS, whereas anticholinesterase compounds increase PS. Then, George *et al.* (27) discovered that bilateral injections of carbachol, a cholinergic agonist, into the PnO and PnC promote PS. It was later shown that PS is induced with the shortest latency when carbachol is ejected in a small area of the dorsal PnO and PnC (4, 26, 54, 92, 99), named peri-locus coeruleus α (peri-LCα) by Sakai *et al.* (76, 77). Sakai and coworkers from Jouvet's laboratory (77-79, 81) found that the great majority of the pontine neurons with a tonic activity specific to PS (PS-on neurons) were localized in the peri-LCα. They recently divided these neurons in two populations (79): the first population of neurons are: 1) located in the dorsal and rostral peri-LCα, 2) inhibited by carbachol, a cholinergic agonist and project rostrally to the intralaminar thalamic nuclei of the thalamus, the posterior hypothalamus and the basal forebrain; the second population of PS-on neurons are: 1) excited by carbachol, 2) distributed in all parts of the peri-LCα and 3) project caudally to the nucleus reticularis magnocellularis (Mc) localized in the ventromedial bulbar reticular formation (76, 77). Based on these and other results, it has been proposed that the first type of neurons are cholinergic and responsible for the cortical activation during PS, whereas the second type of neurons are glutamatergic and generate the muscle atonia observed during this sleep state via descending excitatory projection to glycinergic pre-motoneurons within the Mc (10, 24, 25, 39, 59, 79, 81). Supporting this hypothesis, the great majority of the neurons in the peri-LCα projecting to the Mc are not cholinergic (59), glutamate release in the Mc increases specifically during PS (51) and injection of non-NMDA glutamate ago-

nists in the Mc suppresses muscle tone (55). In addition, spinal-projecting PS-on neurons have been recorded in the Mc (75, 87) and cytotoxic lesion of this structure induced a decrease in PS quantities and an increase in muscle tone during PS (35). Further, intracellular recordings of motoneurons combined with strychnine applications demonstrated that glycine is responsible for the tonic hyperpolarization of the spinal, hypoglossal and trigeminal motoneurons (10, 52, 88, 101) and we have shown that the Mc contains a large contingent of glycinergic neurons (24, 25, 73). These glycinergic neurons directly project to spinal motoneurons (36) while those of the parvocellular and parvocellular alpha nuclei directly project to the trigeminal motor nucleus (57, 74). In addition, we recently showed that glycinergic neurons from these nuclei express Fos after the induction of PS (6). Moreover, following induction of PS by carbachol injections in the peri-LCα, Fos-labeled cells in the Mc have been shown to project to the trigeminal motor nucleus (67).

On the other hand, a number of results indicated that the onset of PS was due to a reciprocal inhibitory interaction between the PS-on neurons and monoaminergic PS-off neurons. Hobson and McCarley were the first to draw in detail this hypothesis in the mid seventies (34, 65). They were followed by Sakai who proposed a slightly revised model (77). This well-accepted hypothesis was formulated following the findings that serotonergic neurons from the raphe nuclei and noradrenergic neurons from the locus coeruleus cease firing specifically during PS i.e have a mirror activity to PS-on neurons (1, 3, 34, 66). Supporting this theory, drugs enhancing serotonin and noradrenergic transmission in particular monoamine oxidase inhibitors and serotonin and norepinephrine reuptake blockers specifically suppress PS (30, 39, 46). However, the site(s) where the monoamines in particular serotonin exert their PS-suppressing effect remain (s) to be unambiguously identified. Indeed, applications of norepinephrine, epinephrine or benoxathian (an α2 agonist) into the peri-LCα inhibit PS but that of serotonin has no effect (11, 12, 91). In addition, norepinephrine via α2-adrenoceptor inhibits the non-cholinergic PS-on neurons but has no effect on the cholinergic PS-on neurons from the peri-LCα while serotonin has no effect on both types of neurons (79). Monoamines could also act on PS-on neurons localized in other structures than the peri-LCα like the Mc (59) or the pedunculopontine tegmental (PPT) and laterodorsal tegmental cholinergic nuclei (LDT) (37). The PPT and LDT have been indeed reported to contain PS-on neurons although the great majority of the neurons from these nuclei are tonically active both during waking (W) and PS (14-16, 50).

In conclusion, a large number of evidence supports the hypothesis that the onset and maintenance of PS is due to reciprocal inhibitory interactions between PS-on cholinergic neurons and PS-off monoaminergic neurons. However, we recently obtained results in rats indicating that GABAergic and glutamatergic neurons might be more important players than cholinergic and monoaminergic neurons. These results were obtained with a new model combining single unit recordings, precise and limited local pharmacology by micro-iontophoresis in unanesthetized head-restrained rats and anterograde and retrograde tracing combined with Fos and neurochemical identification of labeled-cells (6, 7, 13, 28, 29). In the following, we

detail these results and propose a new theory on the neuronal network responsible for PS.

EVIDENCE THAT GABAERGIC PS-OFF NEURONS PLAY A MAJOR ROLE

We recently found that a long-lasting PS-like hypersomnia can be pharmacologically induced with a short latency in the head-restrained rats by iontophoretic applications of bicuculline or gabazine, two $GABA_A$ antagonists specifically into a very small area of the dorso-lateral pontine tegmentum (6). We also recorded neurons in this region specifically active during PS and excited by bicuculline or gabazine iontophoresis (5). This region has been denominated the sublaterodorsal nucleus (SLD) by Swanson (89). It approximately corresponds to the dorsal subcoeruleus nucleus in Paxinos and Watson atlas (71) and seems to be the equivalent in rats of the cat peri-LCα. Our results have been recently reproduced in freely moving rats (72, 82) and are in agreement with a recent study in cats showing that pressure injection of bicuculline and to a lesser extent phaclofen (a $GABA_B$ antagonist) in the dorsal portion of the nucleus pontis oralis (which roughly corresponds to the peri-LCα) induces a strong increase in PS quantities with short latencies, whereas the application of muscimol (a $GABA_A$ agonist) or baclofen (a $GABA_B$ agonist) induced W (97, 98). These and our data imply that the onset of PS-on neurons of the SLD is mainly due to the removal of a tonic GABAergic input present during W and SWS. Combining retrograde tracing with cholera toxin B subunit (CTb) and GAD immunostaining, we recently tried to identify the GABAergic neurons at the origin of this input (7). Our results suggest that the GABAergic innervation of SLD neurons arises both from interneurons and distant neurons located in the pontine and deep mesencephalic reticular nuclei and to a minor extent hypothalamic and medullary structures (7). These results are in agreement with previous studies indicating that the GABAergic neurons responsible for the tonic inhibition during W and SWS of the PS-on neurons from the SLD could be within the SLD itself and/or in the pontine and deep mesencephalic reticular nuclei. A recent study by Xi *et al.* (97) indeed suggested that GABAergic interneurons might be the best candidates for the inhibition of PS-on SLD neurons. They found in cats that administration of antisense oligonucleotides against glutamic acid decarboxylase (GAD) mRNA in the nucleus pontis oralis (NPO), a region corresponding to the peri-LCα, produces a significant decrease in W and an increase in PS. On the other hand, Maloney *et al.* (63) found in rats that the number of Fos expressing GABAergic neurons in the rostral pontine reticular nucleus decreased following PS rebound, suggesting that GABAergic neurons from this structure are active during W and SWS and inactive during PS. Finally, it has been shown in cats (84, 85) and rats (5) that muscimol injections in the most ventrolateral part of the periaqueductal gray and in the region of the deep mesencephalic reticular nucleus just ventral to it induce a strong increase in PS quantities. More recently, Sakai *et al.* (81) reported that muscimol applications limited to the region of the deep mesencephalic reticular nucleus just ventral to the peri-

aqueductal gray induced an increase in PS quantities while those in the ventrolateral periaqueductal gray had no effect. We reported a strong non-GABAergic projection to the SLD from the ventrolateral periaqueductal gray and a mixed GABAergic and non-GABAergic projection from the region of the deep mesencephalic reticular nucleus just ventral to the periaqueductal gray (7). Altogether, we propose that GABAergic neurons located in the most dorsal part of the deep mesencephalic reticular nucleus, the pontine reticular nucleus and in the SLD itself project to and directly inhibit the PS-on neurons from the SLD specifically during W and SWS.

EVIDENCE THAT GLUTAMATERGIC NEURONS TONICALLY EXCITE PS-ON NEURONS OF THE SLD DURING ALL VIGILANCE STATES

We recently showed that kainic acid (a glutamate agonist) iontophoretic application into the SLD induces an activation of PS-on neurons and is consistently associated with a transient PS-like state followed by W and an increase in muscle activity (6). Further, the PS-like state induced by bicuculline iontophoresis in the SLD was reversed by the application of kynurenate (6). In agreement with our results, it has been shown in cats that the administration of kainic acid in the peri-LCα using microdialysis induces a PS-like state (70). Altogether these results suggest that PS-on neurons in the SLD receive a tonic glutamatergic input during all sleep-waking states. They further suggest that following the removal of the tonic GABAergic input at the onset of PS, the unmasked glutamatergic input would be responsible of the tonic activity of the SLD PS-on neurons during PS. The glutamatergic neurons providing a constant excitatory input to SLD PS-on neurons should be located in the brainstem although forebrain glutamatergic neurons could also participate. Indeed, a PS-like state persists in the "pontine cat" indicating that the structures responsible for the onset and maintenance of PS are restricted to the brainstem (44). Such glutamatergic inputs can arise from the numerous non-GABAergic neurons projecting to the SLD localized in the ventrolateral periaqueductal gray, the mesencephalic, pontine and parvocellular reticular nuclei. Additional studies are necessary to determine which one of these structures provides a glutamatergic input to the SLD PS-on neurons.

The afferents to the SLD from the primary motor area of the frontal cortex, the bed nucleus of the stria terminalis and central nucleus of the amygdala could also participate in the activation of the SLD PS-on neurons. Indeed, descending pyramidal cortical cells are known to be glutamatergic. In addition, Maquet *et al.* (64) found that regional cerebral blood flow is positively correlated with PS in the amygdaloid complex. Furthermore, electrical stimulation of the central nucleus of the amygdala increases the frequency of pontine waves recorded in or just dorsal to the SLD during PS (18). From these and our results, it might be hypothesized that the frontal cortex and the central nucleus of the amygdala and the functionally related bed nucleus of the stria terminalis provide excitatory glutamatergic projections to PS-on neurons from the SLD.

EVIDENCE THAT ACETYLCHOLINE DOES NOT PLAY A CRUCIAL ROLE IN THE ACTIVATION OF THE PS-ON NEURONS OF THE SLD

We found that carbachol iontophoresis into the rat SLD induced a W state with increased muscle activity and that SLD PS-on neurons do not respond to carbachol iontophoresis (36). These results indicate important species differences between rats and cats in the pharmacological sensitivity of the pontine PS-on neurons. In agreement with our results, following carbachol administration into the rat pontine reticular formation, the enhancement of PS was of small magnitude (8, 31, 86, 93) or not reliably obtained (21). In cats, however, PS is induced almost immediately after the carbachol injection and the episodes last longer than in control PS. The effective sites in rats were widely distributed in the pontine reticular formation. In contrast, the most effective site in cats is the peri-LCα that corresponds to the rat SLD (92). The absence of effect of carbachol ejection in the SLD does not rule out a role of cholinergic processes in PS onset and maintenance in the rat. It is indeed possible that PS-on neurons in the SLD have muscarinic and/or nicotinic receptors, but that the activation of these receptors by carbachol is unable to modify their activity due to the strong GABAergic tonic inhibition revealed in our study. Supporting this hypothesis, it has been shown that carbachol applications in the region of the SLD are able to induce with a short latency a long period of atonia in anesthetized or decerebrate rats models (23, 90) in which the GABAergic inhibitory tone on SLD neurons could be decreased or even absent. Another possibility is that the cholinergic system plays an important role in PS in rats via an action on populations of neurons controlling PS localized in other pontine regions than the SLD. Supporting this idea, an increase in the number of PPT and LDT cholinergic neurons containing Fos has been observed following PS recovery (62). Further, a strong enhancement in PS quantities was found following carbachol pressure ejection in the most ventral part of the oral pontine reticular formation (17, 26).

EVIDENCE THAT GABAERGIC PS-ON NEURONS ARE RESPONSIBLE FOR THE INACTIVATION OF MONOAMINERGIC NEURONS DURING PS

According to the classical "reciprocal interaction" model (65, 77), the cessation of firing of the noradrenergic and serotonergic neurons at the onset of PS is the result of active PS-specific inhibitory processes originating from PS-on cells. These neurons were first hypothesized to be cholinergic and localized in the peri-LCα, LDT and PPT. However, acetylcholine excites LC noradrenergic neurons and is only weakly inhibitory on serotonergic DRN neurons (33, 53). It has therefore been suggested that they might use GABA or glycine, rather than acetylcholine, as an inhibitory neurotransmitter (40, 60).To test this hypothesis we determined the effect of iontophoretic applications of bicuculline and gabazine (two $GABA_A$ antagonists)

and strychnine (a glycine antagonist) during W, SWS, and PS on the activity of LC noradrenergic and DRN serotonergic cells in the head-restrained unanesthetized rat (13, 28, 29).

Iontophoretic application of bicuculline, gabazine or strychnine during SWS or PS induced a tonic firing in LC noradrenergic and DRN serotonergic neurons (13, 28, 29). In addition, application of these antagonists during W induced a sustained increase in discharge rate. These results indicate the existence of tonic GABA and glycinergic inputs to the LC and DRN that are active during all vigilance states. Importantly, we found that when the strychnine effect occurred during transitions between PS and W, the discharge rate of the LC or DRN neurons further increased at the onset of W. In contrast, in the same situation but after bicuculline administration, the discharge rate of a given neuron was unchanged at the transition between PS and W. These results strongly suggest that the release of GABA but not that of glycine is responsible for the inactivation of LC noradrenergic neurons and DRN serotonergic during PS. At variance with our results, Levine and Jacobs (56) found in cats that the iontophoretic application of bicuculline reversed the typical suppression of neuronal activity of DRN serotonergic neurons during SWS but not during PS. In addition, Sakai and Crochet (80) did not find in cats an effect of bicuculline microdialysis infusion on DRN serotonergic neurons during PS and hypothesized that our results were due to a non-specific excitatory action of bicuculline. This is unlikely since we reproduced the effect of bicuculline with gabazine, another specific $GABA_A$ antagonist (unpublished results). Further, our results are supported by those of Nitz and Siegel (68, 69) who found in cats with the microdialysis technique a significant increase in GABA release in the DRN and LC during PS as compared to W and SWS and, in contrast, no detectable changes in glycine concentrations. Based on these and our results, we therefore suggest that during W, the LC and DRN cells are under a tonic GABAergic inhibition which increases during SWS and even further during PS, and that the increase in GABAergic inhibition is responsible for the inactivation of these neurons during the sleep states. In contrast, the glycinergic tonic inhibition would be constant across the sleep-waking cycle and, thus, control the general excitability of LC and DRN neurons.

Our results obtained with double-staining experiments indicate that the LC and DRN receive GABAergic inputs from neurons located in a large number of distant regions from the forebrain to the medulla (29, 61). Indeed, we observed a substantial number of GAD-immunoreactive neurons in the preoptic area, the lateral hypothalamic area, the mesencephalic and pontine periaqueductal gray and the dorsal paragigantocellular reticular nucleus that project to the LC and DRN (29, 61). Based on physiological and electrophysiological data (see above), we expect that one or several of these GABAergic afferents are "turned on" specifically at the onset of and during PS episodes and are responsible for the inhibition of brainstem monoaminergic neurons during PS. Although it has recently been proposed that GABAergic neurons located in the extended ventrolateral preoptic nucleus might also be involved (58), previous results highly suggest that brainstem GABAergic neurons are mostly involved. Indeed, it is well known that PS-like episodes occur in pontine or decere-

brate cats (47). Moreover, it has been shown in decerebrate animals that PS episodes induced by carbachol injections in the pons are still associated with a cessation of activity of serotonergic neurons of the raphe obscurus and pallidus nuclei (96). Among the brainstem GABAergic afferents revealed in our study, several are common to the DRN and the LC and are therefore good candidates for this role. We observed substantial GABAergic projections to the LC and DRN from the ventrolateral periaqueductal gray and the dorsal paragigantocellular nucleus (29, 61). In agreement with these results, local application of bicuculline blocked the dorsal paragigantocellular-evoked inhibition of LC neurons (22) and focal iontophoretic application of NMDA in the ventral periaqueductal gray induced bicuculline sensitive IPSPs in DRN serotonergic neurons (38). The hypothesis that the GABAergic inhibition is coming from neurons located in the periaqueductal gray is further supported by two recent studies. Yamuy *et al.* (100) showed that after a long period of PS induced by pontine injection of carbachol, a large number of Fos positive cells are visible in the DRN and a region lateral to it. Moreover, Maloney *et al.* (62) observed after a PS rebound induced by deprivation, an increase in Fos-positive GAD immunoreactive neurons in the periaqueductal gray. To directly determine among the GABAergic afferents to the LC, those active during PS, we recently combined iontophoretic application of CTb in the LC with Fos staining in rats deprived of PS, rats with enhanced PS during rebound after PS deprivation, and control rats. Using this method, we observed a large number of CTb and Fos double-immunostained neurons in the dorsal paragigantocellular reticular nucleus and a substantial number in the ventro-lateral periaqueductal gray and the lateral paragigantocellular reticular nucleus specifically after PS rebound (94). From these results, we propose that the GABAergic neurons responsible for the inhibition of the LC noradrenergic neurons during PS are mainly but not exclusively localized in the dorsal paragigantocellular reticular nucleus. To further test this hypothesis, we recorded the spontaneous activity of neurons from the dorsal paragigantocellular reticular nucleus across the sleep-waking cycle in head-restrained rats. Neurons with an activity specific to PS (PS-on neurons) were found within this nucleus (32), further supporting that it contains the GABAergic neurons responsible for the cessation of activity of the noradrenergic neurons of the LC during PS. This hypothesis is also supported by a recent study showing that electrical stimulation of the area of the dorsal paragigantocellular reticular nucleus induces an increase in PS quantities (49).

CONCLUSION: A NEW NETWORK MODEL FOR PS ONSET AND MAINTENANCE (Fig. 1)

In conclusion, based on our results, we propose that the onset and maintenance of PS is due to the activation of PS-on glutamatergic neurons from the SLD. During W and SWS, they would be hyperpolarized by tonic GABAergic inputs arising from GABAergic PS-off neurons localized in the SLD itself and the deep mesencephalic and pontine reticular nuclei. Noradrenergic and serotonergic PS-off neurons would

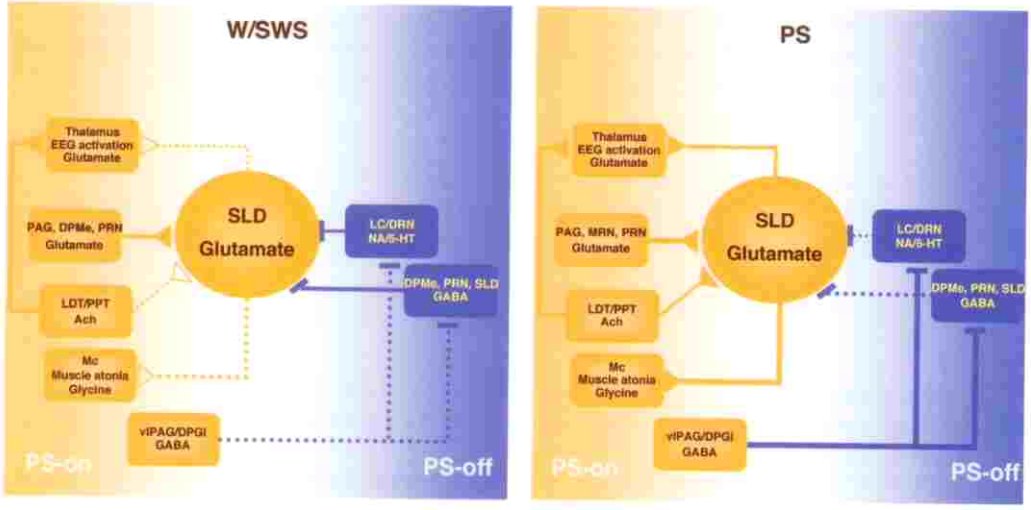

Fig. 1. *Model of the network responsible for PS onset and maintenance.*

The onset and maintenance of PS would result from the activation of PS-on glutamatergic neurons from the SLD. The activation of these neurons would be due to the removal of tonic inhibitions arising from the monoaminergic PS-off neurons and GABAergic PS-off neurons localized in the SLD itself and the deep mesencephalic and pontine reticular nuclei. The cessation of activity of the PS-off neurons would be due to a tonic inhibition issued from GABAergic PS-on neurons localized in the dorsal paragigantocellular reticular nucleus and the ventrolateral periaqueductal gray. Abbreviations: DRN, dorsal raphe nucleus; 5-HT, serotonin; LC, locus coeruleus; NA, norepinephrine; LDT, laterodorsal tegmental nucleus; Ach, acetylcholine; Mc, magnocellular reticular nucleus; Gly; glycine; DPMe, deep mesencephalic reticular nucleus; PAG, periaqueductal gray; DPGi, dorsal paragigantocellular reticular nucleus; PPT, pedunculopontine nucleus; PRN, pontine reticular nucleus; SLD, sublaterodorsal nucleus; Glu, glutamate; Pef/HLA perifornical/lateral hypothalamic area; Hcrt, hypocretin (orexin).

also participate in the hyperpolarization of SLD neurons particularly during W. The cessation of activity of the monoaminergic neurons at the onset of and during PS would be due to an active inhibition by PS-on GABAergic neurons localized in the dorsal paragigantocellular reticular nucleus and the ventrolateral periaqueductal gray. Although the exact mechanism of the cessation of activity of the GABAergic PS-off neurons remains to be identified, we propose that the GABAergic PS-on neurons inhibiting the monoaminergic neurons could, at the same time, inhibit the GABAergic PS-off neurons.

The activation of the SLD PS-on neurons at the onset of PS would be due to the strong glutamatergic excitatory input present during all vigilance states blocked during W and SWS by the inhibitory inputs from the GABAergic and monoaminergic PS-off neurons. It would arise from one or several of the non-GABAergic brainstem afferents to the SLD (e.g. the periaqueductal gray, the deep mesencephalic and pontine reticular nuclei and the parvocellular reticular nucleus).

Ascending SLD PS-on glutamatergic neurons would induce cortical activation via their projections to intralaminar thalamic relay neurons in collaboration with W/PS-on cholinergic and glutamatergic neurons from the LDT and PPT, mesencephalic

and pontine reticular nuclei and the basal forebrain. Descending PS-on glutamatergic SLD neurons would induce muscle atonia via their excitatory projections to glycinergic premotoneurons localized in the magnocellular and parvocellular reticular nuclei.

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