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Synaptic transmission of baro- and chemoreceptors afferents in the NTS second order neurons

Daniela Accorsi-Mendonça, Benedito H. Machado *

Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, 14049-900, Ribeirão Preto, SP, Brazil

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ABSTRACT

Second order neurons in the *nucleus tractus solitarius* (NTS) process and integrate the afferent information from arterial baroreceptors with high fidelity and precise timing synaptic transmission. Since 2nd-order NTS neurons receiving baroreceptors inputs are relatively well characterized, their electrophysiological profile has been accepted as a general characteristic for all 2nd-order NTS neurons involved with the processing of different sensorial inputs. On the other hand, the synaptic properties of other afferent systems in NTS, such as the peripheral chemoreceptors, are not yet well understood. In this context, in previous studies we demonstrated that in response to repetitive afferents stimulation, the chemoreceptors 2nd-order NTS neurons also presented high fidelity of synaptic transmission, but with a large variability in the latency of evoked responses. This finding is different in relation to the precise timing transmission for baroreceptor 2nd-order NTS neurons, which was accepted as a general characteristic profile for all 2nd order neurons in the NTS. In this brief review we discuss this new concept as an index of complexity of the sensorial inputs to NTS with focus on the synaptic processing of baro- and chemoreceptor afferents.

1. Baroreceptor afferent transmission in the 2nd-order NTS neurons

The arterial baroreceptors play a key role on a beat-to-beat adjustments of the autonomic function to the cardiovascular system in order to keep the arterial blood pressure in a narrow range of variation and several studies explored the neurochemical mechanisms involved with the neurotransmission of baroreceptors afferents in the NTS (Talman et al., 1980; Machado et al., 2000; Machado, 2001; Pilowsky and Goodchild, 2002). Afferents of the aortic baroreceptor, via aortic depressor nerves (ADN), reach the central nervous system (CNS) and the terminals of its fibers in *tractus solitarius* (TS) establish synaptic contact with 2nd-order neurons located in intermediate and caudal *nucleus tractus solitarius* [NTS (Contreras et al., 1982; Mendelowitz et al., 1992)]. A series of studies explored the putative neurotransmitter involved in the synaptic transmission of baroreceptors afferents at the NTS level. The presence of glutamate and vesicular glutamate transporters (VGLUTs) was demonstrated in ADN afferent terminals in the NTS (Schaffar et al., 1990; Lin and Talman, 2006), as well as receptors for glutamate in the NTS (Chan et al., 1998; Lacassagne and Kessler, 2000). There is also evidence that removal of nodose ganglion, where the cell bodies of ADN neurons are located, reduces glutamate release in the NTS (Meeley et al., 1989) and that baroreceptor 2nd-order NTS neurons were excited by local microinjections of glutamate (Zhang and Mifflin, 1997).

The involvement of L-glutamate as a putative excitatory neurotransmitter released by the afferents of baroreceptors in the NTS was originally described by Talman et al. (1980) in experiments performed in anesthetized rats. In studies performed in both anesthetized and awake rats, we documented that different anesthetics affect the neurotransmission at the NTS level (Machado and Bonagamba, 1992). Experiments using brainstem slices also showed that NTS neurons and the modulation of synaptic transmission are strongly affected by urethane, one of the most common anesthetic used in cardiovascular experiments (Accorsi-Mendonça et al., 2007).

In a series of experiments performed in awake rats to study the glutamatergic neurotransmission of the baroreflex in the NTS, we verified that bilateral microinjections of non-selective (kynurenic acid) or selective (NMDA, AP-5) glutamate ionotropic receptors antagonists into NTS were effective in blocking the bradycardia in response to baroreflex activation or to microinjections of L-glutamate or a selective agonist (NMDA) into the NTS, but not the sympathoinhibitory component of the responses (Colombari et al., 1994, 1997; Canesin et al., 2000; Frigero et al., 2000; Machado et al., 2000; Machado, 2001; Antunes and Machado, 2003; Takaoka and Machado, 2003; Almado and Machado, 2005). Therefore, these functional studies evaluating the neurotransmission of baroreceptors afferents in the NTS of unanesthetized rats indicate how complex the processing of the baroreceptors afferents at the NTS level under physiological conditions and *in vitro* studies is without anesthetics, such electrophysiological studies using brainstem slices are useful to provide a better understanding about the synaptic

* Corresponding author at: Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900, 14049-900, Ribeirão Preto, SP, Brazil. Tel.: +55 16 3602 3015; fax: +55 16 3602 0221.
E-mail address: bhmachad@fmrp.usp.br (B.H. Machado).

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transmission of afferents inputs at the NTS level. For these reasons, we are using whole cell patch clamp technique to analyze the synaptic transmission of NTS neurons in brainstem slices.

The glutamate, released by the terminals of TS afferent fibers, evokes post-synaptic excitatory current (TS-eEPSC) in 2nd-order NTS neurons, which was abolished by glutamate ionotropic receptor antagonist, demonstrating that the synaptic transmission between TS fibers and 2nd-order NTS neurons involves the stimulation of post-synaptic non-NMDA and NMDA receptors (Andresen and Yang, 1990; Aylwin et al., 1997; Kato and Shigetomi, 2001; Bonham and Chen, 2002; Accorsi-Mendonça et al., 2009). The synaptic neurotransmission of baroreceptors afferents in the NTS also involves the stimulation of metabotropic glutamate receptors in NTS, since repetitive TS stimuli activates pre-synaptic Groups II and III metabotropic glutamate receptors, which decrease the evoked glutamate release, producing a negative feedback of excitatory activity (Chen et al., 2002). In contrast, the Group I metabotropic glutamate receptors, located in post-synaptic neurons, are stimulated after glutamate release, inducing depolarization and increasing the number of action potentials (Sekizawa and Bonham, 2006). In accordance with studies by Chen et al. (2002) and Sekizawa and Bonham (2006) the activation of metabotropic glutamate receptors produces an autoregulatory modulation of glutamatergic transmission during baroreflex activation.

The time between the action potential of pre-synaptic terminal and the evoked response in post–synaptic neuron (timing transmission) is considered one of the most critical features of signal processing and integration in neural communication because neurons transmit information not only by their firing rate but also by the temporal organization of their discharge (Ferster and Spruston, 1995). In relation to the time of synaptic transmission of 2nd-order NTS neurons, Miles (1986) proposed a correlation between timing process and complex neuronal network in the NTS. The latency of TS-eEPSCs, corresponding to the time between stimulus and the appearance of synaptic response, was used by Miles (1986) to classify NTS neurons receiving mono- or polysynaptic inputs from TS in brainstem slice of guinea pigs. In that study, Miles (1986) arbitrarily assumed that NTS neurons receiving direct input from TS presented TS-eEPSCs with low variation in latency (<0.5 ms) after repetitive stimuli of TS. As a consequence of this arbitrary criterion determined by Miles (1986), other studies evaluating the NTS of rats also assumed that TS-eEPSCs with short latencies and low variation was enough to complete characterization of all 2nd-order NTS neurons (Rogers et al., 1993; Andresen and Yang, 1995; Chen et al., 1999; Kato and Shigetomi, 2001).

The use of lipophilic tracer to identify the afferents terminals in the NTS allowed the precise identification of 2nd-order NTS neurons. By applying fluorescent tracer around the surface of ADN, it was possible to visualize the NTS neurons receiving baroreceptor labeled afferent fibers in brainstem slices (Mendelowitz et al., 1992). In this scenario, it is important to note that Brophy et al. (1999) documented the presence of some ADN fibers sensitive to pO_2 level but not sensitive to increase in the blood pressure. However, other studies suggested that ADN of rats does not contain a functionally significant number of chemoreceptor afferent fibers and consequently these ADN chemosensitive afferent fibers cannot generate a chemoreflex-like response (Kobayashi et al., 1999; Barros et al., 2002). Therefore, in all our experiments we assumed that NTS 2nd order neurons receiving labeled ADN fibers represent inputs from peripheral baroreceptors.

Using the combination of electrophysiology and fluorescent tracing techniques, Doyle and Andresen (2001) demonstrated that anatomically labeled baroreceptor 2nd-order NTS neurons presented short-latency for TS-eEPSCs with small variance [standard deviation of latency (SD of latency)], indicating that the timing precision of baroreceptor afferents transmission. In that study by Doyle and Andresen (2001) it was suggested that SD of latency is the most reliable synaptic parameter to indicate monosynaptic contacts and arbitrarily the authors determined that the SD of latency should be lower than 0.1 ms to identify 2nd-order NTS neurons. However, the value of SD of latency to identify the monosynaptic connection in the NTS was also expanded from 0.1 to 0.2 ms in subsequent studies related to baroreceptor 2nd-order NTS neurons (Doyle et al., 2002; Bailey et al., 2006).

Recently, using the same criterion described by Doyle and Andresen (2001) we also verified that anatomically labeled baroreceptor 2nd-order NTS neurons presented a low SD of latency (<0.2 ms) under different experimental conditions [Accorsi-Mendonça et al., 2011 (see Fig. 1)]. This is an important issue because it validates our experimental approach to properly evaluate the temporal parameter of ADN-NTS 2nd order NTS neurons as previously described by Doyle and Andresen (2001). Therefore, afferents from arterial baroreceptors can release glutamate in the synaptic cleft to excite 2nd-order NTS neurons in a temporally precise pattern, as revealed by their low SD of latency of TS-eEPSCs. This precise temporal profile of synaptic currents is probably related to the transmission of afferent information, since the action potentials recorded in baroreceptors 2nd-order NTS neurons in response to ADN stimulation also presented a low variability of latency (Scheuer et al., 1996).

The activation of aortic baroreceptors, during each systole, triggers synchronized action potentials in the aortic depressor nerve, which are also synchronized with evoked currents in baroreceptor 2nd-order NTS neurons (see Fig. 2). Hence, the successive evoked glutamate release with low variability of latency, by TS fibers during each systolic activation of arterial baroreceptors, determine a direct relationship between pulsatile arterial pressure, ADN afferent inputs, synaptic transmission in the NTS and reflex modulation of sympathetic and parasympathetic outflows to the cardiovascular system. This functional organization remarkably provides a uniform and direct high-fidelity afferent transmission during baroreceptor afferent activation, which is consistent with a system that in rats works in a very efficient manner in order to keep the arterial blood pressure within a quite narrow range of variation.

2. Chemoreceptor afferent transmission in the 2nd-order NTS neuron

Although the low SD of latency of TS-eEPSCs has been demonstrated as a temporal profile of baroreceptor 2nd-order NTS neurons, this synaptic characteristic may not be the same for other NTS neurons receiving inputs from a different peripheral sensorial system. In spite of this possibility, several previous studies about NTS neurons and neurotransmission of autonomic reflexes suggested that other afferent inputs, such as the peripheral chemoreceptors afferents, also presented a low SD of latency in NTS 2nd-order neurons, similar to baroreceptors afferents (Kline et al., 2007, 2010; Zhang and Mifflin, 2007; Andresen and Peters, 2008).

Since our laboratory is deeply involved with the neurotransmission of peripheral chemoreceptor afferents in the NTS, in a series of previous experiments, we carefully characterized the temporal profile of chemoreceptor 2nd-order NTS neurons in order to understand the synaptic processing of this complex neuronal network under normal and mainly under hypoxic conditions, when its activation is essential for the animal's survival.

The glomus cells, located in the carotid body (CB), are the most important pO_2 sensitive group of cells and are in close contact with peripheral chemoreceptors afferents in the carotid sinus nerve (McDonald, 1983). However, other structures composed by cells morphologically similar to glomus cells named *paraganglia* have been described in head, neck, thorax and abdomen but not in the carotid bifurcation of rodents (McDonald and Blewett, 1981; Powley et al., 1983; Domeij et al., 1987; Dahlqvist et al., 1991; Berthoud et al., 1995). In our experiments we are convinced that the recorded chemoreceptor 2nd-order NTS neurons received only the afferent fibers from CB but not from *paraganglia* because we applied fluorescent tracer (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) around carotid body (CB), a region where the *paraganglia* cells are not present. Few days later, using brainstem slices and

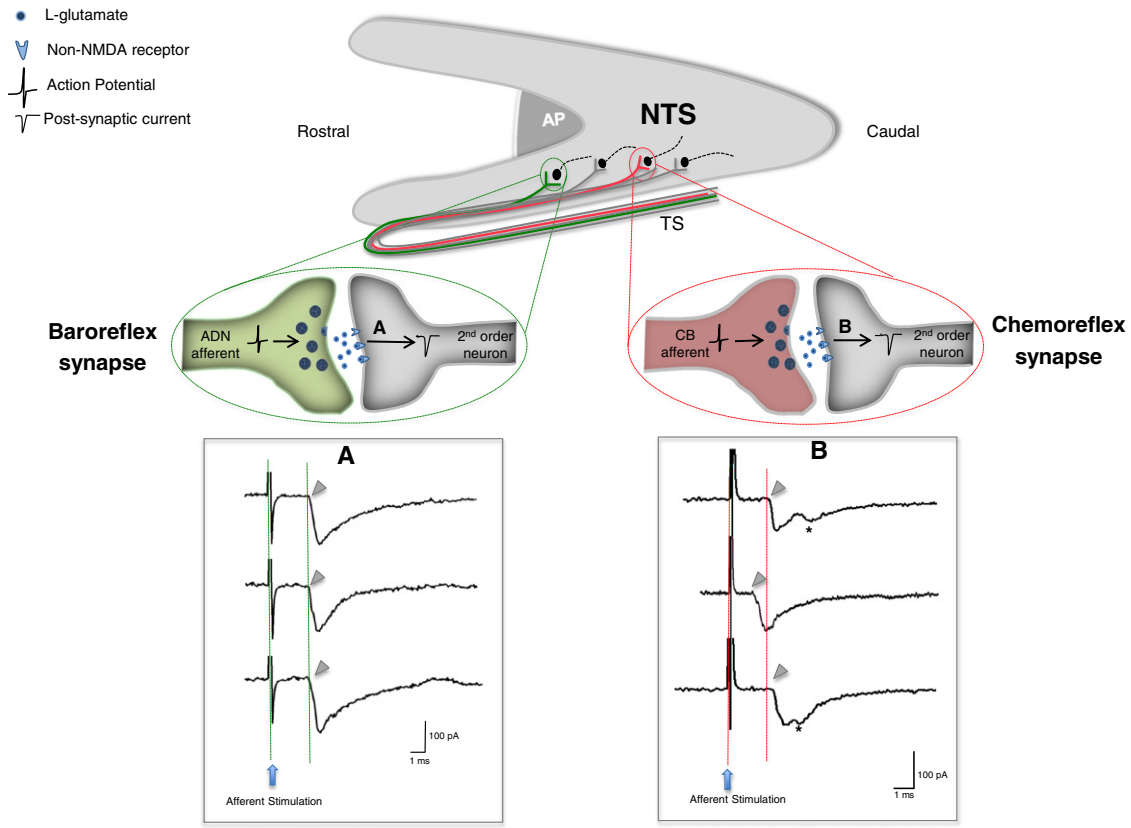

Fig. 1. Baro- and chemoreceptor afferent transmission in the 2nd-order NTS neurons. Schematic illustration showing the TS fibers entering in the NTS and the baro- and chemoreceptor synapses between peripheral afferents and 2nd-order NTS neuron. **Panel A:** three consecutive TS-eEPSCs recorded in baroreceptor 2nd-order NTS neuron. Note that all TS-eEPSCs (gray arrows) begins with similar latency (green lines); **Panel B:** three consecutive TS-eEPSCs recorded in chemoreceptor 2nd-order NTS neuron. Note that TS-eEPSCs (grey arrows) begins with large variability in latency (red lines) and the presence of polysynaptic responses (asterisks). ADN = aortic depressor nerve; CB = carotid body.

fluorescence microscopy, we visualized and recorded the electrophysiological properties of NTS neurons receiving labeled CB inputs. In spite of similar amplitude, the TS-eEPSCs of chemoreflex 2nd-order NTS neurons presented higher latency (4 ± 0.2 vs 3.3 ± 0.3 ms) and higher SD of latency (0.49 ± 0.03 vs 0.19 ± 0.02 ms) when compared with baroreceptors 2nd-order NTS neurons (Accorsi-Mendonça et al., 2011). This difference in the temporal parameters demonstrates that chemoreceptor 2nd-order NTS neurons do not present a timing precision of evoked neurotransmission as that described for baroreceptor 2nd-order NTS. It is also important to note that chemoreceptor 2nd-order NTS neurons frequently present asynchronous currents in TS-eEPSCs, which are not observed in baroreceptor 2nd-order neurons [(Accorsi-Mendonça et al., 2011) see Fig. 1]. These asynchronous currents are probably related to multiple synaptic contacts established by TS afferent fibers and different chemoreceptor 2nd-order NTS neurons. Probably these multiple peaks do not affect the latency of chemoreceptor 2nd-order NTS neurons, since when we were able to discriminate both primary (early) and secondary (late) currents in TS-eEPSCs, the variability of latency of primary asynchronous current for chemoreceptors 2nd-order neurons was also higher than the baroreceptor 2nd-order NTS neurons (Accorsi-Mendonça et al., 2011). Similar to baroreceptor afferents, L-glutamate also seems to play a key role in the neurotransmission of the chemoreceptors afferents in 2nd-order NTS neurons, since the application of DNQx, a glutamatergic ionotropic receptors antagonist, blocked the evoked current in chemoreceptor 2nd-order NTS neurons in brainstem slices (Accorsi-Mendonça et al., 2011).

The peripheral chemoreceptors afferents establish synaptic contact with a large variety of 2nd-order NTS neurons, which send projections to several other nuclei in the brainstem, pons, hypothalamus and cortex in order to provide autonomic, respiratory and behavioral adjustments, which are critical for individual self-protection during hypoxic stress (Fig. 2). There is anatomical and functional evidence that NTS neurons sending direct projections to the ventral lateral medulla (VLM) are involved with the sympathoexcitatory as well as in the respiratory components of the chemoreflex (Ross et al., 1985; Urbanski and Sapru, 1988; Koshiya et al., 1993; Granata, 1994; Aicher et al., 1996; Moraes et al., 2012). In this context, we evaluated whether or not NTS 2nd-order neurons receiving afferents from CB and sending projections to the VLM also presented a high variability of latency of TS-eEPSCs, as observed in chemoreflex 2nd-order NTS neurons without the identification of their target projections. Two different fluorescent tracers were used to label afferent fibers from CB, with the application of DiI (Molecular Probes, USA) around the CB and the neuronal projection from NTS to ventral lateral medulla with the microinjection of GreenRetro Beads into the RVLM (LumaFluor, USA) a few days prior to the electrophysiological experiments using brainstem slices. With these approaches we cannot assure whether or not these neurons retrogradely labeled in the NTS are those related to the pathways of the sympathoexcitatory or the respiratory responses to chemoreflex activation, but there is no doubt that these 2nd-order NTS neurons are sending projections to the VLM and probably are integral to the chemoreflex pathways.

In double-labeled NTS neurons (chemoreceptor 2nd-order NTS neurons sending projections to VLM) the latency and SD of latency of evoked glutamatergic response were comparable to those observed in chemoreceptor 2nd-order NTS neurons, demonstrating that the two subpopulations of chemoreflex NTS neurons analyzed in our studies (NTS neurons receiving inputs from CB and NTS neurons

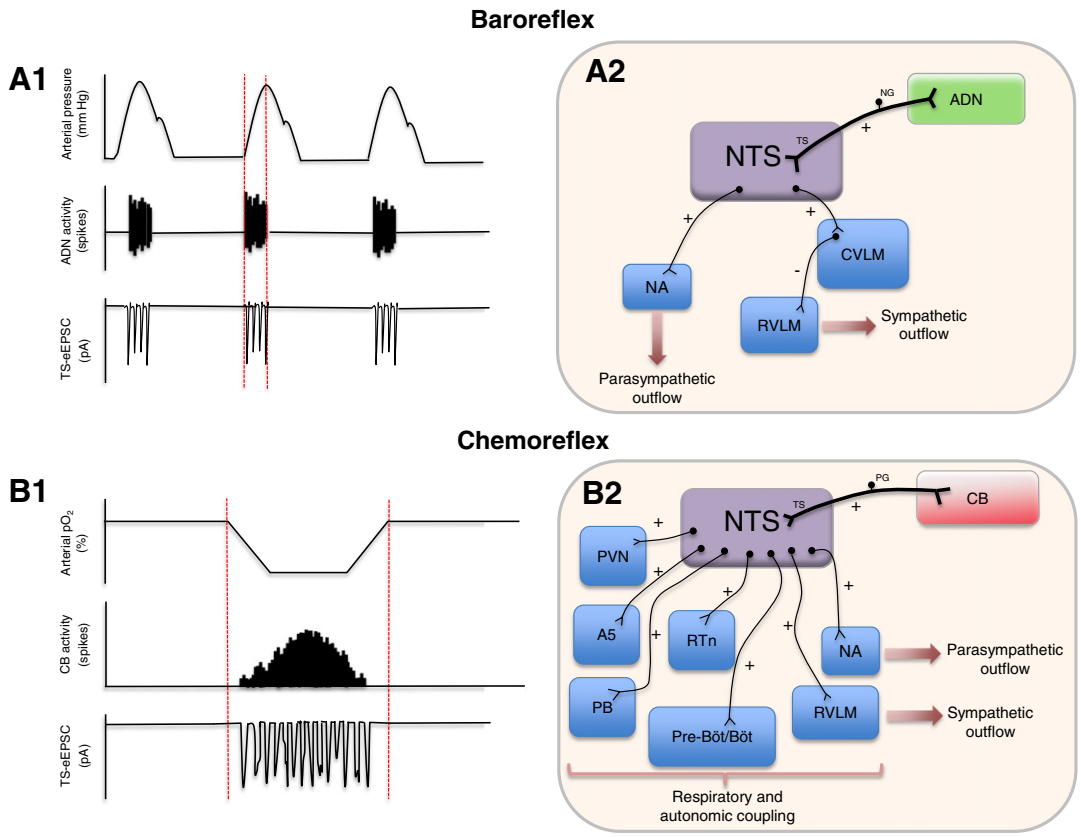

Fig. 2. Activation of baro- and chemoreceptors afferents and their synaptic connections in the NTS. **Panel A1:** schematic illustration showing a relationship between arterial pulse pressure, ADN activity and TS-eEPSCs of baroreceptor 2nd-order NTS neuron. Note that during each systolic increase in arterial pressure there is a simultaneously increase in the ADN activity and the appearance of evoked response in the 2nd-order NTS neuron. **Panel A2:** schematic illustration showing the afferents of ADN in the NTS and its connections with nuclei involved with the generation of sympathetic and parasympathetic activities. **Panel B1:** schematic illustration showing the relationship between arterial pO_2, CB nerve activity and TS-eEPSCs of chemoreceptor 2nd-order NTS neuron. Note that during normal pO2 (normoxia condition), there is no CB activity or evoked current in 2nd-order NTS neuron. A decrease in the arterial pO_2 (hypoxia condition) produces an increase in the CB nerve activity and the appearance of evoked responses in the 2nd-order NTS neuron. **Panel B2:** schematic illustration showing the afferents of CB nerve in the NTS and its connections with nuclei involved with the generation of sympathetic, parasympathetic and respiratory activities. ADN: aortic depressor nerve; NG: nodose ganglion; NA: nucleus ambiguus; CVLM: caudal ventral lateral medulla; RVLM: rostral ventral lateral medulla; CB: carotid body; PG: petrosal ganglion; PVN: paraventricular nucleus of the hypothalamus; PB: parabrachial nucleus; RTn: retrotrapezoid nucleus; A5: A5 area; Pre-Bötl/Bötl: Pre-Bötzinger–Bötzinger complex.

receiving inputs from CB and sending projections to VLM) present higher temporal variability of excitatory transmission compared to baroreceptor 2nd-order NTS neurons. In order to make a consistent comparison between baro- and chemoreceptors afferents in the NTS, in the case of arterial baroreceptors we evaluated the 2nd-order NTS neurons receiving inputs from the ADN and sending projections to the caudal ventrolateral medulla (Accorsi-Mendonça et al., 2011), since a retrograde tracer was previously microinjected into the CVLM.

We then explored the synaptic parameters involved in the higher latency and SD of latency observed in chemoreceptor 2nd-order NTS neurons. It has been described that in hippocampal neurons monosynaptically connected, the latency of evoked response is determined by pre-synaptic release probability [(Pr), (Boudkkazi et al., 2007)]. Based upon this concept, we compared the Pr values of baro- and chemoreceptors afferents in the NTS. Experiments to estimate the Pr demonstrated that chemoreceptor afferents presented a significantly higher Pr (0.77 ± 0.02, $n = 5$) compared to baroreceptor afferents (0.56 ± 0.07, $n = 8$), suggesting that Pr, an intrinsic property of fibers, may determine the temporal profile observed in chemoreceptor 2nd-order NTS neurons (Accorsi-Mendonça et al., 2011). Moreover, we cannot rule out the possibility that other synaptic factors may also contribute to the observed temporal differences between chemo- and baroreceptor 2nd-order NTS neurons. In this context the following possibilities must be considered: a) that chemoreceptor 2nd-order neurons present synaptic contact not only at the cell body, but also along the dendritic tree producing a longer latency and higher SD of latency, reflecting the differential activation of distal and proximal or somatic synapses; b) baroreceptors afferents are composed by different types of neuronal fibers: myelinated and unmyelinated [A and C fibers, respectively (Fazan et al., 1997)]. The baroreceptor afferents (A and C fibers) are differently activated by the frequency and intensity of stimulus and both evoke glutamatergic currents in 2nd-order NTS neurons with distinct latencies (Fan and Andresen, 1998; Bailey et al., 2002). However, there is no available information about the currents evoked by chemoreceptors A and C fibers in 2nd-order NTS neurons. We can also speculate that stimulation of baro- or chemoreceptors afferents may activate different groups and ratio of A and C fibers, which may contribute for the observed differences in the timing of synaptic processing at 2nd-order NTS neurons.

3. Functional implications of distinct timing processing and perspectives

The finding that chemoreceptor 2nd-order NTS neurons present different timing processing after afferent stimulation contributes to our better understanding about the mechanisms of visceral and sensorial transmission in the brainstem, since it demonstrates that the synaptic activity described to baroreceptor 2nd-order NTS neurons is not a general characteristic for all 2nd-order NTS neurons. These differences in between baro- and chemoreceptors 2nd-order NTS

neurons may reflect an intrinsic heterogeneity of these sensory afferents, and the understanding of the synaptic mechanisms generating this variability will provide fundamental information about the central processing of these two important cardiovascular and respiratory reflexes in normal as well as in pathophysiological conditions such as obstructive sleep apnoea and hypertension, in which the synaptic mechanisms of both reflexes at the NTS level are deeply altered.

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