Repetitive paired stimulation of nasotrigeminal and peripheral chemoreceptor afferents cause progressive potentiation of the diving bradycardia

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Rozloznik M, Paton JF, Dutschmann M. Repetitive paired stimulation of nasotrigeminal and peripheral chemoreceptor afferents cause progressive potentiation of the diving bradycardia. Am J Physiol Regul Integr Comp Physiol 296: R80–R87, 2009.—Markers of the mammalian diving response are protective apnea and bradycardia. These cardiorespiratory adaptations can be mimicked by stimulation of the trigeminal ethmoidal nerve (EN5) and reflect oxygen-conserving mechanisms during breath-hold dives. Increasing drive from peripheral chemoreceptors during sustained dives was reported to enhance the diving bradycardia. The underlying neuronal mechanisms, however, are unknown. In the present study, expression and plasticity of EN5-bradycardias after paired stimulation of the EN5 and peripheral chemoreceptors was investigated in the in situ working heart-brain stem preparation. Paired stimulations enhanced significantly the bradycardic responses compared with EN5-evoked bradycardia using submaximal stimulation intensity. Alternating stimulations of the EN5 followed by paired stimulation of the EN5 and chemoreceptors (10 trials, 3-min interval) caused a progressive and significant potentiation of EN5-evoked diving bradycardia. In contrast, bradycardias during paired stimulation remained unchanged during repetitive stimulation. The progressive potentiation of EN5-bradycardias was significantly enhanced after microinjection of the 5-HT3 receptor agonist (CPBG hydrochloride) into the nucleus tractus solitarii (NTS), while the 5-HT3 receptor antagonist (zacopride hydrochloride) attenuated the progressive potentiation. These results suggest an integrative function of the NTS for the multimodal mediation of the diving response. The potentiation or training of a submaximal diving bradycardia requires peripheral chemoreceptor drive and involves neurotransmission via 5-HT3 receptor within the NTS.

AQUATIC MAMMALS REQUIRE SPECIFIC modulation of cardiorespiratory functions to permit sustained breath-hold dives. These modulations are summarized as the diving response, which is initiated by the activation of trigeminal sensory afferents from the forehead, eyes, nasal cavity, and lips (6, 9, 12, 18, 21, 26, 42, 44). The hallmarks of the diving response are centrally mediated apnea and glottal constriction to protect the lower airways from invasion of liquids. The respiratory response is accompanied by bradycardia, peripheral vasoconstriction, spleen contraction, and redistribution of blood volume toward the vital organs (e.g., heart, brain). These cardiorespiratory adjustments during breath-hold diving are geared toward oxygen conservation to delay the rapid progression of asphyxia (9, 17, 18). The fundamental cardiorespiratory reflex pattern of the diving response is further modulated by peripheral inputs from chemoreceptors, pulmonary stretch receptors, and baroreceptors (see Ref. 9), but also by training (19, 45). Of particular interest is the influence of increased chemoreceptor drive during breath hold. Under normal physiological conditions, increased peripheral chemoreceptor drive causes air hunger and therefore limits breath-hold time (3, 45). However, previous studies demonstrated that increasing peripheral chemoreceptor drive during experimental dives enhanced the bradycardia. This was interpreted as a mechanism to further boost oxygen-conserving components of the diving response as blood oxygen levels dwindled (10, 17, 18, 45). The underlying neuronal mechanisms of the secondary modulation of the diving bradycardia are not understood.

Previous studies identified the trigeminal ethmoidal nerve (EN5), a branch of the ophthalmic division of the trigeminal nerve, as crucial afferent for the initiation of the diving response in rat (12, 42). The EN5 targets anatomically defined areas of the ponto-medullary brain stem (16, 31, 34, 35). Physiological investigations revealed that the main targets involved in the mediation of the diving response included the caudal nucleus of the spinal trigeminal tract (36), the nucleus of solitary tract (15), the pontine A5 (31), Kölliker-Fuse nucleus (14, 16), and cardiorespiratory centers (C1 region and rostral respiratory group) within the ventrolateral medulla oblongata (12, 30). However, central neuronal mechanisms underlying the processing of multimodal inputs during the diving response are still unknown.

In the present study, we investigated the influence of peripheral chemoreceptor inputs on the expression of the diving response in the in situ working heart-brain stem preparation (WHBP) of rat. The ponto-medullary neural networks required for the mediation of the diving response are anatomically and functionally conserved in the arterially perfused and precollicular-decerebrated WHBP. Previously published data showed that the diving response can be triggered by electrical stimulation of the EN5 in the WHBP (12, 13). The in situ diving response strongly corresponds to the cardiorespiratory pattern observed in the unanesthetized rat (26). Thus, the WHBP can be considered as a useful experimental rat model to study neuronal mechanisms underlying the mediation, modulation, and plasticity of the diving response.

Here, we show that submaximal paired stimulation of the EN5 and peripheral chemoreceptors caused a highly significant facilitation of the bradycardia compared with the response of stimulation of the EN5 without coactivation of chemoreceptor afferents. Repetitive paired stimulation followed the protocol...
of classical conditioning and caused a progressive potentiation of the diving bradycardia triggered by EN5 stimulation alone. Finally, we performed microinjections of serotonin type 3 receptors (5-HT3R) agonists and antagonists into the NTS to investigate a pharmacological modulation of the observed progressive potentiation of the diving bradycardia. Thereby, we were aiming for neural population in the NTS, since anatomical data strongly suggest first-order convergence of EN5 (4, 34) and chemoreceptor (27) afferents in this nucleus, and 5-HT3R in the NTS were targeted because previous data have shown their importance in particular for the mediation of bradycardic responses within the NTS (7, 8, 25, 46, 47).

MATERIALS AND METHODS

Experiments were performed on juvenile rats (Sprague-Dawley, 70–100 g, male or female). We employed the WHBP, which was described in full detail previously (38). All experiments were approved by the committee for animal welfare and ethics of the Georg-August-University, Göttingen.

WHBP

Rats were anesthetized deeply with halothane. Once respiration was depressed and the animal failed to respond to a noxious pinch of the tail or a toe, the rat was transected below the diaphragm and exsanguinated. In chilled Ringers solution gassed with 95% O2-5% CO2 (carbogen), rats were decerebrated at the precollicular level, thereby making the animal insentient. The cerebellum was removed. After these initial procedures, which took 4–5 min, the preparation was transferred to a recording chamber. The descending aorta was cannulated and perfused with carbogen-gassed Ringers containing Ficoll (1.25%) at 31°C and at a flow rate of 28–32 ml/min via a double-lumen catheter. For perfusion, a peristaltic pump (Watson Marlow model 505s) (1.25%) at 31°C and at a flow rate of 28–32 ml/min via a double-lumen catheter. For perfusion, a peristaltic pump (Watson Marlow model 505s) was used. The perfusate was filtered and passed through bubble traps to remove gas bubbles and dampen both perfusion pump- and cardiac-generated pulsations. Perfusion that leaked from the preparation was collected, reoxygenated, and recirculated. Two to five minutes after the start of perfusion, rhythmic contractions of the diaphragm resumed. Respiratory-related movements were abolished by using vecuronium bromide (0.3 µg/ml). The perfusate contained (in mM): 125 NaCl, 24 NaHCO3, 2.5 CaCl2, 1.25 MgSO4, 4 KCl, 1.25 KH2PO4, 10 n-glucose, and Ficoll (Sigma) to maintain osmotic clot pressure. The osmolality of the perfusate [artificial cerebrospinal fluid (ACSF)] was 298 ± 5 mosmol/l, and on gassing with carbogen the pH was 7.35 ± 0.05.

Recording of Respiratory Activity and Cardiovascular Parameters

Perfusion pressure within the aorta was monitored via one port of the double-lumen catheter (see above) connected to a pressure transducer. The other lumen was used to perfuse the preparation. Perfusion pressure was set to 70–90 mmHg by adjusting the flow rate. In all experiments, a phrenic nerve was cut at the level of the diaphragm, and the discharge was recorded from its central end by using a glass suction electrode containing Teflon-isolated silver wire. The indifferent electrode was placed into the orbital cavity. Electrical stimulation was performed with a 10-s stimulus train (20 Hz, 100-µs pulse width, stimulus intensity 0.5–2 V). To stimulate peripheral chemoreceptors, sodium cyanide (NaCN; 50–200 µl, 0.01%) was injected into the perfusion circuit just ahead of the double-lumen aortic cannula. Volumes of injected NaCN were adjusted to induce a mild increase in respiratory frequency and mild bradycardia before starting an experimental protocol (50–200 µl, 0.01%). To investigate the impact of chemoreceptor activation on the magnitude of the diving bradycardia we used paired stimulation of the EN5 and peripheral chemoreceptors. Because of the transit time of the NaCN in the perfusate, the interaction of the reflex heart rate response evoked by stimulation of EN5 and peripheral chemoreceptors was performed by injecting NaCN 1 s before electrical stimulation of the EN5 to ensure simultaneous activation. At the beginning of the experimental protocol EN5 and peripheral chemoreceptors were stimulated separately to determine the magnitude of the individual reflexly evoked bradycardia. Subsequently, we determined whether the bradycardic response during paired stimulation of trigeminal and peripheral chemoreceptor afferents was enhanced compared with EN5 stimulation alone. Using these predetermined stimulation parameters, we investigated the effect of repetitive paired stimulation (10 trials; see Fig. 1A) on the expression and strength of EN5-evoked bradycardia. Thereby, each trial consisted of alternating EN5-stimulation followed by paired stimulation of the EN5 and peripheral chemoreceptor stimulation (NaCN). The EN5 and paired stimulation were separated by 30 s in this protocol.

Subsequent analysis revealed that maximal or submaximal stimulation of either the EN5 or the chemoreceptors are major determinants of the impact of paired stimulation on the magnitude and plasticity of diving bradycardia. Therefore, the data on repetitive, alternate EN5 and paired stimulation are presented in different experimental groups, which were classified according the initial stimulation strength (see RESULTS).

Experimental Protocol 2: Microinjections

The NTS was previously proposed to be the integrative center for the mediation of chemoreceptor-mediated modulation of the diving bradycardia (17). Since 5-HT3-R were reported to have implications in NTS neurotransmission associated with the mediation of bradycardia (7, 8, 25, 46, 47), we microinjected the 5-HT3R agonist [N-(3-chlorophenyl)-imidodiacarbonimidic diamide (CPBG) hydrochloride, n = 3; Tocris] or antagonist (zacopride hydrochloride, n = 3; Tocris). Both drugs were used at a concentration of 10 mM (dissolved in perfusion medium without Ficoll), which was shown to modulate effectively reflexly evoked bradycardic responses following NTS microinjections in vivo (7, 8, 25, 46, 47). For NTS microinjections, we used the oex at the caudal part of the fourth ventricle as a reference surface landmark for positioning of a multibarrel micropipette (external diameter 10–30 µm) with the aid of a binocular dissecting microscope. All drugs were microinjected from the dorsal medullary surface into the commissural NTS at a depth of 300–400 µm. Injected volumes were assessed by measuring the movement of the meniscus through the pipette. The distance of the movement of the meniscus was controlled with a binocular microscope fitted with a precalibrated eyepiece reticle. The barrels of the microinjection micropipettes were filled with either 5-HT3-R agonist or antagonist, vehicle (ACSF), and 2% Pontamine sky blue (Sigma) for the subsequent identification of the injection site.

To examine the role of serotonin in the peripheral chemoreceptor and EN5-evoked bradycardia, we used a different experimental protocol. This was necessary since in the initial protocol the repetitive EN5-stimulation and coactivation were separated by 30 s. Thus, the timing of the initial protocol did not allow for microinjections. Therefore, the repetitive stimulations were changed to the following protocol. A stimulation trial included one EN5 stimulation followed by four paired stimulations of EN5 and peripheral chemoreceptors (see Fig. 2A). The individual stimulations were separated by a 3-min
interval. During the first trial, we performed control microinjections of medium (ACSF) between the first and second paired stimulation of the trial (see Fig. 2A) to exclude injection volume effects in NTS. If microinjections of ACSF caused no significant changes in heart rate and respiratory activity, we injected the same volume of drug between the second and third paired stimulation of the initial stimulation trial: Left: changes in HR and PNA to stimulation of the EN5 alone. Right: changes in HR and PNA during paired stimulation. Middle: overlays of the HR response to EN5-stimulation trials. E: group data (ANOVA with Fisher’s post hoc test; ANCOVA) \( *P < 0.05; **P < 0.01 \). HR changes during 1st trial: 292.8 ± 15.6 to 214 ± 21.5 beats/min; 5th trial: 291.9 ± 17.7 to 178.1 ± 19.3 beats/min; 10th trial: 278.1 ± 27.4 to 140.2 ± 30.7 beats/min.

**Statistical Tests**

We analyzed the magnitude of reflex bradycardias and apnea duration to EN5-stimulation and paired stimulation of peripheral chemoreceptors and EN5. EN5-evoked changes in perfusion pressure caused by vasoconstriction proved unreliable and were not analyzed in the present study. The baseline HR values were measured 30 s before each test stimulation (e.g., EN5, peripheral chemoreceptor, or paired stimulation). The EN5-evoked bradycardia was determined from the mean fall in HR that occurred during the 10-s stimulation.
period. The progressive changes in heart rate responses during repetitive stimulations were analyzed with regression analysis using the ANCOVA test. For pairwise comparisons, we applied ANOVA followed by Fisher's post hoc test (e.g., direct effects of the microinjected 5-HT₃R agonist or antagonist on heart rate). All data are expressed as means ± SE. Statistical test were performed with SYSTAT (version 11) software package. Differences were taken to be significant at the 95% confidence interval.

RESULTS

Progressive Potentiation the EN5-Evoked Bradycardia

We studied the influence of repeated, paired stimulation of EN5 (20 Hz, 100 μs, 10 s) and peripheral chemoreceptor (NaCN, 50–200 μl, 0.01%) on the heart rate response elicited by EN5-stimulation alone (for details see MATERIALS AND METHODS). The initial paired stimulation of EN5 and chemoreceptor afferents decreased heart rate from 292.8 ± 15.6 to 82.7 ± 14.2 beats/min (−71.7%) and exceeded the sum of mean bradycardia evoked by submaximal stimulation of peripheral chemoreceptors (292.8 ± 15.6 vs. 271.4 ± 18.2 beats/min; −7.3%) and EN5-stimulation alone (292.8 ± 15.6 vs. 214 ± 21.5 beats/min; −26.9%; P < 0.01). Repetitive stimulations (10 trials, n = 5 preparations) of the EN5 followed by a paired stimulation of EN5 and chemoreceptor afferents (Fig. 1A) revealed a progressive and significant potentiation of the EN5-evoked bradycardia (Fig. 1, B–D). Regression analysis revealed a
significant progressive potentiation of the EN5-evoked bradycardia (1st trial: −26.9%; 5th trial: −39%; 10th trial: −49.6%; ANCOVA; *P < 0.01; Fig. 1E). In contrast, the bradycardia triggered by paired stimulations remained constant (1st trial: −71.7%; 5th trial: −72.9%; 10th trial: −75.8%; ANCOVA not significant [n.s.]). Finally, repetitive stimulations did not cause changes in baseline heart rate (see Fig. 1E) or EN5-evoked apnea duration (1st trial: 10 ± 0.4 s; 10th trial: 10 ± 0.3 s).

In a second group of experiments (data not shown), we performed maximal stimulation of the EN5 (*n* = 8), which caused a mean decrease in heart rate of 277.3 ± 12.8 to 134.7 ± 25.9 beats/min (−51.4%, compared to −26.9% after submaximal stimulation) in combination with submaximal stimulation of peripheral chemoreceptors (277.3 ± 12.8 to 269.5 ± 12.5 beats/min, −2.8%). Here the paired stimulation of EN5 and chemoreceptor afferents revealed no facilitation of the bradycardia, but summation of bradycardia response evoked by separate stimulation of EN5 and chemoreceptor afferents. During the stimulation protocol (Fig. 1A) no significant potentiation of the EN5 evoked bradycardia was observed (1st trial: 277.3 ± 12.8 to 134.7 ± 25.9 beats/min = −51.4%; 5th trial: 274.5 ± 12.1 to 105 ± 22.8 beats/min = −61.7%; and 10th trial: 266.7 ± 13.1 to 98.6 ± 19.9 beats/min = −63%; ANCOVA, n.s.). The same accounts for the magnitude of the bradycardia during paired stimulation (1st trial: −58%; 5th trial: −67.7%; 10th trial: −65.2%; ANCOVA, n.s.). Also in these experiments, the baseline HR and the apnea duration associated with 10 s EN5-stimulation was not affected (first trial: 10 ± 0.5 s; 10th trial: 10 ± 0.4 s).

Finally, in the last experimental series (*n* = 3, data not shown), we injected larger volumes of NaNCl (e.g., >200 µl), which caused a maximal stimulation of peripheral chemoreceptors. In these cases, paired stimulation of chemoreceptors and EN5 produced disruption of EN5-evoked apnea accompanied by unpredictable fluctuations in HR. Thus, the physiological response pattern of the diving response was not maintained after maximal stimulation of peripheral chemoreceptors.

In summary, our results demonstrate that only submaximal activation of both the EN5 and peripheral chemoreceptors was capable to produce a progressive potentiation of the EN5-evoked diving bradycardia.

**NTS Microinjection of 5-HT3R Agonists and Antagonists**

**Modulated Progressive Potentiation of the EN5-Evoked Bradycardia**

The microinjection studies required modification of our experimental protocol (Fig. 2A; see MATERIALS AND METHODS), since in the initial protocol (Fig. 1A) the repetitive EN5-stimulation and coactivation were separated by only 30 s, which did not give sufficient time to complete the microinjections. According to our previous findings, we only used submaximal stimulation of EN5 and chemoreceptor afferents in these experiments, which evoke facilitation of the bradycardia during paired stimulation of EN5 and peripheral chemoreceptors (see Fig. 2B).

**Control experiments for the microinjection studies.** Initially, we validated the new protocol (*n* = 3 preparations). As seen before, a progressive and significant potentiation of the EN5-evoked bradycardia was observed during the modified stimulation protocol (ANCOVA *P < 0.001; Fig. 2E). In response to EN5 stimulation HR changed from 292.8 ± 15.6 to 214 ± 21.5 beats/min (−38.2%) during the 1st trial, from 291.9 ± 17.7 to 178.1 ± 19.3 beats/min (−54.3%) during the 5th trial, and from 278.1 ± 27.4 to 140.2 ± 30.7 beats/min (−65.7%) during the 10th trial. Thus, the modified stimulation protocol yields a similar progressive potentiation of the EN5-evoked bradycardia, as observed in the previous protocol, including no change in the evoked 10-s apnea (first trial: 10 ± 0.3 s; 5th trial: 10 ± 0.1 s).

**5-HT3R agonist.** Ten minutes after microinjection of 5-HT3R agonist (CPBG hydrochloride, 10 mM, 20 nl) no significant change was observed for the EN5-evoked bradycardia (297.5 ± 8.4 vs. 242.9 ± 20.8 beats/min; −18.3%; n.s.) compared with that evoked prior to the microinjection (291.9 ± 7.7 vs. 232.4 ± 12.2 beats/min; −20.1%; Fig. 3C). In the third trial, EN5-evoked bradycardia was increased, and HR dropped from 294.5 ± 9.1 to 203.4 ± 19 beats/min (−30.91%). During the final fifth stimulation trial, the EN5-evoked HR response was further increased (295.3 ± 9.8 to 137.7 ± 33.8 beats/min; −53.4%; Fig. 3D). Analysis of the linear regression revealed a highly significant potentiation of the EN5-evoked bradycardia (ANCOVA, *P < 0.001; Fig. 3E).

**5-HT3R antagonist.** Microinjection of 5-HT3R antagonist zacopride hydrochloride (10 mM, 20 nl, *n* = 3) into the commissural NTS had the opposite effect. Instead of augmentation a progressive attenuation of the EN5-evoked, diving bradycardia was observed. The first EN5-evoked bradycardia
after microinjection was already decreased (297.7 ± 12.89 beats/min vs. 180.96 ± 42.5 beats/min; −39.2%) compared with that evoked prior to the microinjection (293.4 ± 7.8 vs. 154.6 ± 40.22; −47.3%). During the stimulation protocol, the HR response to EN5-stimulation further decreased during the third (300.1 ± 14.3 vs. 195.9 ± 38.8; −34.7%) and fifth stimulation trial (300.7 ± 13.1 vs. 225.3 ± 38.1 beats/min; −25.07%). The progressive attenuation of the EN5-evoked bradycardia was highly significant (P < 0.001 ANCOVA).

Comparative analyses of the effects of 5-HT3R agonist and antagonist microinjections. None of the used drugs revealed significant progressive changes in the strength of bradycardia evoked by paired stimulation of EN5 and chemoreceptors (data not shown). To compare progressive changes in the EN5-evoked bradycardia during control and after drug injections, we normalized the magnitude of the bradycardia in response to EN5-stimulations. Compared with control protocols, the slope of the linear regression of the normalized EN5-evoked bradycardia was increased after microinjection of CPBG hydrochloride and was decreased after zacopride hydrochloride (Fig. 3A). The histological analysis showed that all injection sites were placed into the commissural subnucleus of the NTS (Fig. 3B).

DISCUSSION

Modulation of the Diving Bradycardia by Activation of Synaptic Inputs from Peripheral Chemoreceptors

In diving mammals, the role of chemoreceptors is seen to maintain and magnify the diving bradycardia in the late stage of a dive when the afferent drive from peripheral chemoreceptors is increased (3, 6, 17, 18). The present study revealed a potent facilitation of the diving bradycardia by simultaneous submaximal activation of EN5 afferents and peripheral chemoreceptors (arterial bolus injection of NaCN), while the reflex apnea was maintained. A full expression of the diving bradycardia after maximal stimulation the EN5, however, could be not facilitated, and only additive effects of the EN5- and peripheral chemoreceptor-evoked bradycardia were observed. We conclude that a maximal diving bradycardia during EN5-stimulation alone cannot be further facilitated, since the vagal inputs to heart may be already close to their maximum and reaches a magnitude as it was described for diving mammals (6, 9, 26). Nevertheless, the summation of chemoreceptor- and EN5-evoked bradycardia still illustrates the influence of chemoreceptor drive on the magnitude of bradycardia as it occurs in diving mammals. In diving mammals (e.g., seals) an increasing chemoreceptor drive is seen to be particularly crucial for the persistence of the diving bradycardia during prolonged dives, but not for the initiation of the bradycardia (6, 9). However, a maximal activation of peripheral chemoreceptors can break the protective apnea and thus, would cause drowning.

Here, we show for the first that repetitive paired stimulation of EN5 and peripheral chemoreceptors caused a progressive potentiation of EN5-evoked diving bradycardia, while the apneic response was maintained. The activation of convergent synaptic inputs is, thereby, a prerequisite for the maintenance of full expression of the diving response during repetitive stimulations, since previously we have shown that repetitive stimulation of the EN5 alone caused habituation of the protective apnea and bradycardia (11).

The progressive potentiation of the diving bradycardia evoked by EN5 stimulation observed in the present study, therefore, was most likely induced by repetitive paired stimulations, which followed the experimental protocol of classical conditioning, as a form of associative learning (for excellent reviews see Refs. 40 and 41). The repetitive coactivation of closely linked and convergent synaptic inputs clearly caused a facilitation of diving bradycardia. Thus, we suggest that the repetitive activation of convergent synaptic inputs during paired stimulation caused a permanent synaptic enhancement within the primary pathways for the nasotrigeminal inputs. Thereby, the chemoreceptor input serves as an unconditioned stimulus that causes synaptic enhancement within the primary or conditioned pathway, which mediates the diving response. Therefore the observed potentiation of the diving bradycardia is consistent with the definition of classical conditioning (see Ref. 40). Classical conditioning may indeed reflect an important mechanism underlying increased autonomic responses during dives in response to regular training as it is seen in humans (19). We speculate that the observed progressive potentiation of the diving bradycardia may relate to a form of plasticity in autonomic control circuits, such as facilitation of respiratory motor outputs in response to intermittent hypoxia (33).

Convergence of the Trigeminal Ethmoidal and Chemoreceptor Afferent Inputs in Ponto-Medullary Autonomic Control Nuclei

Anatomical data showed that the NTS receives primary synaptic inputs from the EN5 (4, 34) and the same nucleus is the primary relay for afferents from peripheral chemoreceptors (27). Thus, the first convergent synapses for EN5 and peripheral chemoreceptor afferents may be found within the NTS. Thus, the NTS may be clearly one of the integrative centers for the mediation of multimodal afferent inputs (37) activated not only during the diving response. Nevertheless, second order convergence of the two functional different afferent inputs certainly can be located at any autonomic nuclei within ponto-medullary autonomic networks. Thereby, the parabrachial/Kölliker-Fuse complex (PB/KF) could be of particular importance for the mediation of multimodal synaptic inputs activated during breath-hold diving. The PB/KF receives dense synaptic inputs from the primary termination field of the EN5 within the caudal nucleus of spinal trigeminal tract (26) as well as from the NTS, which relays the chemoreceptor afferents. Indeed, it was shown that the KF is involved in the mediation of the diving bradycardia (14) and also integrates trigeminal and respiratory information (48). In turn, the descending projections of the KF target the medial and commissural subnuclei of the NTS and, thus, could have secondarily contributed to the mediation of the progressive potentiation of the diving bradycardia observed in our studies. Therefore, the role of the KF in the mediation and plasticity in response to multimodal afferent synaptic inputs during breath-hold diving should be investigated in future experiments.
5-HT₃ Receptors in the NTS are Involved in the Mediation of the Progressive Enhancement of Diving Bradycardia

The NTS is densely innervated by serotonergic terminals (28, 43), and 5-HT₃Rs are expressed in the NTS (22, 33). In contrast to all other 5-HT receptor isoforms, the 5-HT₃Rs are ligand-gated ion channels (5, 24) and show a voltage-dependent Ca²⁺ block (2, 50, 51). Previously, the 5-HT₃R was shown to be involved in mediating the reflex-evoked bradycardia following stimulation of cardiopulmonary afferents (23, 47), arterial baroreceptors (8), and carotid chemoreceptors (8, 46). We now showed that microinjection of a 5-HT₃R agonist into the commissural NTS augmented the progressive facilitation of the diving bradycardia, whereas the antagonist attenuated this effect. This indicates that serotonergic neurotransmission via 5-HT₃Rs in NTS may be essential for this form of plasticity. However, a potential spillover to cardiac vagal motoneurons located ventral to the NTS cannot be fully excluded in the present study. Furthermore, involvement of other 5-HT-receptor subtypes is feasible due to potential side effects of the used drugs. Nevertheless, several neuronal mechanisms for a 5-HT₃R-mediated regulation of the strength of the diving bradycardia are conceivable. Activation of presynaptic 5-HT₃Rs can increase the amplitude and frequency of spontaneous postsynaptic potentials in the NTS, but also direct postsynaptic depolarization has been reported (20). A recent study suggested that both mechanisms are of physiological relevance in the NTS. Stimulation of 5-HT₃Rs caused direct postsynaptic excitation, but experimental evidence for a facilitation of the release of glutamate was also found (23). Both mechanisms can account for the progressive potentiation of the diving bradycardia after repetitive coactivation of EN5 and peripheral chemoreceptors. The progressive potentiation of the diving bradycardia clearly reflects a plasticity phenomenon associated with the activation of two different synaptic inputs. Therefore, it is tempting to propose a coincidence detector function for the 5-HT₃Rs via the proposed voltage-dependent Ca²⁺ block (2, 50, 51). Analogous to Mg²⁺ block of NMDA-R in hippocampal long-term potentiation, a removal of a Ca²⁺ block of the NMDA receptors after the synchronous activation of two convergent synaptic inputs could open the gate for a long-term potentiation-like mechanism in the NTS. However, the multisynaptic form of long-term potentiation cannot be excluded with the experimental approach used in the present study, since a more precise definition of the plasticity phenomenon would require electrophysiological analyses.

Perspectives and Significance

We suggest that the described potentiation of the diving bradycardia is of physiological relevance. It shows that specialized cardiorespiratory adaptations, such as the diving response, can be learned. This is particularly obvious in humans where a diving bradycardia is weak in breath-hold-untrained subjects, whereas in highly trained breath-hold divers it can be very prominent and reach a drop in heart rate of ~90% (19).

In addition, our findings may have some clinical implications. For instance the diving response is discussed to be a trigger for unexpected death in the sudden infant death syndrome (29). Thereby, the neural mechanisms involved in the potentiation of the diving bradycardia described in our study could contribute to fatal heart arrest in sudden infant death syndrome victims. Furthermore, the hypertension together with a chronic cardiopulmonary risk observed in obstructive sleep apnea patients might be a result of increased autonomic reactions to repetitive asphyxic exposures. A potential potentiation of bradycardic responses in this disease state is suggested by profound bradycardias during apneic periods in obstructive sleep apnea patients (1).

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REFERENCES

PROGRESSIVE POTENTIATION OF THE DIVING BRADYCARDIA