Intrinsic and Synaptic Long-Term Depression of NTS Relay of Nociceptin-Sensitive and Capsaicin-Sensitive Cardiopulmonary Afferents Hyperactivity

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ABSTRACT

The nucleus tractus solitarius (NTS) in the caudal medulla is a gateway for a variety of cardiopulmonary afferents important for homeostatic regulation and defense against airway and cardiovascular insults, and is a key central target potentially mediating the response habituation to these inputs. Here, whole-cell and field population action potential recordings and infrared imaging in rat brainstem slices *in vitro* revealed a compartmental pain-pathway-like organization of capsaicin-facilitated vs. nocistatin-facilitated/nociceptin-suppressed neuronal clusters in an NTS region which receives cardiopulmonary A- and C-fiber afferents with differing capsaicin sensitivities. All capsaicin-sensitive neurons and a fraction of nociceptin-sensitive neurons expressed NMDA receptor-dependent synaptic long-term depression (LTD) following afferent stimulation. All neurons also expressed activity-dependent decrease of excitability (intrinsic LTD), which converted to NMDA receptor-dependent intrinsic long-term potentiation after $GABA_A$ receptor blockade. Thus, distinct intrinsic and synaptic LTD mechanisms in the NTS specific to the relay of A- or C-fiber afferents may underlie the response habituation to persistent afferents hyperactivity that are associated with varying physiologic challenges and cardiopulmonary derangements — including hypertension, chronic cough, asthmatic bronchoconstriction, sustained elevated lung volume in chronic obstructive pulmonary disease or in continuous positive-airway-pressure therapy for sleep apnea, metabolic acidosis, and prolonged exposure to hypoxia at high altitude.

INTRODUCTION

Excitatory neurotransmission by a spiking neuron comprises two sequential steps: (i) conversion of presynaptic spikes into excitatory postsynaptic potentials (EPSPs); and (ii) integration of the EPSPs into neuronal spikes. Both steps may exhibit activity-dependent plasticity. Synaptic plasticity such as synaptic long-term potentiation (LTP) and depression (LTD) has been widely thought to underlie learning and memory and other experience-dependent modifications of brain function (42, 57). Intrinsic plasticity (LTP or LTD) refers to the modifiability of "EPSP-spike coupling" as measured by the changes in time-averaged probability of neuronal firing or ensemble-averaged population spike amplitude (field population action potential (FPAP) (77)) for given unit EPSP (9, 37, 75). Accordingly, we define neurotransmission plasticity as the resultant of synaptic and intrinsic LTP or LTD, as measured by the changes in FPAP for given presynaptic activation.

The synaptic plasticity effect has been extensively studied in the past two decades mostly in the hippocampus, neocortex, cerebellum and other higher brain structures. Much less is known about intrinsic plasticity and the combined effects of both on neurotransmission plasticity. Even less well understood is whether synaptic and/or intrinsic plasticity might play a role in brainstem structures that modulate vital physiologic or defensive functions.

The nucleus tractus solitarius (NTS) in the caudal dorsolateral medulla is a gateway for primary afferents that mediate cardiorespiratory regulation and defense against a variety of cardiopulmonary insults (2, 4, 7, 18, 34, 47). Neurons in the medial (mNTS) and commissural (comNTS) aspects of NTS demonstrate synaptic short-term depression (STD) without (type I) or with synaptic LTD (type II) following afferent stimulation *in vitro* (76). Such synaptic STD and LTD in the NTS have been suggested to underlie the habituation (variously known as central adaptation, resetting, short-term depression, roll-off, temporal differentiation or high-pass

frequency filtering) of the response to sustained cardiopulmonary afferent inputs (38, 51, 52, 54, 66, 72). The induction of synaptic LTD is dependent on postsynaptic N-methyl-D-aspartate receptor (NMDAR) activity and intracellular Ca^{2+} levels similar to those in the hippocampus and neocortex (40, 42). Paradoxically, newborn mice with defective NMDARs due to genetic deletion of NMDAR subunit 1 robustly express an anomalous (NMDAR-independent) form of synaptic LTD in all NTS neurons *in vitro* while those mutant animals also demonstrate progressive respiratory failure *in vivo* (53), raising the specter that abnormal development of synaptic LTD in the NTS may predispose newborns to cardiorespiratory dysregulation. However, little information is currently available as to (i) the afferent phenotypes of type I and type II NTS neurons; and (ii) whether these neurons also express intrinsic LTP or LTD in addition to synaptic LTD.

In the present study, we used capsaicin and the nociceptin family of neuropeptides as functional probes to assess the afferent phenotypes of these neurons. The capsaicin-sensitive TRPV1 receptor (formerly vanilloid receptor 1), a heat and ligand-gated non-selective cation channel involved in nociception and hyperalgesia, is expressed throughout the length of nonmyelinated C-fibers and lightly myelinated Aδ-fibers of some primary afferents (6, 64). TRPV1-rich afferent fibers and terminals abound in rat NTS (20, 68); a subset of NTS neurons has been shown to exhibit capsaicin sensitivity (13, 31) thus providing a specific marker for tracking the connectivity of C- and Aδ afferent fibers to NTS neurons.

The neuropeptide nociceptin/orphanin FQ (N/OFQ, or nociceptin), an endogenous ligand for the opioid-like orphan receptor NOP1 (formerly ORL1) (46, 58), is known to modulate pain transmission and a variety of behavioral processes (reviewed in (45)). NOP1 receptors have been identified on sensory afferent fibers in NTS (41); microinjection of N/OFQ into various NTS subregions *in vivo* elicits distinct cardiovascular responses (63, 65). In addition, two derivatives

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of N/OFQ have also been found to modulate pain transmission: nocistatin (NST), an anti-nociceptin neuropeptide (49) and rat prepro-N/OFQ$_{154-181}$ (murine prepro-N/OFQ$_{160-187}$, ppN/OPQ), which produces complex anti-nociceptive or pro-nociceptive actions. These N/OFQ derivatives have been shown to modulate nociception by activating distinct capsaicin-sensitive or capsaicin-insensitive nociceptive fibers through different G protein-coupled signaling mechanisms (26), suggesting a capsaicin- and nociceptin-specific organization of certain pain pathways.

To correlate structural and functional phenotypes, we employed the technique of FPAP recording of spike-based neurotransmission (77) combined with whole-cell patch clamp recording and infrared imaging in rat brainstem slices *in vitro* to discern intrinsic and synaptic plasticity in mNTS. Our results revealed distinct expressions of intrinsic and synaptic LTD in type I and type II mNTS neurons that were specific to the relay of A- or C-fiber afferents. These findings identify the mNTS as potential pharmacological targets for modulating CNS habituation to adverse cardiopulmonary afferents hyperactivity.

MATERIALS AND METHODS

All experimental protocols were reviewed and approved by the M.I.T. Committee on Animal Care in compliance with the Animal Welfare Act and in accordance with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

Transverse brainstem slice preparation

Brainstem slices were obtained from 10 to 21-day old Sprague-Dawley rats (Charles River Laboratories) as described previously (76, 77). Briefly, under isoflurane anesthesia the animal

was decapitated and craniotomized. The brainstem was rapidly removed and cut into transverse slices (350 μm thick) on a Stoelting tissue chopper or a Vibratome 3000. Typically, the slice at the obex level was discarded and the next one or two slices just caudal to the obex were used for study. Throughout the procedure, exposed brain surfaces were irrigated with chilled and carbogen-saturated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) containing in mM: NaCl, 125; KCl, 3; $CaCl_2$ 2.5; $NaHCO_3$, 26; $MgSO_4$, 1.5; NaH_2PO_4, 1.5; glucose, 10; pH adjusted to 7.4 when necessary. After incubation in ACSF at room temperature (20-22°C) for at least 1 hr, the brainstem slice was transferred to a recording chamber on a microscope stage (Olympus, BX50WI), stabilized under a nylon mesh and submerged and continuously superfused with carbogen-saturated ACSF at room temperature.

A fine glass micropipette (tip diameter 1.8-2.0 μm, impedance 5-8 MΩ) filled with ACSF was micromanipulator-positioned (Newport, MX 100L) at the tractus solitarius (TS), which could be readily identified under the microscope as a small dark patch in the dorsomedial medulla. Electrical pulses (duration 0.1 ms) generated by a computer-triggered isolator (A.M.P.I., ISO-Flex) were delivered through this micropipette to the TS for focal afferent stimulation (Fig. 1a). In some experiments a tungsten microelectrode (Microprobes, tip diameter 1-2 μm, impedance 1 MΩ) was used for stimulation in place of glass micropipette. A silver/silver chloride ground pellet was placed in the recording chamber as reference electrode.

Field population action potential (FPAP) recording

The technique of FPAP recording from mNTS is as previously described (77). The NTS was imaged under DIC with an infrared CCD camera (Model VCB-3574IR, Sanyo) and the image was digitized with a video converter (Model PL-A544, PixeLINK). Under this image guidance, a

glass micropipette (tip diameter 4-8 μm, impedance 0.7-1.0 MΩ) filled with ACSF was micromanipulator-positioned (Sutter Instruments, MP-285) at mNTS regions that were 100-300 μm medial to the TS (Fig. 1a). To obtain maximal FPAP response, the tip of the recording electrode was placed at sites where densely packed neurons (cluster) were observed (Fig. 1b). The recorded FPAP signal was amplified (Axopatch 200A, Axon Instruments), low-pass filtered (5 kHz) and the resultant digitized data were stored on a computer. The stimulation intensity was initially set at 10 V for maximal activation of afferent fibers in the TS. Once a mNTS site with stable FPAP recording was located, the stimulation intensity was reduced to 1/2 the minimum voltage needed to evoke maximal FPAP volleys and was kept at this value (typically 3.5-5.0 V) throughout the experiment (Fig. 1c). Changes in the amplitude of FPAP at this stimulation intensity delivered at a nominal frequency of 0.1 Hz was used as a measure of changes in neurotransmission efficacy before and after low-frequency (5 Hz) afferent stimulation.

Whole-cell recording

In some experiments, whole-cell patch clamp recordings (Axopatch 200A, Axon Instruments) were obtained using a micropipette (resistance 4-10 MΩ) back-filled with solution containing (in mM): KCl 130, $CaCl_2$ 0.4, EGTA 1.1, $MgCl_2$ 1, NaCl 5, potassium HEPES 10, Mg^{2+}-adenosine triphosphate 2, and Na^{2+}-guanosine triphosphate 0.1, pH 7.2-7.3. After a neuron was approached under visualization via an infrared CCD camera and a gigaohm seal was formed, the cell membrane was ruptured by applying additional negative pressure to the micropipette to obtain whole-cell patch clamp under current clamp condition. Once in the whole-cell mode, a stabilization period of ~10 min was allowed before recordings began. Membrane characteristics were monitored periodically by injection of hyperpolarizing current pulses (−0.01

nA) through the patch electrode.

Whole-cell recorded EPSPs (Fig. 1b,c) had short latencies (1.06 ± 0.62 ms, n=13) and could faithfully follow a high-frequency test stimulus train (50 Hz, 7-12 pulses). Consequently, they were considered as monosynaptic responses to TS stimulation (12, 48).

Chemicals

(-)-D(-)-2-amino-5-phosphonopentanoic acid (D-AP5, Sigma) was dissolved in ACSF and diluted to 50 µM. Bicuculline (BIC, Sigma) was dissolved in dimethylsulfoxide (DMSO) and diluted with ACSF to 20 µM. Capsaicin (Sigma) was dissolved in ethanol and diluted with ACSF to 100 nM. N/OFQ, NST, ppN/OFQ (all from Phoenix Pharmaceuticals, Belmont, CA, USA) were dissolved in ACSF, each diluted to 300 nM. The ppN/OFQ was a C-terminal neuropeptide (rat prepro-N/OFQ$_{154-181}$ or murine prepro- N/OFQ$_{160-187}$) derived from the same precursor to N/OFQ and NST (26, 43, 60).

Data analysis

The baseline amplitude of evoked FPAP or EPSP responses in each slice was obtained by averaging 60 consecutive responses after stimulation for 10 min at 0.1 Hz. Data from each slice were normalized to their baseline value and averaged every 2 min before the group means ± S.E. were calculated. Differences between responses were evaluated by means of Student's t test at the 5% significance level unless otherwise specified.

RESULTS

Characteristics of evoked FPAP in mNTS

In the transverse brainstem slice preparation (Fig. 1a), the relatively short distance between the tips of the stimulation and recording electrodes (100-300 μm) keeps afferent propagation delay minimal, allowing synchronized activation of second-order neurons in mNTS for ensemble averaging into FPAP. The dominant FPAP volley was a negative wave with a short onset latency of 1.01-1.75 ms (1.21 ± 0.10 ms, mean ± SE, n=12), which was consistent with monosynaptic activation. Occasionally, the dominant wave was followed by a small positive volley with delayed onset latency of 2.92-3.37 ms (3.14 ± 0.23 ms, n=4). The negative volley was abolished after α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid (AMPA) receptor blockade and the positive volley was abolished after type A γ-aminobutyric acid receptor (GABA$_A$R) blockade; both volleys disappeared upon reversal of stimulation polarity or bath application of Na$^+$ ion channel blocker tetrodotoxin (TTX), further supporting their correspondence to excitatory/inhibitory neurotransmission rather than direct electrical depolarization/polarization of cell bodies (77). Because of the relatively sparse and irregular spatial organization of NTS neurons, only population spikes could be detected reliably with low-impedance electrodes although summated subthreshold EPSP's (if any) may contribute a minor component to the FPAP response (Fig. 1b). The amplitude of FPAP varied with stimulation intensity in a graded fashion above a threshold of 2.6-3.0 V required to trigger neuronal firing (77), indicating its correspondence to population spike rather than unit spike (Fig. 1c).

FPAP recording revealed type I and type II neurotransmission LTD in mNTS

Afferent stimulation at 5 Hz for 3 min (relative to 0.1 Hz during control periods) resulted in

two types of neurotransmission LTD in mNTS neurons as indicated by FPAP recordings (Fig. 2 *upper*). In type I (n=7), the FPAP response demonstrated a post-stimulation depression (to 84±2% of baseline) and a STD-like partial recovery within the first 5 min, culminating in a sustained depression (to ~90%; $p < 0.05$) for over 30 min. In type II (n=5), following an initial depression and STD-like recovery within the first 2 min as with type I, the FPAP response slipped to a nadir (70±2%, mean ± SE) at ~25 min before returning to 74±2% at 30 min.

In all slices, either type I or type II response might turn up (one recording per slice) and never any mixed response in between, i.e, with LTD levels intermediate between the type I and type II LTD levels. Analysis of variance showed that the within-group variability for each response type was negligible compared with the between-group difference ($p < 0.01$) indicating that there was no mixing of type I or type II responses in each recording. The graded and type-specific properties of FPAP seen in Fig. 2 and all data et seq. imply that mNTS neurons expressing type I or type II neurotransmission LTD were segregated into functionally homogeneous clusters with little mutual interference.

Synaptic and intrinsic components of type I and type II neurotransmission LTD

The type I and type II neurotransmission LTDs with STD-like partial recovery presently observed are reminiscent of the synaptic STD and LTD in type I and type II NTS neurons reported previously (76). To investigate whether synaptic LTD and/or intrinsic LTD contributed to type I and type II neurotransmission LTD, we repeated the above experiment under NMDAR blockade with or without GABA$_A$R blockade (Fig. 2 *lower*).

Blockade of NMDAR with D-AP5 (50 μM) revealed only type I neurotransmission LTD (along with STD-like initial partial recovery within the first 2 min) in all slices with none

indicating type II (n=5, <0.05). Bath application of D-AP5 (50 µM) together with BIC (20 µM) abolished all forms of neurotransmission LTD (both type I and type II) in all neurons (n=7, p < 0.05), further indicating that D-AP5 eliminated type II response.

These data suggest that type I and type II mNTS neurons (without and with synaptic LTD) could be readily distinguished by the corresponding type I or type II neurotransmission LTD revealed by FPAP recording. Type I neurotransmission LTD was GABA$_A$R-dependent (but NMDAR-independent) whereas type II neurotransmission LTD had both NMDAR-dependent and NMDAR-independent components. The NMDAR-dependent component probably reflected the synaptic LTD in type II neurons reported previously (76), which is abolished after D-AP5 application. It follows that type I neurotransmission LTD and the NMDAR-independent component of type II neurotransmission LTD were not synaptic LTD (since neither of them was abolished by D-AP5) but represented a novel intrinsic LTD component that was common to both type I and type II neurons.

Intrinsic LTD converts to NMDAR-dependent LTP after GABA$_A$R blockade

In contrast to synaptic plasticity, which is induced solely by excitatory postsynaptic NMDAR and non-NMDAR activities, intrinsic plasticity may also be subject to concurrent inhibitory activities that modulate neuronal excitability. To test this hypothesis, we repeated the 5-Hz TS stimulation experiment with or without GABA$_A$R blockade (Fig. 3a). Bath application of BIC (20 µM) had little effect on the initial depression of FPAP post-stimulation. The ensuing FPAP response demonstrated two types of neurotransmission plasticity similar to type I and type II LTD but slowly shifting upwards, so much so that the type I LTD converted to LTP (109.0±0.8%, n=6) after 20 min post-stimulation while the type II LTD was significantly reduced throughout the post-stimulation period (91.2±0.7% at 50 min, n=8).

The neurotransmission LTP manifested in type I neurons under BIC is ascribable to intrinsic LTP, since synaptic LTP was not induced by 5-Hz TS simulation in mNTS neurons under ACSF or BIC (Fig. 3b). Such an intrinsic LTP component may also account for the upward shift of the neurotransmission LTD in type II neurons under BIC (Fig. 3a *lower*). Interestingly, the intrinsic LTP in both type I and type II neurons were abolished (or diminished) by NMDAR blockade under BIC even though intrinsic LTD per se was not abolished by NMDAR blockade without BIC (Fig. 2 *lower*). These observations taken together suggest that in both type I and type II neurons, induction of intrinsic LTD is dependent on $GABA_AR$ activity (but *not* NMDAR activity) whereas induction of intrinsic LTP is dependent on NMDAR activity *and the absence of* $GABA_AR$ activity. The expressions of intrinsic LTP and intrinsic LTD in mNTS neurons are therefore determined by a delicate balance of NMDAR and $GABA_AR$ activities.

Capsaicin sensitivity in a subgroup of type II neurons

So far, the classification of type I and type II neurons has been based on the identification of synaptic or intrinsic LTD. Presynaptically, mNTS neurons receive both capsaicin-sensitive and capsaicin-insensitive afferents from cardiopulmonary sensory receptors (13, 31). To trace capsaicin-sensitive afferent projections to type I and type II neurons, we applied the TRPV1-selective agonist capsaicin after inducing LTD in mNTS neurons under normal ACSF or BIC-enriched ACSF. In both cases, bath application of capsaicin (100 nM) facilitated the evoked FPAP (Fig. 3a) or EPSP (Fig. 3b) in a subgroup of type II neurons but had no effects on all type I and other type II neurons ($p < 0.01$). Thus, capsaicin sensitivity provides a physiologic marker that selectively identifies a subgroup of type II neurons.

Nociceptin/prepro-nociceptin/nocistatin sensitivities in type I and other type II neurons

Another class of nociceptive neuromodulators is the family of nociceptin-related neuropeptides including N/OFQ, NST and ppN/OFQ (26, 43, 46, 49, 58, 60). To investigate whether mNTS neurons were sensitive to nociceptin and its derivatives as with capsaicin, we repeated the 5-Hz stimulation experiment with ppN/OFQ, NST, N/OFQ and capsaicin added sequentially to the bath after inducing LTD under normal ACSF. Type II neurons that were sensitive to capsaicin (n=5) did not respond to ppN/OFQ, NST or N/OFQ (Fig. 4a *upper*). However, in all type I (n=4) and other type II neurons (n=3) that were capsaicin-insensitive, the FPAP response was suppressed by N/OFQ and ppN/OFQ and facilitated by NST (all 300 nM) (Fig. 4a *lower*). Similar mutually exclusive sensitivities to capsaicin or nociceptin family of neuromodulators were also found in naïve mNTS neurons not subjected to 5-Hz stimulation (Fig. 4b), with all FPAP recordings responding to either capsaicin (p<0.01, n=5, Fig. 4b *upper*) or N/OFQ, NST and ppN/OFQ (p<0.01, n=7, Fig. 4b *lower*) but not both.

mNTS neurons are segregated into capsaicin-sensitive and nociceptin-sensitive clusters

Infrared imaging in 15 brainstem slices revealed that FPAP was discernible provided the tip of the recording electrode was positioned in or around (< 50 μm from) some densely packed neuronal clusters (Figs. 5, 6). FPAPs recorded from imaged clusters were sensitive to either N/OFQ (n=11) or capsaicin (n=4) at varying locations in mNTS (Fig. 5) and such specificity was expressed uniformly within each cluster (Fig. 6). In some imaged slices in which the perimeters of the neuronal clusters were traced, the sizes of nociceptin-sensitive and capsaicin-sensitive clusters were estimated to be roughly 160-190 μm (n=7) and 75-85 μm (n=3) in diameter, respectively (Figs. 5, 6). The separation between neighboring clusters was > 100 μm in most cases. Although the gap between neighboring clusters was not always totally void of neurons, the corresponding cell density was usually too low to produce discernible FPAPs. The remarkable

consistency of FPAP recording and infrared imaging results provides strong evidence that mNTS neurons are segregated into functionally homogeneous capsaicin-sensitive or nociceptin-sensitive clusters which are separated by sparse nonspecific cells in between.

DISCUSSION

Our results show that second-order relay neurons in mNTS are segregated into nociceptin-sensitive or capsaicin-sensitive clusters (Fig. 7). Each cluster demonstrates either NMDAR-independent (type I) or NMDAR-dependent (type II) neurotransmission LTD, which are comprised of $GABA_A$R-dependent intrinsic LTD without or with NMDAR-dependent synaptic LTD. Suppression of $GABA_A$R activity converts the intrinsic LTD to NMDAR-dependent intrinsic LTP. All type I neurons and ~50% of type II neurons are suppressed by N/OPQ and ppN/OPQ and facilitated by NST; the remaining type II neurons are facilitated by capsaicin.

Critique of methodology

Compared to intracellular or whole-cell recordings widely used for studying synaptic plasticity, our FPAP recording technique allowed stable long-term monitoring of ensemble-averaged transmission of action potentials, which was requisite for studying intrinsic plasticity. Our whole-cell and FPAP recordings in a transverse brainstem slice (which allowed short-latency synchronous neuronal firing requisite for FPAP recording) revealed similar mNTS neuronal sensitivities to capsaicin as those reported previously using whole-cell recordings in a longitudinal brainstem slice preparation (13, 31).

Because neuronal structures in unstained brainstem slices *in vitro* are not well demarcated, the NTS region under study could extend beyond mNTS to encroach on other subnuclei, such as

the intermediate and central NTS and part of the comNTS, which are all located medial to the TS (14, 74). Since similar sensory afferent fibers tend to terminate in the same NTS subnucleus (1, 73) and many NTS neurons are coupled via gap junctions (11, 24), it is not surprising that neurons in a given cluster are functionally homogenous. Such a compartmental organization of the NTS with segregated afferent and synaptic phenotypes (Fig. 7) suggests distinct physiological roles played by type I and type II NTS neurons.

Intrinsic and synaptic LTD in NTS: distinct habituation mechanisms for cardiopulmonary afferents hyperactivity

The capsaicin-sensitive type II neurons presently reported can be identified with certain capsaicin-sensitive cardiopulmonary afferents including non-myelinated C-fibers and lightly myelinated small Aδ-fibers — all of which terminate in comNTS and mNTS (10, 13, 31, 34). This class of afferents arises mostly from aortic baroreceptors or vagal bronchpulmonary C-fiber receptors and irritant receptors that are sensitive to noxious stimuli, temperature, or inflammatory and immunological mediators. These afferents participate in certain physiologic functions that may benefit from synaptic and intrinsic LTD in NTS relay neurons. For example, activation of bronchopulmonary C-fibers (such as during chronic airway inflammation and asthma) may elicit burning chest pain sensations and a variety of physiologic reactions that tend to suppress cardiorespiratory function and promote cough and bronchoconstriction (35). Although such acute effects may serve an immediate defensive function, prolonged respiratory hyperresponsiveness could cause cardiorespiratory insufficiency and physical discomfort that are detrimental to survival. These adverse sequelae of chronic bronchopulmonary insults may be mitigated by a combination of synaptic and intrinsic LTD in the NTS.

Similarly, the synaptic and intrinsic LTD in capsaicin-sensitive type II NTS neurons provide possible cellular correlates of the putative central baroreflex resetting (habituation) (17, 23) which preserves the baroreflex sensitivity to short-term blood pressure fluctuations in chronic hypertension (22). Conversely, recent studies show that animals at risk of heatstroke hypotension are protected by an augmented baroreceptor reflex sensitivity via heat shock protein-mediated up-regulation of glutamate receptors in NTS neurons (7). Such self-adaptive up- or down-regulation of NTS relay of baroreceptor afferent inputs helps to restore normal baroreflex sensitivity in the face of sustained hypo- or hypertension.

The afferent origins of capsaicin-insensitive NTS neurons are less clear. Since capsaicin-sensitive type II neurons are linked to capsaicin-sensitive non-myelinated C-fiber and lightly myelinated small Aδ-fiber afferents, the remaining type II neurons likely receive capsaicin-insensitive C- or Aδ-fiber afferents. Indeed, recent studies have identified a capsaicin-insensitive subclass of vagal bronchopulmonary C-fiber afferents (33) and capsaicin-insensitive Aδ-fiber afferents that arise from rapidly-adapting bronchopulmonary stretch receptors (59, 69). Accordingly, it may be concluded that type II neurons likely receive predominantly C- and Aδ-fiber afferents which could be either capsaicin-sensitive or −insensitive, whereas type I neurons most likely receive myelinated A-fiber (Aα and possibly some Aδ) afferents which are exclusively capsaicin-insensitive (Fig. 7). The latter arise mostly from carotid baro/chemoreceptors and vagal pulmonary slowly-adapting stretch receptors that mediate cardiorespiratory regulation. Thus, type I neurotransmission LTD (NMDAR-independent intrinsic LTD) provides a possible neural substrate for cardiorespiratory habituation to baro/chemo- or volume-related afferents hyperactivity which may occur in a variety of physiologic challenges such as sustained elevated lung volume in chronic obstructive pulmonary

disease or continuous positive-airway-pressure therapy for sleep apnea, metabolic acidosis, exposure to sustained hypoxia at high altitude, etc.

The suggested association of type I behavior with A-fiber afferents important for homeostatic regulation is further supported by several lines of evidence. First, the type I (NMDAR-independent) neurotransmission LTD presently observed is analogous to the NMDAR-independent habituation effects previously demonstrated in two respiratory reflex mechanisms: the carotid chemoreflex modulation of the hypoxic ventilatory response and vagal Hering-Breuer lung inflation reflex modulation of the respiratory rhythm (66, 72). Second, mutant mice which exhibit anomalous (NMDA receptor-independent) synaptic LTD in nearly all NTS neurons suffer progressive respiratory depression shortly after birth, suggesting that synaptic LTD (which is normally absent in type I neurons) may be deleterious to respiratory regulation when expressed in these neurons (53).

Taken together, these observations support the notion that, whereas intrinsic LTD is common to all NTS neurons and is a universal mechanism of habituation for all cardiorespiratory afferents, synaptic LTD in type II NTS neurons is specific to C-fiber afferents and provides a booster mechanism for the habituation of noxious inputs associated with hypertension, chronic airway inflammation, asthmatic bronchoconstriction and other cardiopulmonary derangements. Since NMDARs are clearly expressed in both type I and type II neurons, the absence of NMDAR-dependent synaptic LTD in type I neurons suggests that these neurons lack the intracellular calcium-dependent cascade of reactions that mediate synaptic LTD in type II neurons.

GABA$_A$R and NMDAR activities differentially regulate intrinsic LTD and intrinsic LTP

In the transverse brainstem slice, electrical stimulation of TS evokes complex EPSP and inhibitory postsynaptic potential (IPSP) in NTS neurons as recorded intracellularly (16) or extracellularly (77). The inhibitory response reflects the activation of local GABAergic interneurons, which play an important role in cardiorespiratory regulation (55, 71). Recent studies suggest that glutamatergic inputs in NTS may up- or down-regulate GABA release in inhibitory interneurons via distinct presynaptic metabotropic glutamate receptors (30). The present results show that GABAergic inputs, in turn, could modulate NTS excitatory transmission by promoting intrinsic LTD and suppressing intrinsic LTP in both type I and type II neurons. The intrinsic LTD is $GABA_AR$-dependent but NMDAR-independent, and is consistent with the reported LTP of inhibitory transmission in NTS (16). By contrast, the intrinsic LTP is NMDAR-dependent and is expressed only in the absence of $GABA_A$ activity. Thus, the preferential expression of intrinsic LTD is determined by a fine balance between $GABA_AR$ and NMDAR activities. Disruption of this balance may lead to intrinsic LTP and hence, exacerbation of the adverse responses to cardiopulmonary afferents hyperactivity.

Capsaicin-nociceptin divisions of NTS: parallelism with pain pathways

Although visceral sensation and nociception have traditionally been considered as distinct sensory modalities, they exhibit many similarities both at the level of central representation and resultant behavioral and autonomic responses (19, 62). At the afferent level, vagal C-fibers and somatosensory C-fibers are akin to one another morphologically, physiologically and pharmacologically (36). Not surprisingly, certain visceral afferent effects bear close resemblance to those of nociception. For example, the hyperresponsiveness to inflammatory mediators during cough and asthmatic bronchoconstriction has been likened to primary hyperalgesia resulting from sensitization of capsaicin-sensitive pain pathways (5, 67, 69). In addition to peripheral

sensitization at the sensory receptor terminals or cell bodies, capsaicin-sensitive cardiopulmonary afferents may also contribute to central sensitization plasticity through the release of substance P or other neurokinins at their central ends in NTS (3, 69). In pain pathways, central sensitization refers to the synaptic LTP-like increases in synaptic efficacy in somatosensory neurons in the spinal dorsal horn resulting in hyperalgesia (25, 28), whereas synaptic LTD in these neurons may lead to hypoalgesia (32, 56). The present finding of synaptic and intrinsic LTD in capsaicin-sensitive type II neurons provides a central mechanism of hypoalgesia-like habituation that could attenuate the adverse cardiorespiratory reflexes secondary to visceral nociceptive afferent hyperactivity.

Adding to the parallelism between nociception and visceral sensation, recent studies (8, 15, 44) showed that capsaicin-mediated bronchoconstriction and respiratory hyperresponsiveness to inflammatory mediators are inhibited by N/OFQ -- a peptide nociceptive neuromodulator that produced bidirectional dose-dependent hyperalgesic or analgesic effects in pain pathways (45, 50, 61). These previous findings suggest that capsaicin-sensitive and nociceptin-sensitive pathways may interact in modulating pain and respiratory hyperresponsiveness.

Surprisingly, the present study demonstrates exclusively capsaicin-sensitive or nociceptin-sensitive neurons in NTS indicating that the capsaicin receptor TRPV1 and N/OFQ receptor NOP1 probably do not co-localize at the central ends of these afferents. Thus, any central mechanism of N/OFQ inhibition of capsaicin-induced cough (44) is likely to occur in post-NTS sites. The responsiveness of capsaicin-insensitive NTS neurons to N/OPQ, NST and ppN/OPQ suggests a co-localization of NOP1 with the receptors to NST and ppN/OPQ in capsaicin-insensitive but not capsaicin-sensitive TS afferents. This finding is in contrast to the distinct expressions of NST and ppN/OPQ sensitivities in corresponding capsaicin-sensitive or capsaicin-insensitive nociceptive pathways (26, 70).

On the other hand, it is not certain whether such segregation of capsaicin and nociceptin sensitivities in NTS neurons extends to the peripheral ends of the corresponding afferents. Thus, peripheral neurogenic mechanisms of N/OFQ inhibition of capsaicin-sensitive bronchoconstriction and respiratory hyperresponsiveness (15, 29, 44) could involve the interplay of TRPV1 and NOP1 on the same sensory neuron or distinct capsaicin-sensitive and nociceptin-sensitive sensory neurons or their receptor terminals. There is evidence that capsaicin-insensitive somatosensory pathways can be indirectly sensitized by capsaicin-sensitive pathways, producing secondary hyperalgesia effects via high-threshold Aδ afferents or allodynia effects via Aβ afferents (32, 39). In a similar fashion, secondary hyperalgesia or allodynia-like sensitization of capsaicin-insensitive vagal afferents (e.g. rapidly adapting pulmonary stretch receptor afferents) or their central relays via nociceptin-sensitive NTS neurons could contribute to the respiratory hyperresponsiveness and bronchoconstriction secondary to the activation of capsaicin-sensitive afferents. This possible mechanism of secondary plasticity involving nociceptin-sensitive, capsaicin-insensitive vagal-NTS pathways is consistent with the suggested allodynic effect of N/OFQ (21, 27, 45).

Conclusion

In conclusion, we have shown that second-order mNTS neurons are segregated into neuronal clusters that receive distinct nociceptin-sensitive or capsaicin-sensitive cardiorespiratory afferents, a neuronal organization that bears close resemblance to that of pain pathways. Nociceptin-sensitive neurons are either type I or type II (labeled as type IIA) neurons, whereas capsaicin-sensitive neurons are exclusively type II (labeled as type IIC) neurons (Fig. 7). Both type I and type II neurons exhibit NMDAR-independent intrinsic LTD, a novel form of neurotransmission plasticity that is distinct from the previously reported NMDAR-dependent

synaptic LTD in type II neurons (53, 76). Thus, intrinsic LTD constitutes the neurotransmission LTD in type I neurons whereas both intrinsic LTD and synaptic LTD contribute to the neurotransmission LTD in type II neurons. Intrinsic LTD is $GABA_AR$-dependent but NMDAR-independent, whereas intrinsic LTP is NMDAR-dependent and is expressed only in the absence of $GABA_A$ activity. Thus, the expressions of intrinsic LTD and LTP are determined by a fine balance between $GABA_AR$ and NMDAR activities. We suggest that intrinsic LTD and synaptic LTD provide distinct afferents-specific cellular mechanisms of response habituation to hyperactivity of cardiorespiratory A- and C-fiber inputs. These findings identify intrinsic and synaptic plasticity in the NTS as potential pharmacological targets to allay the sequelae of varying physiologic challenges and cardiopulmonary derangements, such as hypertension, chronic cough, asthmatic bronchoconstriction, hypoxia, metabolic acidosis, and sustained elevated lung volume in chronic obstructive pulmonary disease or continuous positive-pressure therapy for sleep apnea.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Fig. 1. Recording of evoked FPAP and EPSP. a: Schematic showing electrical stimulation at the TS and recording from mNTS with transverse brainstem slice preparation. **b:** Schematic illustration of extracellular multi-unit recording of FPAP compared with whole-cell single-unit recording of EPSP from mNTS second-order relay neurons. **c:** *upper*, EPSP obtained by whole-cell current clamp recording; *middle*, FPAP recorded with extracellular low-resistance electrode; *bottom*, three recordings of FPAPs evoked at 2.9 V (top trace), 3.9 V (middle trace), and 4.9 V (bottom trace) recorded from the same site and superimposed to show that the amplitude of FPAP is graded by the stimulation intensity instead of all-or-none (see (77) for further details). The response is deemed monosynaptic if the time between stimulation and peak response is within 3-5 ms (76, 77).

Fig. 2. Two types of neurotransmission LTD and their intrinsic and synaptic components in mNTS neurons. *Upper* panel: Type I and type II neurotransmission LTD as indicated by FPAP (means ± SE) after 5-Hz TS stimulation in ACSF. *Lower* panel: 5-Hz TS stimulation under NMDAR blockade by D-AP5 resulted in only one type of neurotransmission LTD (open circles) which is identical to type I neurotransmission LTD expressed in ACSF (*half-tone line*, redrawn from *upper* panel for comparison). Simultaneous NMDAR and GABA$_A$R blockade by D-AP5 and BIC suppressed neurotransmission LTD in all neurons (filled circles).

Fig. 3. GABA$_A$ receptor blockade converts intrinsic LTD to intrinsic LTP in capsaicin-sensitive and capsaicin-insensitive mNTS neurons. a: FPAP recordings. *Upper* panel: Capsaicin selectively facilitated neurotransmission in ~50% of type II neurons (n=3) but none of

the type I (n=4) or remaining type II neurons (n=3). *Lower* panel: Blockade of $GABA_A$R with BIC converted intrinsic LTD to LTP in type I neurons and reduced neurotransmission LTD in type II neurons. The proportions of type I and type II neurons are similar to those reported previously using whole-cell recording (76). **b:** Corresponding EPSP recordings obtained with a whole-cell patch clamp electrode instead of extracellular electrode. The proportions of type I and type II neurons are different than (**a**) or previously reported (76) because recordings that were unstable during the capsaicin test at the end were excluded.

Fig. 4. Distinct sensitivities to capsaicin and nociceptin family of neuropeptides in mNTS neurons. a: FPAP response of all type I neurons (n=4) and capsaicin-insensitive type II neurons (n=3) were suppressed by N/OFQ and ppN/OFQ, and facilitated by NST (*lower* panel), whereas FPAP response of capsaicin-sensitive type II neurons were unaffected (*upper* panel). **b:** Similar effects (as measured by changes in normalized FPAP relative to ACSF) were obtained in separate slices for naive mNTS neurons not subjected to 5-Hz afferent stimulation.

Fig. 5. FPAPs recorded from separate neuronal clusters in mNTS demonstrate distinct sensitivities to nociceptin or capsaicin. In this brainstem slice, two separate neuronal clusters were observed with infrared imaging. Cluster 1 (~200 × ~150 μm) was larger than cluster 2 (diameter ~100 μm), but neurons were more densely packed in the latter. FPAP recorded from cluster 1 was depressed by nociceptin (N/OFQ), but not affected by capsaicin. FPAP recorded from cluster 2, which was located ~200 μm ventrolateral to cluster 1, was non-responsive to nociceptin, but potentiated by capsaicin.

Fig. 6. FPAPs recorded from different sections within each neuronal cluster show similar responsiveness to capsaicin or nociceptin. *Photos*: In this brainstem slice, infrared imaging identified a large neuronal cluster with diameter of ~200 μm. FPAP recordings were made at three different sites (1, 2, and 3, separated by ~60 μm) within this cluster and at a control site (C) outside the cluster. *Traces at bottom*: FPAPs recorded at sites 1-3 had similar waveforms and latencies; FPAP was not discernible at site C. Bar graphs: Bath application of N/OFQ depressed the FPAPs at sites 1, 2 and 3.

Fig. 7. Compartmental organization of mNTS neurons according to afferent, synaptic and intrinsic phenotypes revealed by whole-cell and FPAP recordings. Second-order relay neurons in mNTS have been previously classified into two main types (76): Type I neurons do not express NMDAR-dependent synaptic LTD, but type II neurons do. The present findings necessitate a sub-classification of type II neurons into A and C groups depending on their specific afferent sensitivities to either nociceptin family of neuropeptides or capsaicin. *Left panel*: Type I neurons are capsaicin-insensitive/nociceptin-sensitive. These neurons likely receive A-fiber (α, β or δ) afferents. Type II neurons are further classified into type IIA and type IIC subgroups. Type IIA neurons probably receive C- or Aδ-fiber afferents that are capsaicin-insensitive/nociceptin-sensitive, whereas type IIC neurons likely receive C- or Aδ-fiber afferents that are capsaicin-sensitive/nociceptin-insensitive. All mNTS neurons express intrinsic LTD (Intr-LTD) regardless of their afferent and synaptic types. *Middle panel*: Under GABA$_A$R blockade the Intrinsic LTD converts to intrinsic LTP (Intr-LTP). *Right panel*: Under simultaneous GABA$_A$R and NMDAR blockade both synaptic LTD and intrinsic LTD/LTP are suppressed.

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a

Recording

Stim.

mNTS

TS

12

b

whole-cell rec.

FPAP rec.

c

EPSP

FPAP

FPAP

Stim.

0.5 mv

2 ms
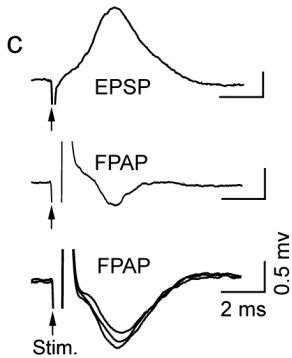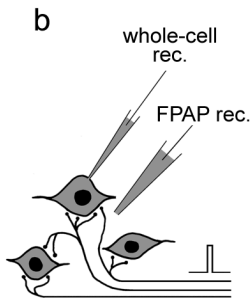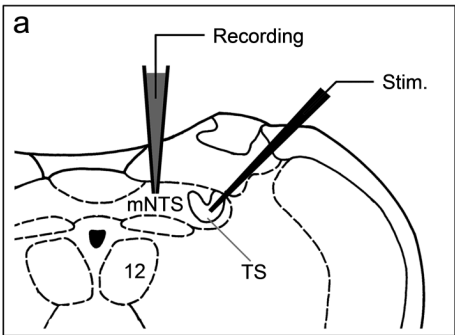
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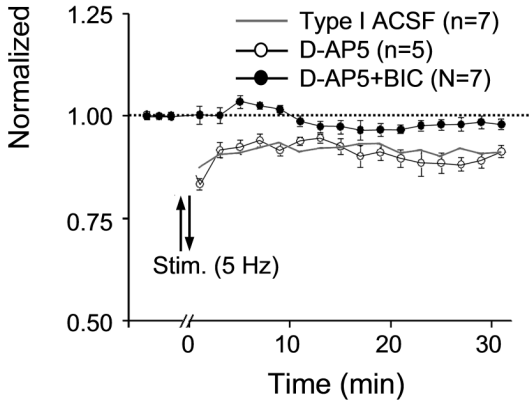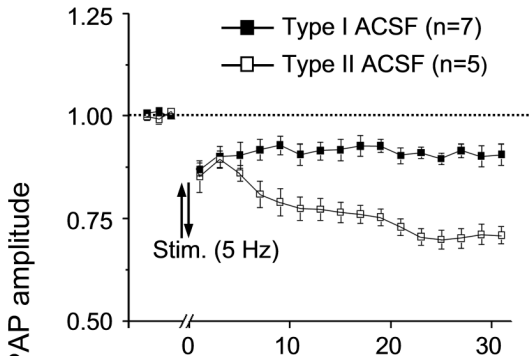
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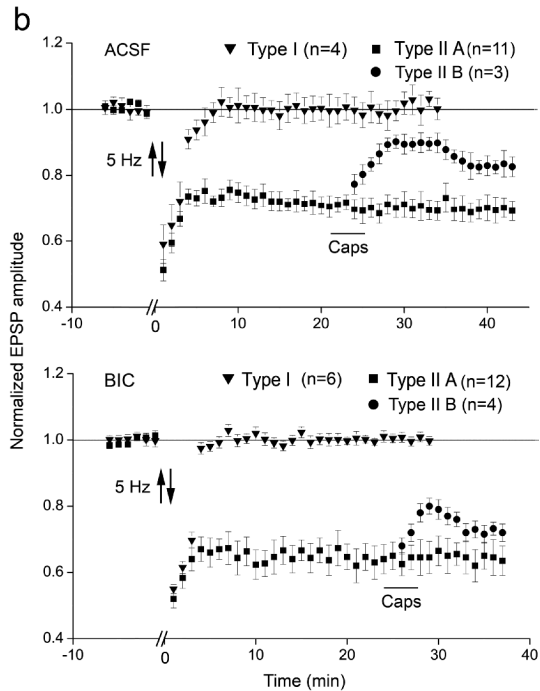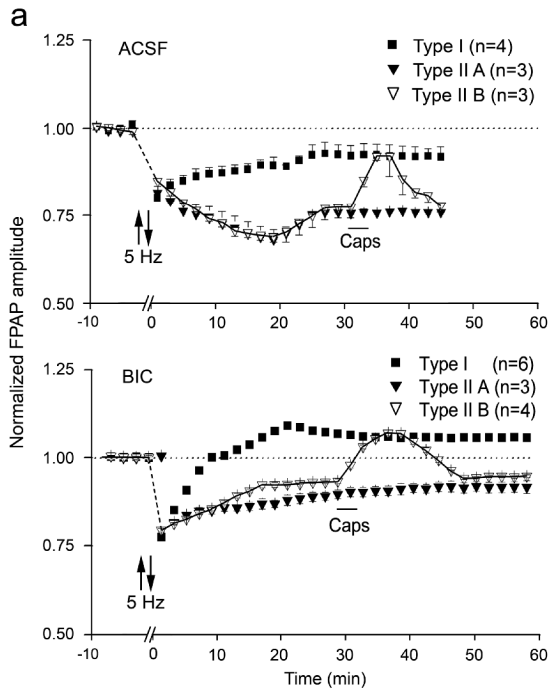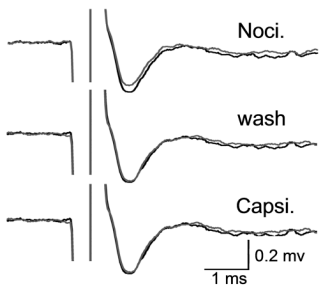
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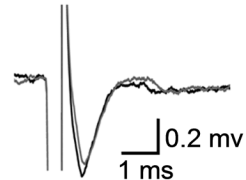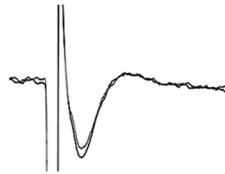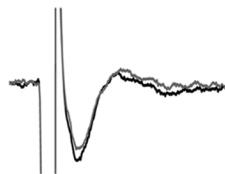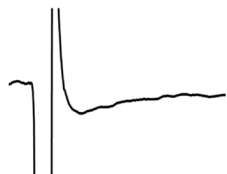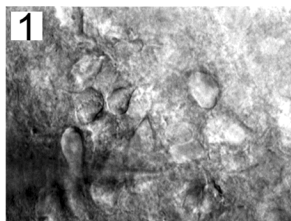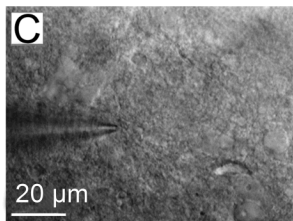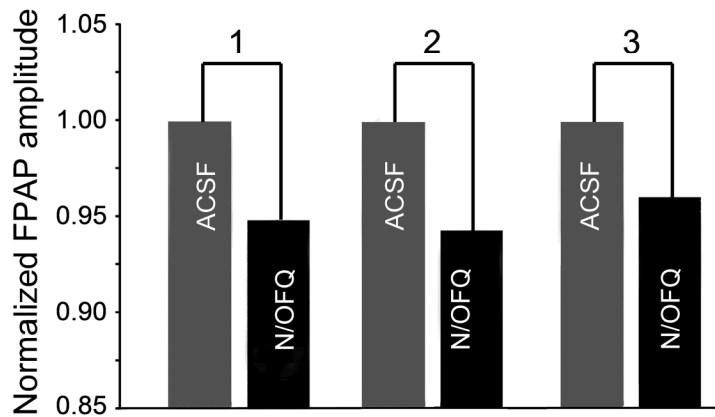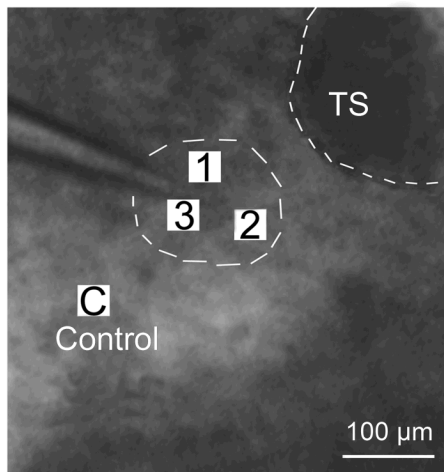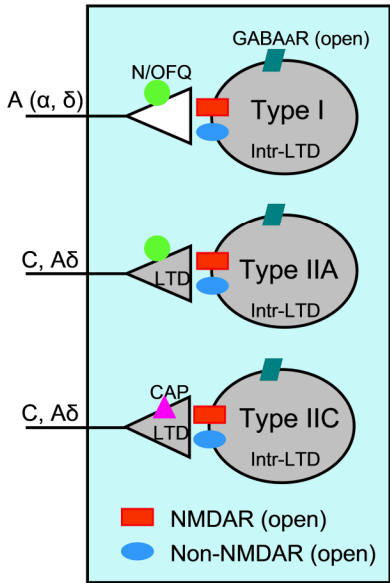Filename: fig_4.tif
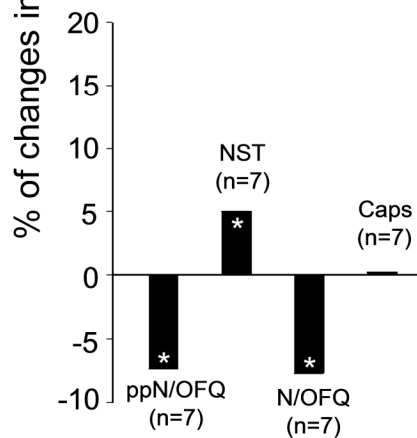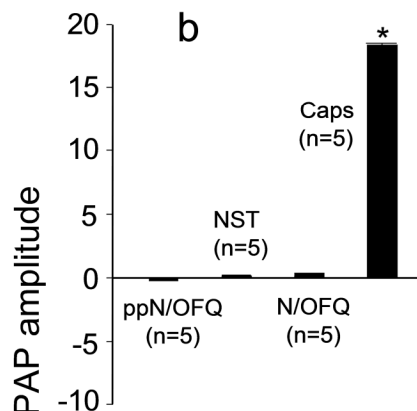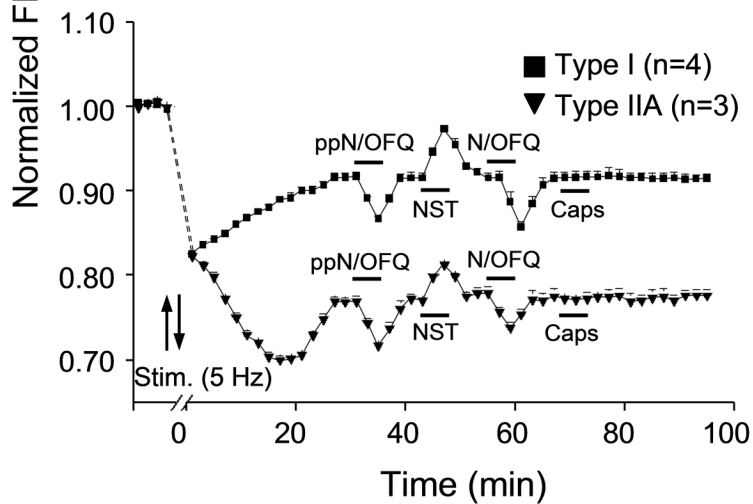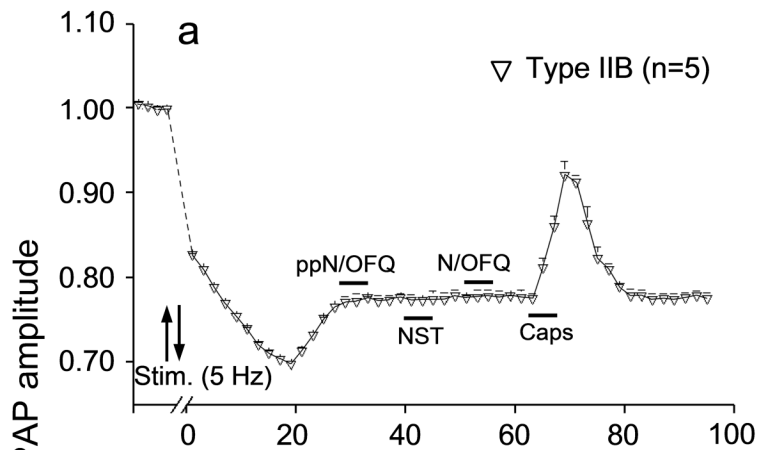
Type of file: figure
Label: Figure 5
Filename: fig_5_small.tif

Noci.

wash

Capsi.

0.2 mv

1 ms

Noci.

wash

Capsi.

0.2 mv

1 ms

1

2

20 μm

1

2

100 μm
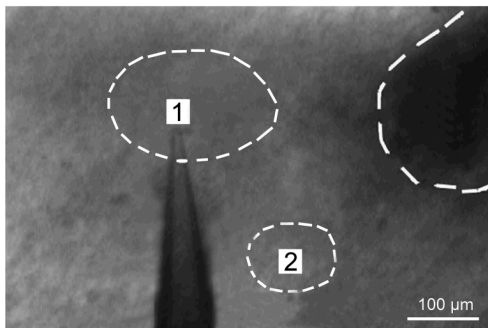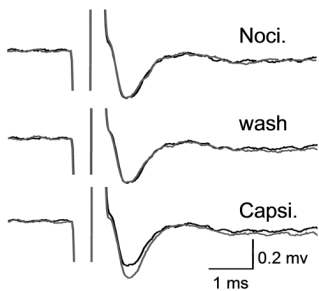
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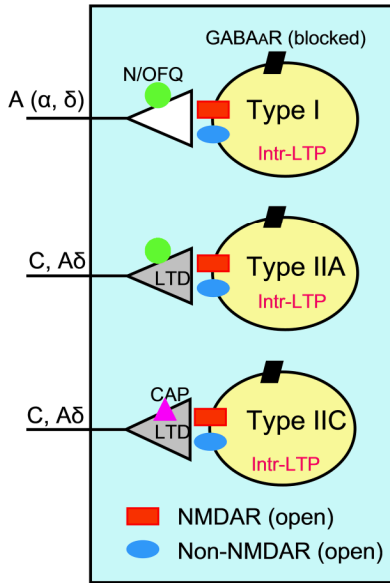Label: Figure 6
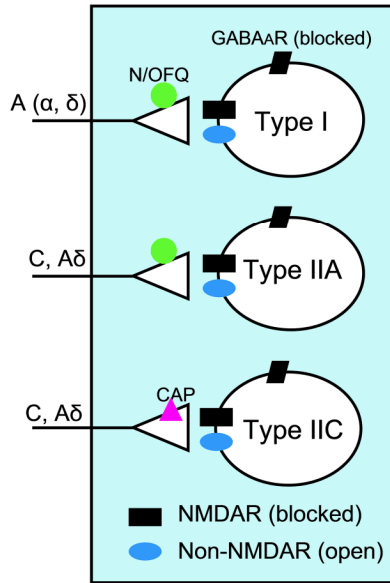Filename: fig_6_small.tif
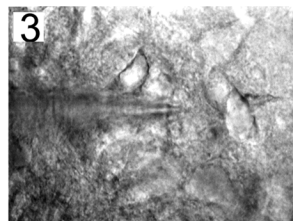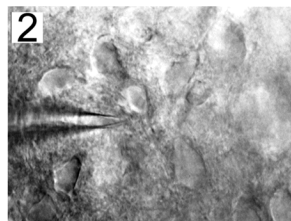
Type of file: figure
Label: Figure 7
Filename: fig_7.tif

ACSF

GABA$_A$R blocked

GABA$_A$R/NMDAR blocked