Title: Frequency-dependent release of substance P mediates heterosynaptic potentiation of glutamatergic synaptic responses in the rat visual thalamus

Abbreviated title: Substance P potentiation in the thalamus

Authors: Sean P. Masterson, Jianli Li1, and Martha E. Bickford*

Anatomical Sciences and Neurobiology, University of Louisville

*Correspondence: Martha E. Bickford, Department of Anatomical Sciences and Neurobiology, University of Louisville, School of Medicine, 500 S. Preston St., Louisville, KY 40292. email: martha.bickford@louisville.edu phone: 502-852-3527 FAX: 502-852-6228

1Current address: Dept of Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines road, La Jolla, CA 92037.

Figures: 9

Tables: 0

Text pages: 33

Keywords: lateral posterior nucleus, tectothalamic, corticothalamic, superior colliculus, synaptic plasticity

Acknowledgements:
We thank Arkadiusz Slusarczyk for his expert technical assistance. This work was supported by NINDS grants R01NS35377 and F31NS052012.

Reviewing Editor: Dr. Sacha Nelson
Abstract

To investigate the interaction between peptides and glutamatergic synapses in the dorsal thalamus, we compared the frequency-dependent plasticity of excitatory postsynaptic potentials (EPSPs) in the tectorecipient zone of rodent lateral posterior nucleus (LPN), which is densely innervated by axons that contain the neuromodulator substance P (SP). Immunocytochemistry and confocal and electron microscopy revealed that neurokinin 1 (NK1) receptors are distributed on the dendrites of LPN cells, while SP is contained in axons originating from the superior colliculus (SC) and is reduced following SC lesions. In vitro whole cell recordings in parasagittal slices revealed that stimulation of the SC or optic radiations (CTX) evoked LPN EPSPs that increased in amplitude with increasing stimulation intensity, suggesting convergence. With 0.5-10Hz stimulus trains, CTX EPSP amplitudes displayed frequency-dependent facilitation, while SC EPSP amplitudes were unchanged. High frequency SC stimulation (100Hz for 0.5 seconds), or bath application of SP, resulted in gradual increases in both SC and CTX EPSP amplitudes to 2 fold or greater above baseline within 15-20 minutes post stimulation/application. This enhancement correlated with increases in input resistance, and both the potentiation and resistance change were abolished in the presence of the NK1 antagonist L-703,606. These results indicate that SP is released when SC-LPN neurons fire at high frequency, and SP acts postsynaptically via NK1 receptors to potentiate subsequent rLPN responses to both cortical and tectal inputs. We suggest that the SP-mediated potentiation of synaptic responses may serve to amplify responses to threatening objects that move across large regions of the visual field.
**Introduction**

The efficacy of sensory information transfer through the dorsal thalamus is subject to state-dependent fluctuations in the membrane properties of thalamic neurons and their synaptic inputs (Sherman, 2001). A particularly robust transformation of sensory signals is mediated by the frequency-dependent plasticity of glutamatergic synapses. For example, the amplitudes of retinogeniculate excitatory postsynaptic potentials (EPSPs) remain stable at low stimulation frequencies (< 1 Hz), but decrease in amplitude as the stimulation frequency is increased (2-20Hz; Turner and Salt, 1998, Chen et al., 2002, Chen and Regehr, 2003). In contrast, the amplitudes of corticogeniculate EPSPs remain stable at low stimulation frequencies, but facilitate with increasing stimulation frequency (Lindstrom and Wrobel, 1990, Turner and Salt, 1998, von Krosigk et al., 1999, Granseth et al., 2002). Higher frequency stimulation (50-500Hz) of corticogeniculate fibers activates metabotropic glutamate receptors, resulting in slow EPSPs that can depolarize the membrane potential of geniculate neurons for 20 seconds or more (McCormick and von Krosigk, 1992). Moreover, tetanic stimulation of corticothalamic fibers in the ventrobasal nucleus results in a long-term (1 hour or more) potentiation of these synapses (Castro-Alamancos and Calcagnotto, 1999).

Further modulation of thalamic transmission is made possible by a rich network of fibers that contain a wide variety of neuropeptides. However, while a number of studies have demonstrated that the membrane properties of thalamic neurons can be significantly altered by the exogenous application of neuropeptides (Cox et al., 1997, Sun et al., 2002, Govindaiah and Cox, 2006, Lee and Cox, 2006, Brill et al., 2007, Lee and Cox, 2008; Paul and Cox, 2010), or by their release from intrinsic interneurons (Sun...
et al., 2003), little is known regarding the conditions under which neuropeptides are released by extrinsic inputs to the thalamus, or their effects on neuronal responses to conventional neurotransmitters. This is an important avenue of investigation because the ability to manipulate thalamic synaptic efficacy could be used to modify abnormal thalamic activity patterns that occur in conditions such as epilepsy or neuropathic pain.

In other areas of the brain, neuropeptides have been shown to amplify glutamatergic postsynaptic responses. For example, in the spinal cord SP release from C fibers is dependent on the frequency of their stimulation, and the binding of SP to neurokinin 1 (NK1) receptors results in the amplification of glutamatergic EPSPs (Adelson et al., 2009). To examine conditions that induce thalamic neuropeptide release and subsequent effects on glutamatergic transmission, we examined the frequency-dependency of corticothalamic and tectothalamic responses in the rat lateral posterior nucleus (LPN). We previously demonstrated that within the tectorecipient zone of the LPN cortical terminals (which contain the type 1 vesicular glutamate transporter, vGLUT1) are located distal to tectal terminals (which contain the type 2 vesicular glutamate transporter, vGLUT2) on the dendrites of projection neurons (Masterson et al., 2009). The LPN is also densely innervated by fibers that contain SP, which likely arise from the superior colliculus (SC; Hutsler and Chalupa, 1991). Thus the LPN provides a model system to examine the interactions of peptides and glutamatergic synapses in the thalamus. Our results demonstrate that substance P release from tecto-LPN axons is frequency-dependent and, via binding to NK1 receptors, can provide a sustained potentiation of both tectal and cortical glutamatergic synaptic responses.
Methods

All procedures conformed to the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the University of Louisville Animal Care and Use Committee.

Tract tracing and lesions

Two adult Long Evans (hooded) rats received bilateral injections of biotinylated dextran amine (BDA; Molecular Probes, Carlsbad, CA) in the SC, and 2 rats received unilateral injections of ibotenic acid (Sigma Chemical Company, St Louis, MO) in the SC. The rats were anesthetized with intraperitoneal injections of ketamine (initially 75mg/kg) and xylazine (initially 8mg/kg), with supplements injected as needed to maintain anesthesia. They were placed in a stereotaxic apparatus and prepared for aseptic surgery. A small craniotomy was made above the SC and BDA (5% in saline, 5 µl) or ibotenic acid (Sigma Chemical Company, St Louis, MO, 10 µl) was injected through a glass micropipette (10 µm tip diameter) using a PV83 pneumatic picopump (WPI, Sarasota, FL). After a survival time of one week, the rats were transcardially perfused with ACSF followed by a fixative solution of 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (PB). The brain was removed from the skull, and a vibratome was used to cut sections in the parasagittal plane to a thickness of 50 µm.

Histochemistry

To examine the distribution of SP and NK1 in the LPN, 8 rats were deeply anesthetized and transcardially perfused with ACSF followed by a fixative solution of 4% paraformaldehyde or 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 (PB). The brain was removed from the skull, and a vibratome was used to
cut sections in the parasagittal plane to a thickness of 50 µm. Sections from these rats, as well as sections from rats that received ibotenic acid injections in the SC, were incubated in either a rat-anti-SP antibody (Accurate Chemical Company, Westbury, NY) diluted 1:500 or a rabbit-anti-NK1 antibody (Chemicon, Billerica, MA) diluted 1:4000 in 0.01M phosphate buffer, 0.9% NaCl (PBS) and 1% normal goat serum. The following day the sections were rinsed in PB and incubated for 1 hour in biotinylated goat-anti-rat or biotinylated-goat-anti-rabbit antibodies (Vector Laboratories, Burlingame, CA) diluted 1:100. They were then incubated for an hour in a solution of 1:100 dilution of avidin and biotinylated-horseradish peroxidase (ABC solution), reacted with nickel-enhanced diaminobenzidine, and mounted on slides or prepared for electron microscopy.

For electron microscopy, sections were postfixed in 2% osmium tetroxide in PB for 1 hour and then dehydrated through a graded series of ethyl alcohol (70-100%) and embedded in Durcupan resin (Ted Pella, Redding, CA) between sheets of Aclar plastic (Ladd Industries Inc., Burlington, VT). A light microscope was used to identify areas of interest, which were excised and mounted on resin blocks. A diamond knife was used to cut ultrathin sections, which were placed on Formvar-coated nickel slot grids, air dried, and stained with a 10% solution of uranyl acetate in methanol for 30 minutes before examination with an electron microscope. SP or NK1-labeled profiles were digitally captured and categorized based on ultrastructural features.

The size of SP-stained profiles was measured using SigmaScan software (SPSS Inc., Chicago, IL) for statistical comparison to previous data (Masterson et al., 2009). The perimeter of each profile was manually traced and the minor axis was calculated. The following description from the SigmaScan manual best describes this calculation: “The
major axis is a straight line connecting the two pixels in the object which have the
greatest distance between them. The minor axis is a straight line which is perpendicular
to the major axis and connects the two pixels in the object with the greatest distance
between them. The pixels used to define these axes are found by sampling the edge pixels
of an object”. We chose the minor axis measurement to compare profile sizes because
this measure best offsets differences in profile size introduced by different labeling
methods. For example, profile measurements of terminals labeled with anterograde
tracers may include small lengths of pre-terminal axons, while immunocytochemical
labeling is more likely to be restricted to the synaptic terminal. Minor axis measurements
are expressed as mean ± SD and compared to previous data sets with unpaired t-tests.

To determine whether tectothalamic terminals contain SP, sections from rats that
received SC BDA injections were incubated overnight at 4°C in a solution of streptavidin
conjugated to Alexa 546 (Molecular Probes, Carlsbad, CA) diluted 1:100 and rat-anti-SP
antibody (Accurate Chemical Company) diluted 1:250 in PBS and 1% normal goat
serum. The following day the sections were rinsed in PB and incubated for 1 hour in a
goat-anti-rat antibody conjugated to Alexa 488 diluted 1:100. After rinsing in PB, the
sections were mounted on slides and viewed with a laser scanning confocal microscope
( Olympus, Center Valley, PA).

**Preparation of LPN slices**

Long Evans (hooded) rats of both sexes (16-45 days old) were anesthetized with
carbon dioxide and decapitated. The brains were hemisected and quickly transferred to a
cold (4°C) oxygenated cutting solution containing (in mM): sucrose 206; KCl 2.5; CaCl2
1; MgSO4 1; MgCl2 1; NaH2PO4 1.25; NaHCO3 26; and d-glucose 10 at a pH of 7.4.
After the tissue was chilled for 3 minutes, parasagittal slices (400μm thick) were cut on a vibratome (Leica VT 100E, Deerfield, IL) and placed back into cutting solution for 20 minutes. The slices were further trimmed into blocks with a razor blade to include the SC, thalamus, and striatum. The slices were then transferred into oxygenated artificial cerebrospinal fluid (ACSF) containing in (in mM): NaCl 124; KCl 2.5; CaCl₂ 2; MgSO₄ 1; NaH₂PO₄ 26; and d-glucose 10, at a pH of 7.4.

**Recording procedures**

After 2 hours of incubation in oxygenated ACSF at 35°C, the slice was placed in a temperature controlled recording chamber and maintained in an interface of warmed (35°C) humidified air (95% O₂-5% CO₂) and ACSF. Bicuculline (10μM, Tocris, Ellisville, MO) and CGP55845 (5μM, Tocris) were routinely included in the ACSF to block GABA_A and GABA_B receptors. In certain experiments, CNQX (20μM, Sigma Chemical Company, St Louis, MO) and/or APV (25μM, Sigma) were added to the ACSF and bath applied to block AMPA/kainate and NMDA receptors respectively. In other experiments substance P (2 μM, Sigma) and/or the NK1 antagonist L703,606 (5 μM, Sigma) were added to the ACSF.

Borosilicate glass microelectrodes (tip resistance, 3.5-7.0 MΩ) were pulled horizontally (P97, Sutter Instruments) and filled with a solution containing (in mM): K-glutamate 115, MgCl₂ 2, ATP 3, GTP 0.3, HEPES 10, KCl 120 and phosphocreatine 10 at a pH of 7.3. Blind whole cell patch-clamp recordings were made in current-clamp mode with an Axoclamp 2B amplifier (MDS Analytical Technologies, Sunnyvale, CA, USA). Positive pressure was maintained while penetrating the tissue, and when a neuron was encountered (as indicated by an increase in electrode resistance), a small negative
pressure was applied to the pipette to rupture a patch of cellular membrane. Recordings were obtained from cells considered to be relay cells. These cells exhibited a low-threshold calcium conductance and a hyperpolarization-activated mixed cation conductance (Li et al., 2003a). Records were digitized at 10 kHz and stored directly on computer. All membrane potential measurements were junction potential (9 mV) corrected.

As schematically illustrated in Figure 3A, to stimulate tectothalamic fibers, a multipolar stimulation electrode (matrix microelectrode; FHC, Bowdoin, ME) was placed in the superficial layers of the SC. Corticothalamic axons (CTX) were stimulated with a second multipolar electrode placed in the optic radiations. Stimulating electrodes were always at least 1 mm from the recording electrode. The electrode array contained 8 tungsten electrodes with a spacing of 115 µm between each electrode. Once a whole cell recording was obtained, SC or CTX stimulation was produced by using any two adjacent electrodes in the arrays. The anode and cathode positions were varied until the best response was achieved.

Current pulses of 50μs were generated with a stimulator (Grass S88, Grass Instrument, Warwick, RI) that was connected to a stimulus isolation unit (World Precision Instruments A365, Sarasota, Fl) which controlled current intensity (100μA-3000μA). Stimulus frequency was controlled by computer using pClamp 8.2 software (MDS Analytical Technologies). To measure short term plasticity, repetitive stimuli were delivered in trains of 20 pulses of variable frequency (0.5-10Hz). The responses to 5 trains delivered in 10s intervals were averaged. To examine long term plasticity, 100 Hz current pulses were delivered for a period of 0.5 sec, and then current pulses were
delivered after 5, 10, 15 and 20 minutes (the average of 5 current pulses in 5s intervals was recorded for each time point). EPSP amplitudes were measured using pClamp 8.2 software. EPSP amplitudes were calculated as the difference between the membrane voltage 2ms before the stimulus and the peak of the synaptic response. For the stimulus trains, the amplitudes of each EPSP of the 20 pulse train were quantified relative to the amplitude of the first EPSP of the train. For long term plasticity experiments, the amplitudes of EPSPs generated after high frequency stimulation were quantified relative to the amplitudes of EPSPs generated just prior to high frequency stimulation.

Depolarization of the membrane potential caused by application of SP or 100Hz SC stimulation was compensated for by current injection to maintain pre-application/stimulation potential levels. Input resistance changes were evaluated by comparing the voltage response to square wave current pulses prior to and 10 minutes following SP application of 100 Hz SC stimulation. Voltage responses were also recorded in response to brief (20ms) application of glutamate (100 µM) from a pipette (8-12 µm tip diameter) placed close to the recording electrode. The glutamate was ejected from the pipette using a PV83 pneumatic picopump (WPI) with the pressure adjusted to elicit a stable pre-SP response of greater than 1 mV. Student t-tests or ANOVA single factor analysis were used to test for statistical significance. Quantitative data are expressed as means ± SD.
Results

*SP is presynaptic, and NK1 is postsynaptic in the LPN*

Immunohistochemical staining for both SP and NK1 was densely distributed in the caudal and lateral regions of the LPN, which we previously identified as the tectorecipient zone of the LPN (Masterson et al., 2009). The SP antibody stained boutons (Figure 1A), while the NK1 antibody stained dendrites and occasional somata (Figure 1B). Double staining with both antibodies revealed that SP-positive boutons were closely associated with NK1 stained cells, and SP-positive boutons did not stain for NK1 (Figure 1C).

To confirm that SP is confined to axon terminals and NK1 receptors are located postsynaptically, we prepared SP and NK1 stained tissue for electron microscopy. An ultrastructural analysis of a sample of 100 profiles stained with the SP antibody revealed that all SP staining was confined to synaptic boutons (identified by the presence of synaptic vesicles, Figure 1D, E). In contrast, a sample of 100 profiles stained with the NK1 antibody revealed that the NK1 receptor was expressed by dendrites postsynaptic to unstained terminals (Figure 1F) and occasional somata. Importantly, no NK1-stained profiles in the LPN contained synaptic vesicles, suggesting that SP activates postsynaptic NK1 receptors in the LPN.

*Tecto-LPN terminals contain SP*

We measured SP-stained terminals in electron micrographs to compare their sizes to that of cortico-LPN and tecto-LPN terminals labeled by anterograde transport, as well as profiles stained for the type 1 and type 2 vesicular glutamate transporters (vGLUT1 and vGLUT2) analyzed in our previous study (Masterson et al., 2009). The minor axis of
100 SP-stained profiles involved in synapses (0.71 ± 0.24 µm) was not significantly different from the minor axis of tecto-LPN terminals involved in synapses (0.76 ± 0.22 µm) or vGLUT2-stained terminals involved in synapses (0.72 ± 0.24 µm). In contrast, synaptic SP-stained profiles were significantly larger than synaptic cortico-LPN terminals (0.43 ± 0.13 µm; p = 5.5 x 10^{-11}) and synaptic vGLUT1-stained terminals (0.43 ± 0.09 µm; p = 1 x 10^{-12}). This suggests that tecto-LPN terminals contain SP and cortico-LPN terminals do not contain SP.

To determine whether tectothalamic axons contain substance P, we stained tissue that contained BDA-labeled tecto-LPN axons for substance P and examined the LPN using a confocal microscope. Individual boutons double-labeled for BDA and SP were identified (Figure 2A), which indicates that at least a subset of tecto-LPN boutons contain substance P. Finally, ibotenic acid lesions of the SC diminished substance P staining in the ipsilateral, but not the contralateral LPN (Figure 2B-E).

**Stimulation of cortico-LPN and tecto-LPN inputs in slices**

As schematically illustrated in Figure 3A, to compare the synaptic responses of LPN neurons to stimulation of their cortical or tectal inputs, whole cell recordings were obtained from neurons in the caudal/lateral LPN (tectorecipient zone, Masterson et al., 2009) in 400 µm thick parasagittal sections maintained in vitro, and EPSPs were recorded following stimulation of the optic radiations (CTX) or SC. All recorded neurons were identified as regular spiking (RS) projection neurons based on their pattern of firing in response to current pulses (Li et al., 2003a). Although our previous anatomical results suggest that projection neurons within the tectorecipient zone receive both tectal input on their proximal dendrites and cortical input on their distal dendrites, not all inputs can be
preserved and/or activated within the reduced slice preparation. Nevertheless, tectal or
cortical EPSPs could be evoked in over half of the recorded cells (47 of 78, or 60.3%, of
cells tested for cortical input, and 53 of 96, or 55.2%, of cells tested for tectal input). We
attribute this relatively high success rate to the use of the 8 electrode arrays that spanned
a distance of 1 mm. While we were not able to move the arrays once a whole cell
recording was obtained, stimulation could be produced between any two electrodes in the
array and the anode and cathode positions were varied to obtain the best response.

**Cortico-LPN and tecto-LPN inputs are glutamatergic and convergent**

We previously determined that stimulation of the optic radiations activates both
NMDA and AMPA/kainate receptors in the LPN (Li et al., 2003b). To test whether these receptors are also activated by the stimulation of tectothalamic fibers, we stimulated the
SC in the presence of the NMDA receptor antagonist APV or the AMPA/kainate receptor
antagonist CNQX (Figure 3B). APV caused a significant decrease in EPSP peak
amplitude (39 ± 12%; n = 4, p = 0.040) and shift in the latency of the peak amplitude
from 11.3 ± 2.1 to 8.5 ± 1.6 ms (n = 4, p = 8.1 x 10⁻⁴). The AMPA/kainate receptor
antagonist CNQX also caused a decrease in EPSP amplitude (60 ± 19%; n = 6, p = 0.016)
and a shift in the latency of peak amplitude (10.4 ± 2.3 to 17.8 ± 2.8 ms, n = 6, p = 3.4 x
10⁻⁵).

As previously reported, all CTX EPSP amplitudes in the tectorecipient zone of the
LPN increased in a graded manner as the stimulation intensity was increased (Li et al.,
2003b). Similarly, SC EPSP amplitudes increased with increasing stimulation current
(Figure 3 C-D, n = 7). In addition, SC EPSP latencies decreased as the stimulation
intensity was increased (Figure 3D), a feature we previously reported for corticothalamic
EPSPs in the tectorecipient LPN (Li et al., 2003b). These results suggest that LPN neurons receive multiple convergent cortical and tectal inputs.

**Tecto-LPN and cortico-LPN EPSPs display distinct short term synaptic plasticity**

The short-term frequency dependency of tecto-LPN and cortico-LPN EPSPs was tested using stimulation intensities that evoked stable EPSPs with initial amplitudes of at least 1 mV. Tecto-LPN fibers were stimulated with 20-pulse trains at frequencies of 0.5, 1, 2, 5, and 10 Hz. For each frequency, an analysis of variance was performed comparing the amplitudes of EPSPs within the pulse-train. Repetitive stimulation of tecto-LPN fibers produced no significant differences between EPSP amplitudes at any frequency (Fig. 4: A and C, n = 40; 10Hz p = 0.85; 5Hz p = 0.90; 2Hz p = 0.90; 1Hz p = 0.99; 0.5Hz p = 0.51; ANOVA single factor). A similar analysis of cortico-LPN EPSPs revealed no significant differences between EPSP amplitudes at frequencies of 0.5 and 1 Hz. However, stimulation frequencies of 2, 5, and 10 Hz showed significant facilitation within the 20-pulse trains (Fig. 4: B and D, n=39; 2-10Hz, p <0.001; 1Hz p = 0.80; 0.5Hz p = 1.0; ANOVA single factor).

**SP increases the amplitude of cortico-LPN and tecto-LPN EPSPs**

We next tested whether the exogenous application of SP affected CTX or SC responses. Control EPSPs were recorded, and then the slice was bathed for 20 minutes with ACSF that contained 2 µM SP. EPSP amplitudes were recorded at 5, 10, 15, and 20 minutes after the initial addition of SP to the ACSF. Application of SP increased the SC EPSP amplitudes in 21/34 cells tested, and CTX EPSP amplitudes in 9/17 cells tested. The average increase in EPSP amplitude of the responding cells, and the time course of these increases, are illustrated in Figure 5.
After 5 minutes exposure to SP, SC responses (white bars in Figure 5, n = 21) increased by an average of 2.1 fold after 5 minutes, 2.4 fold after 10 minutes, 2.7 fold after 15 minutes and 2.4 fold after 20 minutes. All of these post-SP responses were significantly different from responses recorded before SP application (p < 0.05). In the presence of SP, CTX responses (light gray bars in Figure 5, n = 9) increased by an average of 1.4 fold after 5 minutes, 1.7 fold after 10 minutes, 1.6 fold after 15 minutes and 2.2 fold after 20 minutes. All of these post-SP responses were significantly different from responses recorded before SP application (p < 0.05).

**SP effects are mediated by postsynaptic NK1 receptors**

When the NK1 antagonist L703,606 (5 µM) was added simultaneously with the SP, CTX (n = 5, dark gray bars in Figure 5) and SC (n = 9, black bars in Figure 5) EPSP amplitudes were not significantly different from control responses (0 minutes; p > 0.3 for amplitudes 5, 10, 15 and 20 minutes post-SP+L703,606), indicating that the SP effect is mediated through the NK1 receptors that are distributed on LPN cells (Figure 1). A postsynaptic effect is also supported by the fact that 10 minutes after SP application the average input resistance of the recorded neurons (122±77MΩ) increased significantly (235±87MΩ; n=21, p < 0.05). This is illustrated in Figure 6 (A,B) as an increase in the voltage responses to 10 ms 50pA hyperpolarizing current pulses after 10 minutes of bath application of SP. In addition, the amplitude of voltage responses to the brief (20 ms) application of glutamate from the tip of an adjacent pipette increased significantly after 10 minutes of SP bath application (Figure 6C). This increase in the peak amplitude of glutamate responses was correlated with the increase in input resistance (Figure 6D; n = 16, r = 0.68).
SP is released from tecto-LPN terminals by high frequency stimulation

We next tested whether high frequency stimulation (100Hz for 0.5 s) of the SC or CTX resulted in EPSP amplitude changes. In 28/40 cells tested, high frequency stimulation of the SC resulted in a gradual increase in tectal EPSP amplitudes, which peaked 20 minutes post stimulation an average of 2.4 fold above baseline (white bars in Figure 7 histogram). Analysis of all responding cells (n=28) revealed that post-stimulation amplitudes were significantly larger than pre-stimulation amplitudes (p = 0.5 x 10^-4 at 5 minutes, p = 3 x 10^-5 at 10 minutes, p = 1 x 10^-5 at 15 minutes and p = 0.015 at 20 minutes post-stimulation when compared to control). These increases were completely blocked when the NK1 antagonist L703,606 was applied to the bath during the high frequency stimulation, and for the 20 minutes following stimulation during which the EPSP amplitude measurements were obtained (n = 10; black bars in Figure 7 histogram; p = 0.26 at 5 minutes, p = 0.16 at 10 minutes, p = 0.16 at 15 minutes and p = 0.31 at 20 minutes post-stimulation +L703,606 when compared to control).

In our slice preparation, in approximately 20% of the recorded neurons both CTX and SC stimulation electrodes evoked stable EPSPs in the same cell. In these cells we tested whether high frequency SC stimulation affected CTX EPSP amplitudes. In 10/18 cells tested, we found that high frequency SC stimulation resulted in the subsequent facilitation of CTX responses in the same cell (up to 1.9 fold after 20 minutes, light gray bars in Figure 7 histogram). Analysis of all responding cells (n=10) revealed that at 10, 15 and 20 minutes post-stimulation, amplitudes were significantly larger than pre-stimulation amplitudes (p = 0.079 at 5 minutes, p = 0.04 at 10 minutes, p = 0.042 at 15 minutes, p = 0.031 at 20 minutes post-stimulation when compared to control), and this
effect was blocked by the simultaneous application of L703,606 to the bath (n = 6; dark gray bars in Figure 7; p = 0.38 at 5 minutes, p =0.48 at 10 minutes, p = 0.25 at 15 minutes and p = 0.052 at 20 minutes post-stim+L703,606 when compared to control). The effect of high frequency SC stimulation on neuron input resistance was similar to that seen following SP application (from 193±86MΩ to 251±98MΩ; n=28, p < 0.05). We also tested the effects of high frequency CTX stimulation on cells that responded to both SC and CTX stimulation (n= 9). As illustrated in Figure 8, high frequency CTX stimulation did not increase cortical or tectal EPSP amplitudes of LPN neurons >5 minutes post stimulation. In fact, CTX and SC EPSP amplitudes were slightly smaller than control (0 minute) amplitudes and this was found to be significant (p <0.05) at all post-stimulation time points.
Discussion

As schematically illustrated in Figure 9, our results indicate that SC-LPN terminals contain both glutamate and SP. At stimulation frequencies up to 10Hz, glutamate is released, which activates both NMDA and AMPA receptors on postsynaptic neurons. The resulting glutamatergic SC-LPN EPSPs show little frequency-dependent plasticity. In contrast to CTX-LPN EPSPs, which show frequency-dependent facilitation, there was no significant difference between SC-LPN EPSP amplitudes generated by 1, 2, 5 or 10 Hz stimulus trains. However, after high frequency (100Hz) stimulation, SP is released from SC-LPN terminals, which activates NK1 receptors on postsynaptic neurons. The activation of NK1 receptors leads to a subsequent potentiation of both SC- and CTX-evoked responses in the postsynaptic neuron. As discussed below, the unique properties of SC-LPN synaptic terminals may produce amplified responses to threatening visual images.

Tectothalamic synapses: a third type of glutamatergic response

Previous studies of the dorsal thalamus have identified two main types of glutamatergic synaptic responses. The majority of inputs to the dorsal thalamus originate from cortical layer VI (type I inputs), (Guillery, 1969). In vitro studies of a variety of thalamic nuclei have consistently shown that the EPSPs elicited by stimulation of layer VI corticothalamic inputs increase in a graded manner with increasing stimulation intensity, and facilitate at frequencies of 2 Hz or higher (Turner and Salt, 1998, Granseth et al., 2002). In contrast, EPSPs generated by stimulation of ascending sensory inputs, or inputs that originate from cortical layer V (type II inputs), show an all-or-none increase in amplitude with increasing stimulation levels, and are depressed at stimulation frequencies
of 2 Hz of higher (Turner and Salt, 1998, Chen and Regehr, 2000, Chen et al., 2002, Granseth et al. 2002; Li et al., 2003c; Reichova and Sherman, 2004; Arsenault and Zhang, 2006).

In the current study, all CTX EPSPs increased in amplitude with increasing stimulation current, and showed a robust frequency-dependent facilitation, consistent with all previous studies of layer VI corticothalamic responses. In contrast, the SC EPSPs in the LPN were unlike either type I or type II synaptic responses in that their amplitudes remained relatively constant at stimulation frequencies up to 10 Hz. Very similar responses have been recorded in the paralaminar thalamic nuclei (adjacent to the medial geniculate nucleus) following stimulation of the SC (Smith et al., 2007).

Like type I corticothalamic inputs, SC EPSPs show a graded increase in amplitude with increasing levels of stimulation current. This likely reflects the convergence of multiple tectal axons onto single LPN neurons. Previous studies indicate that the tecto-LPN projection in the rodent is nontopographic (Mooney et al., 1984), and our previous electron microscopic observations indicate that multiple tectal terminals innervate individual dendrites (Masterson et al., 2009). This supports the idea that LPN cells may integrate converging inputs from multiple tecto-LPN cells.

Smith et al (2007) recently proposed that inputs from the SC and inferior colliculus to the paralaminar neurons be called “integrators” to distinguish them from the type I and type II glutamatergic synaptic responses which Sherman and Guillery (1998) have defined as “modulating” and “driving” inputs respectively. Smith et al further proposed that it may be the collective activities of multiple convergent integrator inputs that are critical for the formation of the postsynaptic neuron’s receptive field. This
concept fits well with the known properties of the LPN; receptive fields within the
tectorecipient LPN are much larger than those recorded in the superficial layers of the SC
where tecto-LPN cells are located (Chalupa et al., 1983, Abramson and Chalupa, 1988,
et al., 2000, Major et al., 2000). In the hamster, Mooney et al (1984) demonstrated that
receptive fields of LPN cells are on average 10 times larger than those of tecto-LPN cells.

Substance P release in the LPN

We also found that tectothalamic synapses are distinguished from type I and type
II glutamatergic synapses in that they release SP when stimulated at high frequencies.
This is similar to the frequency-dependent release of SP in the spinal cord (Go and
Yaksh, 1987, Adelson et al., 2009) substantia nigra (Diez-Guerra et al., 1988) and
intestinal tract (Baron et al., 1983). In the LPN, a recent study demonstrated that SP
reduces a K+ conductance, likely K_{leak}, via NK1 receptors (Paul and Cox, 2010). The
increases in input resistance that we identified following bath application of SP or high
frequency stimulation of the SC are consistent with this action. The net result of SP
release from tectothalamic terminals is an increase in the response of postsynaptic
neurons to all glutamatergic synaptic inputs.

Our studies suggest that when tectothalamic neurons fire at frequencies up to 10
Hz, glutamate is released and LPN neurons reliably respond to stimulus trains, without
facilitation or depression. In contrast, when tectothalamic neurons fire at high frequency,
SP is released in addition to glutamate, which amplifies the subsequent postsynaptic LPN
neuronal responses to both tectal and cortical inputs. In vivo studies indicate that neurons
in the superficial layers of the SC can fire at very high frequencies. In the anesthetized
rat, firing rates of up to 120 Hz have been recorded in superficial layers of the SC (Prevost et al., 2007), and in the awake behaving monkey, firing rates of 250 Hz or more have been recorded in the superficial SC (Wurtz and Mohler, 1976). Thus, our stimulation parameters are well within the normal firing range of SC neurons.

**Functional implications**

A variety of studies have concluded that SP is released in response to stressful stimuli, and SP antagonists have been suggested to be an important therapeutic target for depression and/or anxiety (e.g. Ebner et al., 2009). We hypothesize that the release of SP from tectothalamic terminals may similarly function to increase reactions to threatening visual stimuli.

We recently studied the tectal projections to the dorsal (Pd) and central pulvinar nucleus (Pc) of the tree shrew (Chomsung et al., 2008). Luppino et al (1988) first described these projections as “diffuse” and “specific” respectively. By using a combination of anterograde and retrograde tracing techniques, as well as electron microscopy, we concluded that both the Pd and Pc receive topographic (specific) projections from the SC, and the Pd receives additional nontopographic (diffuse) projections, possibly arising from convergent axon collaterals. Because we also found that the Pd (but not the Pc) projects to the amygdala, we suggested that the diffuse tectopulvinar projections are involved in coding the movement of large or threatening objects to initiate escape responses, while the specific tectopulvinar projections are involved in coding the precise location of small moving objects to initiate orienting or pursuit responses (Chomsung et al., 2008).
Whether a small portion of the rat LPN receives topographic connections from the SC (comparable to the tree shrew Pc) remains an open question. This is difficult to test because the tectorecipient zone of the rat LPN is quite small when compared to the large tectorecipient zones of the tree shrew pulvinar nucleus. However, both anatomical and physiology studies indicate that the majority of the LPN is organized in a nontopographic manner, and that LPN neurons have very large receptive fields (Mooney et al., 1984).

Mooney et al (1984) suggested the LPN neurons respond to visual events, such as the appearance of novel objects, rather than the specific features of visual stimuli. In particular, the majority of LPN neurons that could be antidromically driven from the SC responded best to visual stimuli moving across their receptive fields, similar to the responses of SC-LPN neurons which have been classified as movement-sensitive wide field vertical cells (Mooney et al., 1988). Convergent input from multiple wide field vertical cells likely accounts for the LPN cells’ sensitivity to movement across widespread regions of the visual field. This organization makes the LPN well suited to signal the appearance of potential danger.

There also appears to be good overlap between tecto-LPN projections, regions of the LPN that project to the amygdala (Doron and Ledoux, 1999, 2000), and regions innervated by terminals that contain SP. Similarly, the paralaminar nuclei, which also receive “integrator” inputs from the SC (Smith et al., 2007), project to the amygdala (Doron and Ledoux, 1999, 2000) and are innervated by SP fibers (unpublished data). Thus, the tectorecipient zone of the LPN may be part of a complex of nuclei that receive convergent tectal inputs and project to the amygdala. In this context, our results suggest that the SP-mediated potentiation of synaptic inputs may serve to amplify responses to
threatening objects that move across large regions of the visual field.
FIGURE LEGENDS

Figure 1) SP is presynaptic, and NK1 is postsynaptic in the LPN.  A) An antibody against SP stains terminal boutons in the LPN. B) An antibody against NK1 stains dendrites and occasional somata in the LPN. C) A 12 µm thick confocal image of tissue from the caudal LPN stained with NK1 and SP antibodies. An NK1-stained neuron (purple) is surrounded by SP-stained terminals (green). There was no double-labeling of NK1 and SP, indicating that the NK1 receptors are not located on SP terminals. D-F) Electron micrographs of the LPN illustrate that SP is located in terminal boutons that synapse (white arrows) with dendrites (D,E) and NK1 receptors are present on postsynaptic dendrites (F). Scale bars = 10 µm in A (applies to B), 10 µm in C, and 0.5 µm in D (applies to E and F).

Figure 2) Tecto-LPN terminals contain SP.  A) A large injection of biotinylated dextran amine (BDA) was made in the superficial layers of the SC to label axons in the LPN by anterograde transport. LPN tissue was subsequently treated to reveal the tecto-LPN axons and SP. A 10 µm thick confocal image of the caudal LPN illustrates the overlap of tecto-LPN axons (purple) and terminals that contain SP (green). The white areas of the image indicate tecto-LP terminals that contain SP. B-F) The excitatory neurotoxin ibotenic acid was injected unilaterally into the superior colliculus. SP staining in the LPN contralateral to the ibotenic acid injection was densely concentrated in the caudal portion of the nucleus, as in normal rats (B, boxed region shown at higher magnification in C). The inset shows neurons visualized with the NeuN antibody in the superior colliculus. SP staining was absent in the LPN ipsilateral to the ibotenic acid injection.
injection (D, boxed regions shown at higher magnification in E). The inset shows the lesion in the superior colliculus caused by the ibotenic acid injection in a section stained with NeuN. Scale bars = 5 µm in A, 500 µm in D (also applies to B), 1 mm in D inset (also applies to B inset) and 25 µm in E (also applies to C).

**Figure 3** Cortico-LPN and tecto-LPN inputs are glutamatergic and convergent.

**A)** Schematic illustration of the recording and stimulation sites used to examine tectothalamic and corticothalamic EPSPs in the lateral posterior nucleus (LPN). Parasagittal slices that contained the most lateral regions of the superior colliculus (SC) were used. An array of 8 electrodes (black dots surrounded by white circles) was placed in the SC to activate the cut axons of tectothalamic cells located in more medial regions of the SC. A second array of 8 electrodes was placed just rostral to the LPN to activate the cut axons of corticothalamic cells (CTX). Our previous anatomical studies (Li et al., 2003; Masterson et al., 2009) revealed that only the rostral LPN is innervated by large corticothalamic terminals that originate from layer V cells. The caudal tectorecipient region of the LPN (light gray) is innervated only by small corticothalamic terminals that originate from layer VI. **B)** Tectothalamic EPSPs activate both NMDA and non-NMDA receptors. In the presence of the NMDA receptor antagonist APV, or the AMPA/kainate receptor antagonist CNQX, there was a decrease in EPSP amplitude and a shift in the latency of the peak amplitude. Tectothalamic EPSPs were abolished in the presence of both APV and CNQX. **C)** With increasing stimulation intensity, tectothalamic EPSPs show a graded increase in amplitude, and a small decrease in latency. **D)** Graph shows
the average tecto-LPN EPSP amplitudes and latencies as a function of stimulation intensity for a sample of 7 cells.

**Figure 4)** Tecto-LPN and cortico-LPN EPSPs display distinct short term synaptic plasticity. Stimulation experiments consisted of 20 pulses at 0.5, 1, 2, 5, and 10 Hz. 

A) EPSPs 1, 10 and 20 from a SC stimulation experiment. Stimulation of tectal fibers produced EPSPs with stable amplitudes. B) EPSPs 1, 10, and 20 from a CTX stimulation experiment. Stimulation of cortical fibers produced EPSPs that showed frequency-dependent facilitation. C and D) Each point represents the normalized average of EPSPs evoked in 35 cells by 20 pulses at 0.5, 1, 2, 5 and 10Hz. The EPSP amplitudes were normalized by dividing the EPSP amplitude evoked by each pulse in the train (EPSPn) by the EPSP amplitude evoked by the first pulse of the train (EPSP1). C) Tectothalamic experiments. D) Corticothalamic experiments.

**Figure 5)** Substance P increases the amplitude of cortico-LPN and tecto-LPN EPSPs. Substance P, or substance P and the NK1 antagonist L703,606, were bath applied during *in vitro* whole cell recordings and stimulation of tecto-LPN axons (SC) or cortico-LPN axons (CTX). In A-D the gray trace is the control EPSP (0 minutes) and the darker traces are EPSPs 10 and 20 minutes after application of substance P or substance P and L703,606. A) EPSPs generated by SC stimulation before and after substance P application. B) EPSPs generated by SC stimulation before and after substance P and L703,606 application. C) EPSPs generated by CTX stimulation before and after substance P application. D) EPSPs generated by CTX stimulation before and after in the
substance P and L703,606.  

The histogram illustrates the potentiation of SC and CTX evoked EPSPs at 5, 10, 15 and 20 minutes after substance P application expressed as a percentage of control values (0 minutes).

**Figure 6) SP effects are mediated by postsynaptic NK1 receptors.** Voltage responses of LPN neurons (bottom traces) to 10 ms 50pA hyperpolarizing current pulses (top traces) (**A**) increase after 10 minutes of bath application of SP (**B**). **C** The amplitudes of LPN neuron voltage responses to brief (20 ms) application of glutamate (lower trace) are increased following 10 minutes of bath application of SP (top trace indicated by arrow).

The histogram illustrates the average increase in peak amplitude expressed as a percentage of control amplitudes (n = 16). **D** The increase in response to glutamate application is correlated with an increase in input resistance (r = 0.68).

**Figure 7) High frequency stimulation of tecto-LPN axons increases the amplitude of subsequent tecto- and cortico-LPN EPSPs.** In cells that responded to stimulation of both tecto-LPN (SC) and cortico-LPN (CTX) axons, tecto-LPN axons were stimulated at a frequency of 100 Hz for 0.5 seconds and the amplitudes of subsequent EPSPs evoked by single SC or CTX stimulus pulses were monitored. In A-D the gray trace is the control EPSP and the darker traces are EPSPs 10 and 20 minutes after 100 Hz SC stimulation. **A** EPSPs generated by SC stimulation before and after 100 Hz SC stimulation. **B** EPSPs generated by SC stimulation before and after 100 Hz SC stimulation in the presence of the NK1 antagonist L703,606. **C** EPSPs generated by CTX stimulation before and after 100 Hz SC stimulation. **D** EPSPs generated by CTX stimulation after 100 Hz SC
stimulation in the presence of L703,606. **E)** The histogram illustrates the potentiation of SC and CTX evoked EPSPs at 5, 10, 15 and 20 minutes after 100 Hz SC stimulation expressed as a percentage of control values (0 minutes).

**Figure 8)** High frequency stimulation of cortico-LPN input does not change the amplitude of subsequent tecto- or cortico-LPN EPSPs. In cells that responded to stimulation of both tecto-LPN (SC) and cortico-LPN (CTX) axons, cortico-LPN axons were stimulated at a frequency of 100 Hz for 0.5 seconds and the amplitudes of subsequent EPSPs evoked by single SC or CTX stimulus pulses were monitored. In A and B, the gray trace is the control EPSP and the darker traces are EPSPs 10 and 20 minutes after 100 Hz CTX stimulation. **A)** EPSPs generated by SC stimulation before and after 100 Hz CTX stimulation. **B)** EPSPs generated by CTX stimulation before and after 100 Hz CTX stimulation. At the completion of the experiment, the SC was then stimulated at 100 Hz for 0.5 seconds. The dotted trace (arrow) indicates the subsequent potentiation of the CTX EPSP. **C)** The histogram illustrates that there was no increase in the amplitudes of SC or CTX evoked EPSPs at 5, 10, 15 and 20 minutes after 100 Hz CTX stimulation. Amplitudes expressed as a percentage of control values (0 minutes).

**Figure 9)** Schematic summary of rat LPN glutamatergic synapses. **A)** In the rostral LPN, projection cells receive input to their proximal dendrites from a small number of large terminals with round vesicles (RL profiles, red) that originate from cortical layer V, and input to their distal dendrites from a large number of small terminals with round vesicles (RS profiles, green) that originate from cortical layer VI (Li et al., 2003c).
Stimulation of RL profiles at 10 Hz produces EPSPs that depress (type II responses, red), characteristic of "driver" inputs (Sherman and Guillery, 1998; Li et al., 2003b), while stimulation of RS profiles at 10 Hz produces facilitating EPSPs (type I responses, green), characteristic of "modulator" inputs (Sherman and Guillery, 1998; Li et al., 2003b). B) In the caudal LPN, projection cells receive input to their proximal dendrites from a large number of medium sized terminals with round vesicles (RM profiles, black) that originate from the SC, and input to their distal dendrites from a large number of small terminals with round vesicles (RS profiles, green) that originate from cortical layer VI (Masterson et al., 2009). Stimulation of RM profiles at 10 Hz produces EPSPs that do not depress or facilitate (type III responses, black), characteristic of "integrator" inputs (Smith et al., 2007), while stimulation of RS profiles at 10 Hz produces facilitating EPSPs (type I responses, green), characteristic of "modulator" inputs. C) Glutamate (white squares) is transported into vesicles within RS (green) and RM (black) profiles by the type I (vGLUT1) and type II (vGLUT2) vesicular glutamate transporters respectively (Masterson et al., 2009). When stimulated at frequencies up to 10Hz, glutamate release activates postsynaptic AMPA and NMDA receptors. D) When stimulated at high frequency (100Hz), RM profiles additionally release SP (blue circles) which activates postsynaptic NK1 receptors and increases the amplitudes of subsequent glutamatergic postsynaptic responses (represented by the green and black traces).


Sherman SM, Guillery RW On the actions that one nerve cell can have on another: distinguishing "drivers" from "modulators". Proc Natl Acad Sci U S A 95:7121-7126.1998.


