Submission to *Journal of Neurophysiology*
Category: Innovative Methodology

**Sniffer Patch Laser Uncaging REsponse (SPLURgE): an assay of regional differences in allosteric receptor modulation and neurotransmitter clearance**

Catherine A. Christian and John R. Huguenard

Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, California, USA

Running head: Sniffer patch laser uncaging responses

Corresponding Author: John R. Huguenard, Department of Neurology and Neurological Sciences, Stanford University School of Medicine, MC 5122, Alway Building Room M030, Stanford, CA, 94305 USA. Phone: 1-650-723-5522, Fax: 1-650-723-1080. Email: john.huguenard@stanford.edu

Manuscript information: 30 text pages, 6 figures
Abstract: 250 words

Author contributions: C.A.C. and J.R.H. conception and design of research; C.A.C. performed experiments; C.A.C. analyzed data; C.A.C. and J.R.H. interpreted results of experiments; C.A.C. prepared figures; C.A.C. drafted manuscript; C.A.C. and J.R.H. edited and revised manuscript; C.A.C. and J.R.H. approved final version of manuscript.

Copyright © 2013 by the American Physiological Society.
Abstract

Allosteric modulators exert actions on neurotransmitter receptors by positively or negatively altering the effective response of these receptors to their respective neurotransmitter. \(\gamma\)-aminobutyric acid (GABA) type-A ionotropic receptors (GABA\(_A\)Rs) are major targets for allosteric modulators such as benzodiazepines, neurosteroids, and barbiturates. Analysis of substances that produce similar effects has been hampered by the lack of techniques to assess the localization and function of such agents in brain slices. Here we describe measurement of the Sniffer Patch Laser Uncaging Response (SPLURgE), which combines the sniffer patch recording configuration with laser photolysis of caged GABA. This methodology enables the detection of allosteric GABA\(_A\)R modulators endogenously present in discrete areas of the brain slice, and allows for the application of exogenous GABA with spatiotemporal control without altering the release and localization of endogenous modulators within the slice. Here we demonstrate the development and use of this technique for the measurement of allosteric modulation in different areas of the thalamus. Application of this technique will be useful in determining whether a lack of modulatory effect on a particular category of neurons or receptors is due to insensitivity to allosteric modulation or a lack of local release of endogenous ligand. We also demonstrate that this technique can be used to investigate GABA diffusion and uptake. This method thus provides a biosensor assay for rapid detection of endogenous GABA\(_A\)R modulators, and has the potential to aid studies of allosteric modulators that exert effects on other classes of neurotransmitter receptors, such as glutamate, acetylcholine, or glycine receptors.

Keywords: GABA, neuromodulation, brain slice, electrophysiology, outside-out patch
**Introduction**

Allosteric modulators of neurotransmitter receptors act by altering the effective response of these receptors to their cognate neurotransmitter in either a positive or negative direction (Schwartz and Holst, 2007). Major classes of such modulators for the ionotrophic receptors for γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain, include benzodiazepines, neurosteroids, barbiturates, and redox agents (Pan et al., 1995; Rudolph and Möhler, 2004; Belelli et al., 2006; Calero et al., 2011), which each act at different binding sites on the GABA_A receptor (GABA_A-R). Assorted GABA_A-R subunit combinations confer diverse sensitivity to specific modulators (Pritchett et al., 1989a, 1989b; Macdonald and Olsen, 1994; Hadingham et al., 1996; Wafford et al., 1996; Möhler et al., 2001; Mody and Pearce, 2004). Furthermore, differential expression of endogenous modulators across brain areas and under various physiological states suggests the potential for discrete but functionally powerful effects on overall circuitry.

The GABAergic neurons of the thalamic reticular nucleus (nRT) are a primary source of inhibition in the thalamocortical circuit, and provide dense inhibitory projections to the thalamocortical relay cells in the ventrobasal nucleus (VB) (Jones, 1975; Houser et al., 1980; Crabtree et al., 1998; Steriade, 2005). In addition, nRT neurons inhibit each other (intra-nRT inhibition) via sparse axon collaterals and dendrodendritic connections (Deschenes et al., 1985; Cox et al., 1996; Pinault et al., 1997; Shu and McCormick, 2002).

The GABA_A-R α1 subunit is highly expressed in VB, whereas mature nRT neurons almost exclusively express the α3 subunit (Wisden et al., 1992; Fritschy and Mohler, 1995; Huntsman et al., 1996). In previous work, we observed that the benzodiazepine binding site antagonist flumazenil reduced the duration of spontaneous inhibitory postsynaptic
currents recorded in nRT, but not in VB (Christian et al., 2013), suggesting the nucleus-specific localization of endogenous benzodiazepine binding site ligands (endozepines). Our investigations into this question necessitated a technique to assess differences in localization of allosteric modulators in different areas of the thalamus.

To this end, we developed a biosensor for endogenous allosteric modulators of GABA\(_A\)Rs in brain slices by combining the “sniffer patch” technique (Allen, 1997), which has traditionally been used to detect synaptic neurotransmitter release, with GABA uncaging via ultraviolet laser stimulation, which we have named the Sniffer Patch Laser Uncaging Response (SPLURgE). Some of the primary data from nRT patches were included in a recent report (Christian et al., 2013); here we present and discuss the development and use of this methodology in further detail. First, we illustrate the inability of sniffer patches pulled from VB neurons to detect electrically evoked release of GABA in nRT, thus requiring an alternative source of GABA that can be applied to these patches in a way that is rapid and spatiotemporally precise. Second, we show that a higher concentration of caged GABA is required to elicit uncaging responses in outside-out patches pulled from nRT cells compared to patches pulled from VB cells, consistent with the concentration dependence observed using rapid GABA application in nRT patches (Schofield and Huguenard, 2007). Third, we describe the use of this technique in investigating allosteric modulator expression in the thalamus. Lastly, we demonstrate that this technique may also be used to investigate the dynamics of GABA transporter function. This methodology will be useful for detection of allosteric GABA\(_A\)R modulators and region-specific differences in GABA diffusion or uptake, and should be fruitful for assessment of modulators for other classes of neurotransmitter receptors.
Methods

Animals

All procedures were approved by the Administrative Panel on Laboratory Animal Care at Stanford University. C57BL/6 mice (Charles River Laboratories, Hollister, CA) of either sex at postnatal days (P) 15-35 were used for all experiments. Mice were bred and housed on a 12:12 light:dark photoperiod with food and water available ad libitum.

Brain slice preparation

Brain slices were prepared according to published procedures (Huguenard and Prince, 1994) following deep pentobarbital sodium (55 mg/kg i.p.) anesthesia. Briefly, the brain was rapidly removed and placed in an oxygenated ice-cold sucrose solution containing (in mM): 234 sucrose, 11 glucose, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, and 0.5 CaCl₂ (310 mOsm). 250 μm horizontal brain slices through the somatosensory thalamus were made using a Leica VT1200 microtome (Leica Microsystems, Bannockburn, IL) and incubated in continuously oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 10 glucose, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 1 MgSO₄, 2 CaCl₂, and 120 NaCl (298 mOsm). Slices were kept at ~30-32°C for 1 hr, then transferred to room temperature (~21-23°C) for at least 15 min before recording.

Patch-clamp electrophysiology and laser uncaging

Slices were individually transferred to a recording chamber on the stage of a Zeiss Axioskop fixed-stage upright microscope (Carl Zeiss Inc., Thornwood, NY) continuously superfused at 2 ml/min with oxygenated ACSF at room temperature. Whole-cell patch-
clamp recordings were made using a MultiClamp 700A amplifier with Clampex 9.2 software, and signals digitized using a Digidata 1322A (Molecular Devices, Sunnyvale, CA). Borosilicate glass recording pipettes were prepared using a Model P-97 Flaming/Brown micropipette puller (Sutter Instrument Co., Novato, CA) to 2.5-4 MΩ tip resistance when filled with an isotonic chloride intracellular pipette solution, which contained (in mM): CsCl, 10 HEPES, 2 MgCl₂, 5 QX-314, and pH adjusted to 7.3 with CsOH (290 mOsm). Where indicated, 5 mM Mg-ATP was added to the internal solution immediately prior to use. All recordings were made in the presence of D-(-)-2-amino-5-phosphonovaleric acid (APV, 100 μM, Ascent Scientific, Princeton, NJ) plus 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20 μM, Ascent) to isolate GABA₆R-mediated currents. For uncaging experiments, α-carboxy-2-nitrobenzyl ester (CNB)-caged GABA (100 μM – 1 mM, Invitrogen, Carlsbad, CA) was added to a 15-20 mL recirculating bath ACSF solution. In some cases 1 μM flumazenil (FLZ, Sigma-Aldrich, St. Louis, MO) and/or the GAT antagonists 1,2,5,6-Tetrahydro-1-[[2-[(diphenylmethylene)amino]oxy]ethyl]-3-pyridinecarboxylic acid hydrochloride (NNC 711, 4 μM, Tocris Bioscience, Minneapolis, MN) and 1-[2-[[tris(4-methoxyphenyl)methoxy]ethyl]-(S)-3-piperidinecarboxylic acid ((S)-SNAP 5114, 10 μM, Tocris) were included in the ACSF bath solution. For the experiments in Figure 1, electrical stimuli were delivered via a bipolar tungsten electrode placed in nRT.

Outside-out membrane patches were pulled from neurons using established procedures (Hamill et al., 1981; Sakmann and Neher, 2009). Briefly, a whole-cell recording was established with the membrane holding potential (Vₘ) set at -60 mV. Vₘ was then set at -30 mV followed by slow withdrawal of the patch electrode until a cell-free patch of membrane was isolated on the pipette tip, which ensured stability of the resultant outside-
out patch. The pipette tip was fully removed (>50 μm) from the slice in order to completely
excise the patch from the original cell. The peak patch input resistance was ~1-2 GΩ.
Patches with input resistance <100 MΩ were less stable and either ruptured upon
placement into the slice or exhibited very low amplitude and unstable uncaging responses.
Sniffer patches were placed in the slice to a depth at which nearby cell bodies could be
clearly visualized (~25-50 μm). Where indicated, some responses were recorded from
patches positioned above the slice. For the experiments in Figures 6 and 7, precise patch
placement depth was determined by positioning the tip of the pipette at the surface of the
slice and setting the z-axis value on the micromanipulator (MP-285, Sutter) to 0 for each
patch. 1 ms-duration ultraviolet light pulses (20 μW) were delivered using a 355 nm
wavelength laser beam (DPSS Lasers, Santa Clara, CA) directed into the epifluorescence
port of the microscope and through the back aperture of a 60X water immersion objective.
The pipette tip was placed in the center of the focused laser spot to ensure maximal
photolysis of ambient caged GABA. Placement of the pipette 25 μm below the plane of the
focus was sufficient to completely abolish the uncaged GABAergic current. Final
localization of the patch electrode tip in VB or nRT was confirmed by capturing a video
frame from the microscope using a 2.5X or 10X objective to visualize pipette placement.
Recordings were made in voltage-clamp mode with 20 mV/pA gain, sampled every 40 μs,
and low-pass Bessel filtered at 2 kHz.

Data analysis

Recordings were analyzed using Clampfit 9.2 software (Molecular Devices) and data
were exported to Excel (Microsoft, Redmond, WA) and Origin 7 (Microcal Software,
Northampton, MA) for statistical analysis. In Clampfit, traces were low-pass Boxcar filtered
post hoc at 99 smoothing points. The peak amplitude, half width, 10-90% rise time, and 90-
10% decay time of the responses were calculated using the Statistics protocol in Clampfit.
Two-tailed paired t-tests were used for comparisons of values for the third vs. the first
responses in individual patches and for comparisons of responses obtained up to 50 μm
into the slice with those obtained 50 μm above the slice. The third response was chosen for
comparison because at least 3 responses were readily obtained from each patch.
Comparisons between VB and nRT patches and between responses obtained in control vs.
GAT blockade conditions were made using two-tailed independent t-tests. Comparison of
amplitude ratios obtained at 10-s, 30-s, and 60-s interstimulus intervals (Figure 2) was
performed using one-way ANOVA. A Fisher’s exact test was used for the statistical
comparison in Figure 1D. Statistical significance was set at p<0.05. Data are presented as
means ± SEM.

Results
Patches pulled from VB neurons can detect electrically evoked GABA release in VB, but not
nRT
The ability of outside-out membrane patches pulled from VB neurons to detect
GABA released synaptically (Isaacson et al., 1993; Banks and Pearce, 2000) was tested by
placing these patches back in the slice in either VB or nRT to act as “sniffer” patches (Allen,
1997) (Figure 1A) and electrically evoking GABA release via a stimulating electrode placed
in nRT. VB patches were chosen because previous work using rapid GABA application
indicated that GABA\textsubscript{A}Rs in patches from VB neurons exhibit higher channel density and
affinity for GABA than those from nRT neurons (Schofield and Huguenard, 2007), and thus
would be more likely to detect synaptic GABA release. Indeed, small amplitude (<10 pA)
synaptically evoked responses were detectable in VB (responses observed in 5 of 8 patches
tested; example in Figure 1B), but when patches were placed in nRT, responses were
undetectable (responses were not observed in any of 8 patches; Figure 1C; p<0.05, Fisher
Exact test). The increased probability of detecting synaptic GABA release in VB compared
to nRT is likely due to the greater GABAergic innervation of VB, as compared to the
relatively sparse innervation within nRT (Cox et al., 1996; Pinault et al., 1997).

Because the synaptic release of GABA was not detectable by these means with
patches placed in nRT, we decided to take the approach of providing an external source of
GABA. Although rapid GABA application or puffs could potentially be used in this fashion,
the mechanical shock associated with these methods could compromise the stability and
reproducibility of responses from patch to patch. By contrast, laser uncaging of GABA
results in rapid transients in GABA concentration in the region of the focused beam without
mechanical artifacts. Furthermore, because the caged GABA is applied via the bath solution,
penetration into the extracellular space throughout the depth of the slice and across
different regions facilitates comparison of responses across different brain areas.

Patches pulled from nRT and VB cells show nucleus-specific differences in affinity to laser-
uncaged GABA

The affinity of VB and nRT patches to laser-uncaged GABA was tested by recording
the responses to 1 ms laser stimuli in the presence of different ambient concentrations of
caged GABA. At 100 μM caged GABA, VB patches exhibited high-amplitude (-96.26 ± 8.23
pA) responses that had a rapid onset (10-90% rise time 17.34 ± 3.25 ms) and slower decay profile that could be fit to a double exponential function, typical of postsynaptic currents (n=7 patches; example in Figure 2A). nRT patches, however, showed smaller amplitude (17.56 ± 7.91 pA), slow-onset (121.51 ± 60.99 ms) responses at this concentration (n=3 patches; example in Figure 3A). When the caged GABA concentration was increased to 1 mM, however, nRT patches showed responses that were much higher amplitude (~2-fold, 40.84 ± 10.96 pA) and faster onset (24.95 ± 2.22 ms) that more closely resembled the kinetics and amplitude of IPSCs (n=8 patches; example in Figure 3B). These findings are consistent with nRT patches expressing GABA_A receptors with lower affinity than patches from VB cells, as previously demonstrated using rapid GABA application to outside-out patches (Schofield and Huguenard, 2007), though the duration of uncaging responses in both VB and nRT patches was ~4-5 times longer than those obtained with rapid application. The duration of the response of nRT patches was 2-5 fold longer than that of VB patches (p<0.001) (Figure 2A-B, 3B-C), consistent with the longer time course of nRT spontaneous inhibitory postsynaptic currents (Zhang et al., 1997; Huntsman et al., 1999; Browne et al., 2001; Schofield et al., 2009; Christian et al., 2013) and responses to transient GABA application (Schofield and Huguenard, 2007).

Amplitude of GABA uncaging response in patches progressively decreases with successive stimulations, but decay rate is stable

We observed a progressive decrease in the amplitude of the response of patches pulled from either VB or nRT neurons to GABA uncaging over successive trials when laser stimuli were delivered at 10-s intervals (Figure 2B-C, 3C-D). Initial response amplitude
was variable from patch to patch, potentially resulting from differences in patch input resistance, patch size, and/or the number of receptors located within each patch. The decay properties of responses in these same patches, however, were relatively stable (Figure 2B-C, 3C-D, p>0.1), indicating that several responses can be obtained from the same patch and used in kinetics analyses.

It is possible that the decrease in amplitude reflects desensitization of the receptors, which does not recover within the 10-s window between laser stimuli. When laser pulses were given at either a 30-s (n=4 patches) or 60-s (n=3 patches) interstimulus interval (ISI), however, the progressive reduction persisted (Figure 2D-F). The degree of this reduction was not significantly different between patches stimulated at 10-s, 30-s, or 60-s ISIs (F=0.36, p>0.7). Therefore, it appears that the reduced amplitude over successive trials every 10 s is not due to an insufficient ISI duration.

Rundown of uncaging response amplitude in VB neurons is ameliorated by replenishment of caged GABA

To determine if the rundown of uncaging response amplitudes observed in pulled patches is an artifact of the patch recording configuration, we performed whole-cell recordings of VB neurons in the same conditions of uncaging stimuli as performed on patches. Although the degree of rundown was not as steep as that seen in patches, there was a significant decrease in response amplitude by the 5th stimulus when given at a 10-s ISI (n=8 cells) (Figure 4A). This was not improved by inclusion of 5 mM Mg-ATP (Kaneda et al., 1995) in the pipette internal solution (n=6 cells). We did observe a positive correlation, however, between the degree of rundown and the order in which each cell was
recorded relative to the last application of new caged GABA (p<0.05) (Figure 4B). This suggests that the amplitude rundown is not a result of recording in pulled patches, but that as “liberated” GABA and/or cage compound accumulates in the bath solution, these can interfere with the amplitude of the uncaging responses. These effects can therefore be reduced by minimizing recirculation of the uncaging solution.

GABA transport differentially affects the early and late decay kinetics of SPLURgEs

The results presented in Figures 2-3 describe the responses to laser uncaging obtained when patches are held outside of the slice, and thus away from potential influences of modulation or altered diffusion or uptake. Placement of patches within the slice, which confers the designation of “sniffer patch” (Allen, 1997), allows for investigation of these factors by measurement of the SPLURgE. Properties of SPLURgEs in VB patches were tested by pulling patches from VB neurons and placing them back in the slice either in VB or in nRT, typically ~25-50 µm into the slice, before uncaging GABA onto the patch. Placement of patches in the slice was not detrimental to patch quality or SPLURgE stability (Figure 5A, B; p>0.1 for the ratio of the SPLURgE half width in response to the third laser stimulus to the respective value of the response to the first stimulus, n=5).

We hypothesized that SPLURgE kinetics would be affected by differential GABA diffusion and/or uptake in and out of the slice. Diffusion will be slowed within the slice compared to in free solution due to cellular elements that hinder movement of solutes in the microenvironment of the brain (Syková and Nicholson, 2008). In addition, GABA transporters (GATs), which are present within, but not outside, the slice may play a major role in determining the kinetic profile of the SPLURgE, acting to reduce duration by
speeding the elimination of uncaged GABA. To examine these possibilities, we tested the SPLURgE response in and out of VB, where FLZ-sensitive allosteric modulation does not appear to occur (Christian et al., 2013).

In the following experiments, uncaging responses were obtained as the patch was moved sequentially along the z-axis into the tissue to obtain at least one response at each z-axis position. As patch health permitted, this process was reversed to obtain additional responses. Where possible, multiple responses at each z-axis position were averaged to obtain the mean value for a given parameter at a particular z-axis position. These values were then normalized to the mean value obtained at -50 μm above the slice. In control conditions, the time course of SPLURgEs was accelerated when patches were placed within the slice compared to when patches were recorded above the slice (Figure 5C). When patches were placed into the slice, SPLURgE duration decreased such that the decay time, but not the half width, was significantly reduced (p<0.05) compared to values obtained when the same patches were recorded above the slice (Figure 5E, F). Consistent with the hypothesis that GAT activity affects SPLURgE duration, this relationship was reversed when the GAT-1 blocker NNC 711 and the GAT-3 blocker (S)-SNAP 5114 were included in the bath solution. Under these conditions, SPLURgE response duration was instead increased when patches were placed within the slice (Figure 5D). Specifically, SPLURgE half width displayed a greater degree of enhancement, whereas the decay time was not significantly different from values obtained above the slice (Figure 5E, F). When SPLURgEs were obtained in patches held above the slice, GAT blockade produced no change in either the half width or 90-10% decay time (p>0.5) compared to control conditions. By contrast, when patches were placed within the slice, both the half width and decay time were
significantly increased (p<0.01) by GAT blockade treatment compared to control (Figure 5E, F). These results suggest that diffusion and GAT-mediated GABA uptake differentially affect the early and late decay kinetics of uncaging responses.

SPLURgE duration in VB patches is potentiated when patches are placed in nRT

When patches were placed in nRT, the SPLURgE was significantly potentiated compared to that obtained in patches placed in VB (Figure 6A), suggesting differences in GABA diffusion, uptake, or allosteric modulators between the two nuclei. We have previously demonstrated that this potentiation represents a combination of: 1) allosteric modulation that is sensitive to the benzodiazepine binding site antagonist FLZ, suggesting that this potentiation is due at least in part to the actions of an endogenous positive allosteric benzodiazepine site agonist in nRT; and 2) slower GABA uptake in nRT than in VB (Christian et al., 2013). Our previous work used mice on the 129/SvEvTac background; here we extend this finding to the C57BL/6 strain.

To further calibrate the SPLURgE above vs. within nRT, patches were pulled from VB neurons and recorded when positioned from 25 µm above to 50 µm deep into the slice. In patches recorded in control conditions, SPLURgE duration progressively increased as the patch was placed more deeply in nRT (n=4 patches; Figure 6B-C). In the presence of FLZ, however, the increase in duration was blocked, and SPLURgE 90-10% decay time was significantly decreased with deeper placement within the slice (n=4 patches; Figure 6B-C). These results support and extend our previous observations that the nRT-dependent potentiation of the uncaging response largely reflects actions of endogenous allosteric modulators acting on GABA<sub>AR</sub> benzodiazepine binding sites (Christian et al., 2013).
Furthermore, note that the differential effects of patch placement within the slice on half width vs. 90-10% decay time in the presence of FLZ (i.e., when endozepine modulation is blocked) are similar to what was observed when VB patches were placed in VB (Figure 5), where endozepines do not appear to be released (Christian et al., 2013). Thus it is likely that in nRT, as in VB, the SPLURgE profile that is independent of endozepine modulation reflects a combination of GAT-mediated uptake, preferentially affecting the late decay kinetics, and diffusion, which contributes to the early decay parameter.

**Discussion**

Here we describe a new methodology for detecting region-specific differences in GABA receptor function in brain slices, which we have used to detect localized expression of endozepines, endogenous benzodiazepine site ligands. By combining the sniffer patch recording configuration with GABA uncaging, stereotyped transient GABA increases can be reproduced in different nuclei. In addition to allosteric modulation, this method could potentially be used to investigate regional differences in extracellular space volume fraction, tortuosity, diffusion, and uptake (Syková and Nicholson, 2008). The advantage of providing an exogenous source of GABA is illustrated by the ability of patches pulled from VB neurons to robustly detect responses even under conditions in which synaptic release of GABA was undetectable.

The durations of uncaging responses for patches pulled from neurons in both nuclei were longer than those for spontaneous or evoked IPSCs, consistent with previous studies on patches pulled from thalamic, hippocampal, and cortical neurons (Galarreta and Hestrin, 1997; Jones and Westbrook, 1997; Perrais and Ropert, 1999; Banks and Pearce, 2000;
Schofield and Huguenard, 2007). The longer time course for responses in nRT patches is in accordance with the longer time course of inhibitory postsynaptic currents (Zhang et al., 1997; Huntsman et al., 1999; Browne et al., 2001) in nRT compared to VB, and likely reflects nucleus-specific differences in receptor subunit composition (Wisden et al., 1992; Fritschy and Mohler, 1995; Huntsman et al., 1996) and resultant differences in GABA affinity and unbinding (Schofield and Huguenard, 2007). The mechanism underlying increased duration of currents in patches vs. neurons remains unclear, although alterations in interactions of cytoskeletal elements or intracellular modulators with GABA₆Rs (Wang et al., 1999; Petrini et al., 2003) may play a role.

Both the rise times and duration were longer for uncaging responses than the values previously observed using rapid (2 ms) 1 mM GABA application (Schofield and Huguenard, 2007). Methodological differences between the two studies, such as alterations in patch holding potential and ionic composition of pipette solutions, may account for this. Alternatively, it is more likely that variations in the spatiotemporal profile of GABA application between the two methods underlie these differences. The rapid GABA application method uses a piezoelectric transducer to expose the patch to control or ligand-containing solutions, and the duration of ligand exposure is determined by the rate of switching between the two solutions. As demonstrated here in the SPLURgE method, by contrast, the time course of ligand exposure evoked by UV uncaging can be influenced by factors such as neurotransmitter uptake and diffusion, and is almost certainly longer than that achieved using rapid application, as evidenced by the longer responses obtained with uncaging. For experiments requiring higher spatiotemporal uncaging control, the SPLURgE technique could potentially be modified by using a two-photon laser to uncage the ligand.
onto the patch, although the current lack of information about the precise dynamics of the 
GABA concentration transient remains a limitation of the SPLURgE technique.

Another general limitation of using pulled patches is that the membrane arises from 
the soma and the relative content of synaptic vs. extrasynaptic receptors is not known. In 
thalamic relay neurons, synaptic receptors are composed of $\alpha 1\beta 2\gamma 2$ GABA$_A$ receptor 
heteromers, whereas extrasynaptic are $\alpha 4\beta 2\delta$ (Sur et al., 1999; Porcello et al., 2003; Jia et 
al., 2005). Given that the latter are insensitive to benzodiazepine modulation (Rudolph and 
Möhler, 2004), then the augmentation of GABA$_A$ responses by nRT-derived endozepines in 
thalamic patches indicates that the sniffer patches do contain, at least in part, synaptic-type 
receptors. Nevertheless, the longer transient of GABA availability at the receptors in the 
SPLURgE technique may provide further applications for examination of extrasynaptic 
signaling mechanisms, which are more highly regulated by transporters (Beenhakker and 
Huguenard, 2010).

The amplitude of uncaging responses was consistently observed to progressively 
decrease over subsequent UV stimuli in both VB and nRT patches. In whole-cell recordings 
in VB neurons, which also displayed a significant rundown of uncaging response amplitude, 
the degree of this effect could be ameliorated by replacement of the used caged GABA 
solution (which over time will contain more accumulated GABA, cage molecules, and 
perhaps other by-products) with fresh caged GABA. Therefore, in cases where 
experimenters require as little rundown as possible, the caged GABA solution could be 
replenished before each recording, though this may be prohibitively expensive in many 
cases, especially where high concentrations are required.
The time required to move the pipette to nRT was typically less than 10 s. Given that decay properties of VB patch responses to GABA uncaging are stable across many trials (Figures 2, 4), it is unlikely that this delay accounts for the potentiation of the uncaging current. Furthermore, FLZ largely blocked the potentiation, demonstrating that endozepine actions play a major role in this difference. In addition, once the pipette was positioned over the nRT portion of the slice, the same amount of time was required to place the pipette within the slice as when the patch was placed in VB. It therefore appears that allosteric potentiation of GABAergic currents occurs on a fast time scale depending on the diffusion and binding of a given modulator to the affected GABA_ARs, although the rate of modulation detected by the SPLURgE likely depends both on the combination of GABA_A subunits and allosteric modulators in question. It is conceivable, however, that this technique could be used to understand the dynamics of allosteric modulation in cases in which this interaction occurs on a much slower time scale.

In addition to investigations into allosteric receptor modulation, we also demonstrate that this technique may be used to ascertain properties of GABA diffusion and uptake in brain slices. The altered profile of responses obtained at different positions along the z-axis within and above the slice suggests that GAT activity speeds up the decay kinetics of SPLURgEs by removing uncaged GABA, and conversely, that decreased GABA diffusion slows some SPLURgE decay properties. In control conditions, the half width parameter was not significantly affected by placement in the slice, but the 90-10% decay time was significantly shortened as the patch was placed more deeply into the slice. In GAT blockade conditions, however, SPLURgE half width significantly increased with placement in the slice, whereas decay time was not altered by z-axis position. This finding suggests that the
early decay times of SPLURgEs reflect opposing forces: 1) factors which would increase response duration such as tissue tortuosity, decreased volume fraction (Syková and Nicholson, 2008) and/or a gradient of GABA concentration through the depth of the slice; and 2) GAT-mediated uptake, which acts strongly to decrease response duration. The late decay component, on the other hand, appears primarily determined by the rate of GAT uptake. This is consistent with previous findings that GAT activity plays a critical role in determining the kinetics of GABA<sub>B</sub> IPSCs (Thompson and Gähwiler, 1992; Isaacson et al., 1993; Scanziani, 2000; Beenakker and Huguenard, 2010), which are similarly long-lasting. In addition, slight changes on SPLURgE duration with positioning closer to the surface of the slice (Figure 5) indicate that this technique could potentially be used to investigate properties of the “unstirred layer” at the surface of the slice (Lipinski and Bingmann, 1987; Hall et al., 2012). The powerful effects of GAT activity to decrease SPLURgE duration through removal of extracellular GABA also suggest that the degree of allosteric modulation in nRT (which acts in this case to increase SPLURgE duration) is partially masked by GAT uptake and may in fact be greater than observed by these methods.

Here we have focused on GABA<sub>A</sub>R modulation, but the SPLURgE methodology, with minor modifications, could theoretically be applied to the study of other neurotransmitter receptors as well. For example, glutamate receptors in patches pulled from dendrites (Davie et al., 2006) or somata (O’Connor et al., 1995) could be exposed to uncaged glutamate to detect endogenous modulators mimicking compounds that act allosterically at AMPA, NMDA, and metabotropic glutamate receptors (Fucile et al., 2006; Yang and Svensson, 2008; Mony et al., 2009; Costa et al., 2010; Sheffler et al., 2011). Similarly, acetylcholine, glycine, and serotonin receptors are also targets for allosteric modulation.
In summary, a major advantage of the SPLURgE method, which combines the “sniffer patch” recording configuration with laser photolysis of caged GABA, is relatively rapid assessment of local differences in GABAAR function. Furthermore, the ability to move receptors contained within membrane patches from one brain area to another provides a way to determine whether a lack of modulatory effect on a particular category of neurons or receptors is due to insensitivity to such modulation or a lack of local release of endogenous ligand. This technique should therefore be useful in applications for rapid detection of GABAAR modulators, as well as allosteric modulators that act on other classes of ionotropic receptors.

Acknowledgements

We thank Anne Herbert, Kathy Peng, and Corinne Badgley for assistance with mouse colony maintenance.

Grants

This work was supported by NIH R01 grants NS034774 and NS006477. C.A.C. was supported by NIH Institutional Postdoctoral Training Grant T32 NS007280, an Epilepsy Foundation of America Postdoctoral Research Fellowship, and a Katharine McCormick Advanced Postdoctoral Fellowship from Stanford School of Medicine.
Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.
References


Collins T, Young GT, Millar NS. Competitive binding at a nicotinic receptor transmembrane site of two alpha7-selective positive allosteric modulators with differing effects on agonist-evoked desensitization. Neuropharmacology 61: 1306–1313, 2011.


Figure 1. GABA release in thalamus evoked by electrical stimulation in nRT can be detected by VB membrane patches placed in VB, but not in nRT. A) Example microscope image of a horizontal thalamic slice showing positions of nRT (outlined by white dashed lines) and VB. Calibration bar, 250 μm. IC, internal capsule; Str, striatum. B) Representative averaged evoked GABAergic response (35V stimulation in nRT) of an outside-out sniffer patch pulled from a VB neuron and placed back in VB. Black dot indicates time of electrical stimulation applied to nRT. The illustrated response is typical of that observed in 5 out of 8 patches tested. C) Representative averaged response of a patch pulled from a VB neuron and then placed in nRT (40V stimulation). Example chosen from 8 patches tested. Electrical stimulation artifacts have been truncated for clarity. D) Stacked bar chart illustrating rate of response of sniffer patches pulled from VB neurons to electrical stimulation when placed in VB or nRT. *p<0.05, Fisher’s exact test.

Figure 2. Responses of VB patches to laser photolysis of caged GABA when patches are held above the slice. A) Representative example of uncaging responses in a VB patch in the presence of 100 μM caged GABA. The gray traces represent individual sweeps elicited every 10 s, and the black trace is the composite average. Numbers to the left of gray traces indicate the order of responses elicited. B) Individual uncaged IPSC half width (filled circles) and amplitude values (open triangles) for each sweep in the example in (A). C) Mean + SEM for the ratio of the uncaged IPSC half width (black bar) or amplitude (gray bar) in response to the third laser stimulus to the respective value of the response to the first stimulus, averaged across 6 patches. D) Individual uncaged IPSC amplitude values for an
example VB patch with uncaging responses elicited every 30 s. **E** Individual uncaged IPSC amplitude values for an example VB patch with uncaging responses elicited every 60 s. **F** Mean + SEM for the ratio of the uncaged IPSC amplitude in response to the third laser stimulus to the respective value of the first stimulus, averaged across 4 patches (30-s ISI) or 3 patches (60-s ISI). ISI, interstimulus interval; hυ symbol, 1 ms UV laser stimulus.

*p<0.05, **p<0.01, paired t-test within patches vs. response to first stimulus

**Figure 3.** Responses of nRT patches to laser photolysis of caged GABA when patches are held above the slice. **A** Representative example of averaged uncaging response in an nRT patch in the presence of 100 μM caged GABA. Example chosen from 3 patches tested. **B** Representative example (from 8 patches) of uncaging responses obtained every 10 s in an nRT patch in the presence of 1 mM caged GABA. The gray traces represent individual sweeps, and the black trace is the composite average. Numbers to the left of gray traces indicate the order of responses elicited. **C** Individual uncaged IPSC half width (filled circles) and amplitude values (open triangles) for each sweep in the example in (B). **D** Ratio of the uncaged IPSC half width (black bar) or amplitude (gray bar) in response to the third laser stimulus to the respective value of the response to the first stimulus, averaged across 5 patches. ISI, interstimulus interval; hυ symbol, 1 ms UV laser stimulus. *p<0.05, paired t-test within patches vs. response to first stimulus

**Figure 4.** Uncaged GABA response rundown occurs similarly in whole-cell vs. isolated patch mode, and is not rescued by inclusion of Mg-ATP in the pipette, but the degree of rundown increases progressively with each successive recording after introduction of new
caged GABA solution. A) Mean ± SEM for uncaged IPSC amplitude over successive trials normalized to the amplitude of the first uncaging response in each cell obtained at a 10-s ISI when cells were filled with either control CsCl internal (filled circles) or CsCl with additional 5 mM Mg-ATP (open circles). B) Individual values for each cell of the slope of the line of best fit through the normalized amplitude values over 10 successive trials. Linear regression lines of best fit show positive correlations between recording order after fresh caged GABA replacement, and the slope of rundown response. The solid line indicates the line of best fit for all control values (filled circles), and the dotted line indicates the line of best fit for cells recorded with Mg-ATP in the pipette solution (open circles). *p<0.05, paired t-test within cells vs. response to first stimulus (A); Pearson correlation (B)

Figure 5. GABA diffusion and uptake differentially regulate the early and late decay kinetics of SPLURgEs, respectively. A) Representative example of SPLURgEs in a VB patch in the presence of 100 μM caged GABA when the patch is placed back in the slice in VB. The gray traces represent individual sweeps elicited every 10 s, and the black trace is the composite average. Numbers to the left of gray traces indicate the order of responses elicited. Note here that all responses were obtained with the patch positioned within the slice. B) Individual SPLURgE half width values for each sweep in the example in (A). hυ symbol, 1 ms UV laser stimulus. C-D) Averaged SPLURgE traces from VB patches placed above the slice (black traces) and in the slice in VB (gray traces) in control conditions (C) or under conditions of pharmacological GAT blockade (D). Traces were obtained in the presence of 100 μM caged GABA and normalized to peak amplitude. E-F) Mean ± SEM for SPLURgE half width (E) and 90-10% decay time (F) as a function of z-axis placement of
pipette relative to the surface of the brain slice for VB patches placed back within VB in control conditions (black circles, 4 patches) or in the presence GAT blockers (white circles, 4 patches). Responses are normalized to the values obtained -50 μm above the slice for each patch. hv symbol, 1 ms UV laser stimulus. *p<0.05, **p<0.01 paired t-test within patches vs. response at -50 μm above slice; #p<0.05, ##p<0.01, ###p<0.001 t-test vs. Control at respective position depth; †, p<0.05 paired t-test within patches vs. normalized 90-10% decay time values at respective position depth.

**Figure 6.** SPLURgE potentiation in patches pulled from VB neurons and placed in nRT is flumazenil-sensitive and dependent on depth of patch placement. **A)** Averaged SPLURgE traces from representative individual VB patches placed in VB or nRT in the presence of 100 μM caged GABA, normalized to peak amplitude. **B-C)** Mean ± SEM for SPLURgE half width (B) and 90-10% decay time (C) as a function of z-axis placement of pipette relative to the surface of the brain slice for example VB patches placed in nRT in control conditions (black boxes, 4 patches) or in the presence of 1 μM flumazenil (FLZ, gray boxes, 4 patches). Responses are normalized to the values obtained >-25 μm above the slice for each patch. hv symbol, 1 ms UV laser stimulus. Data are presented as means ± SEM. *p<0.05, **p<0.01 paired t-test within patches vs. response at >-25 μm above slice; #p<0.05, ##p<0.01, ###p<0.001 t-test vs. Control at respective position depth; ††, p<0.01 paired t-test within patches vs. normalized 90-10% decay time values at respective position depth.