Methods for recording and measuring tonic GABA_A receptor-mediated inhibition

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Tonic inhibitory conductances mediated by GABA_A receptors have now been identified and characterized in many different brain regions. Most experimental studies of tonic GABAergic inhibition have been carried out using acute brain slice preparations but tonic currents have been recorded under a variety of different conditions. This diversity of recording conditions is likely to impact upon many of the factors responsible for controlling tonic inhibition and can make comparison between different studies difficult. In this review, we will firstly consider how various experimental conditions, including age of animal, recording temperature and solution composition, are likely to influence tonic GABA_A conductances. We will then consider some technical considerations related to how the tonic conductance is measured and subsequently analyzed, including how the use of current noise may provide a complementary and reliable method for quantifying changes in tonic current.

Keywords: tonic inhibition, GABA_A, extrasynaptic, methods, noise

INTRODUCTION

Tonic inhibition is mediated by extrasynaptic GABA_A receptors of defined subunit composition (usually containing the α5 or δ subunits). These receptors display a high affinity for GABA that allows them to respond to the low ambient levels of GABA present in the extracellular space and generate a persistent "tonic" inhibition. Tonic conductances have been found to be important regulators of neuronal excitability *in vivo* in granule cells of the cerebellum (Chadderton et al., 2004; Duguid et al., 2012) and olfactory bulb (Labarrera et al., 2013). However, most studies of tonic inhibition have been conducted *in vitro* in acute brain slice preparations and these have allowed the identification of tonic GABA_A receptor-mediated conductances in all major brain areas including cortex, hippocampus, thalamus, hypothalamus and brain stem (Farrant and Nusser, 2005; Brickley and Mody, 2012). Thus, recordings of tonic inhibition have been made in many different labs under a variety of different experimental conditions, many of which are likely to impact upon the magnitude of the tonic conductance.

Furthermore, various methods are available to analyse tonic conductances. This diversity of experimental conditions and analytical methods makes comparison of tonic inhibition between studies quite difficult. In this review, we will firstly consider how different experimental conditions, including choice of experimental animal, recording temperature and solution composition, influence the tonic GABA conductance. We will then consider the available methods for measurement and analysis of the tonic conductance, including how the use of current noise may provide a complementary method for analysis of tonic inhibition.

It should be noted that inhibition is a physiological process whereby the probability of a neuron generating an action potential is reduced. Therefore, the phrase "tonic inhibition" should only be used strictly when action potentials become less likely. The action of the high-affinity extrasynaptic receptors that underlie tonic GABA_A conductances may of course be excitatory, depending upon both the reversal potential and the magnitude of the conductance (Farrant and Kaila, 2007; Song et al., 2011). However, here, we use the phrase "tonic inhibition" to describe the activity of extrasynaptic GABA_A receptors, even when the effect on action potential generation has not been quantified, since this has become widely accepted as a convention within the field.

EXPERIMENTAL CONDITIONS FOR RECORDING *in vitro* TONIC INHIBITION

AGE OF ANIMAL

Generally, brain slices are prepared from either mice or rats, although other animals are used in some labs. In most cases, immature animals are used since slices from younger animals seem to harbor a higher proportion of healthy cells compared to adults, and also the lack of fully developed connective tissue makes it easier to visualize and patch cells within the preparation. However, the use of juvenile animals presents other problems, since many of the systems associated with neurotransmitter release, detection and uptake, are not fully developed. Indeed, early recordings of tonic inhibition in cerebellar granule cells demonstrated a developmental increase in tonic GABA conductance over the first few postnatal weeks (Brickley et al., 1996) that mirrored the increased expression of underlying α6-containing receptors (Kaneda et al., 1995; Tia et al., 1996). Later studies investigating developmental regulation of the tonic conductances in dentate gyrus granule cells (DGGCs) and relay neurons of the ventrobasal (VB) thalamus have also shown an increasing tonic conductance over the initial postnatal period (Peden et al., 2008; Holter et al., 2010; Lee and Liou, 2013).

Interestingly, it appears that the subunit composition of the GABA$_A$ receptors responsible for tonic inhibition in DGGCs may also be age-dependent, with potentially a larger α5-mediated component present in immature cells, compared with increased α4δ-GABA$_A$ receptors in the adult (Glykys et al., 2008; Herd et al., 2008; Holter et al., 2010). A very recent study has examined tonic inhibition within the auditory thalamus and shown that in old rats (28–32 months), the tonic conductance displayed by neurons of the medial geniculate body (MGB) is reduced by 50% compared with young adult (3–8 months) animals (Richardson et al., 2013). Thus, the age of the experimental animal is a key factor in determining the magnitude of the tonic inhibitory conductance.

STORAGE AND PERFUSION OF SLICES

The manner in which brain slices are stored prior to their use and perfused with solutions during recording may also be important variables in controlling tonic current amplitudes. It has been demonstrated that the tonic current recorded in hippocampal CA1 pyramidal cells is greater in slices stored in an interface chamber compared with the more commonly used submerged chamber (Glykys and Mody, 2006). It was suggested that this might be due to a decrease in GABA uptake, altered protein phosphorylation or a lower metabolic state of distress for cells stored in the interface chamber.

It is also apparent that the way in which slices are perfused with solution during recording can impact upon neuronal activity and thus may affect tonic GABA inhibition. For whole-cell recording, brain slices are usually placed on a thin glass plate and superfused with artificial cerebrospinal fluid (aCSF) i.e., the slice is submerged in recording solution. Neurons can then be visualized at high resolution using a water-immersion objective. The disadvantage of this set-up is that slices are only effectively perfused from one side and gradients for oxygen and nutrients may develop within the slice, leading to compromised neuronal activity. This can be a particular problem for experiments where high levels of neuronal activity are necessary, for instance, in the generation of network oscillations (Hajos et al., 2004; Hajos and Mody, 2009). Some improvement can be gained by using higher flow rates to improve the oxygen supply to the slice (Hajos et al., 2009).

Although not specifically addressed, perfusion rate (and hence oxygen supply) may also be important for tonic inhibition, since at higher flow rates inhibitory interneurons within the hippocampal CA3 area showed greater firing activity and CA3 pyramidal cells received a higher frequency of inhibitory synaptic input (Hajos and Mody, 2009; Hajos et al., 2009). Since tonic inhibition has been shown to be dependent upon GABA derived from synaptic release (Bright et al., 2007; Glykys and Mody, 2007b), a higher perfusion rate might be expected to be coupled to an enhanced tonic conductance. However, it should also be noted that enhanced flow rate might lead to greater wash-out of ambient transmitter within the slice so the effects of perfusion rate on tonic inhibition are not easy to predict.

RECORDING TEMPERATURE

Whole-cell recordings within brain slices are generally made either at room temperature (20–23°C) or at an elevated temperature, closer to physiological body temperature (37°C). However, stable recordings can be difficult to maintain at higher temperatures and therefore, intermediate temperatures are often used (30–35°C). Thus, recording temperatures can vary over a considerable range. Given the large temperature dependence of enzymatic reaction rates, it is unsurprising that changes in temperature can modulate intrinsic membrane properties and synaptic potentials in neurons (Thompson et al., 1985; Volgushev et al., 2000; Trevelyan and Jack, 2002; Kim and Connors, 2012). With regard to inhibitory transmission, the function of GABA$_A$ receptors is affected by temperature, with increased temperature reducing the apparent affinity for GABA and increasing single GABA channel conductance (Jenkins et al., 1999; Perrais and Ropert, 1999; Millingen et al., 2008). The impact of raised temperature on inhibitory synaptic transmission is manifest as an increased frequency of synaptic currents, correlated with increased firing of inhibitory interneurons (Perrais and Ropert, 1999; Kim and Connors, 2012). Elevated temperature also results in larger IPSCs with faster kinetics (Otis and Mody, 1992; Perrais and Ropert, 1999).

Tonic inhibition is dependent upon various factors, including GABA release and uptake, as well as the activation properties of the underlying extrasynaptic receptors—thus, the overall effect of temperature on tonic GABA$_A$ transmission will reflect a convolution of temperature effects on multiple processes. For example, uptake of GABA is more efficient at physiological temperatures than at room temperature (Mitchell and Silver, 2000). It has been demonstrated that enhanced uptake at physiological temperature constrains the spillover of GABA from synapses and prevents the activation of perisynaptic δ subunit-containing GABA$_A$ receptors in DGGCs (Wei et al., 2003). It is also of note that extrasynaptic GABA$_A$ receptors are subject to functional modulation by phosphorylation and the actions of kinases/phosphatases may be temperature dependent (Machu et al., 2006; Abramian et al., 2010; Tang et al., 2010; Saliba et al., 2012; Bright and Smart, 2013). We have recently discovered that the overall effect of raising the recording temperature from room (21–23°C) to physiological levels (34–36°C) is to cause a 2-fold increase in the tonic conductance in DGGCs (Bright and Smart, 2013). More work is necessary to establish whether tonic currents in other brain areas are also elevated with increased temperature.

COMPOSITION OF RECORDING SOLUTION

The basic composition of the aCSF used to bathe brain slices during recording is generally similar between different laboratories. However, differences arise in the additional ingredients that are sometimes added and these can play an important role in setting the amplitude of the tonic inhibition. The most obvious example of this is the addition of GABA to the aCSF. Tonic inhibition clearly relies upon the availability of an ambient GABA concentration sufficient to activate extrasynaptic GABA$_A$ receptors, and whilst in many brain areas tonic inhibition can be recorded without supplementary GABA, in many studies, a low concentration of GABA is added to the aCSF. There is some discussion as to whether tonic GABA currents recorded under conditions of added GABA are more or less "physiological" than currents recorded in the absence of extra GABA (Glykys and Mody, 2007a). Indeed, it has been suggested that if 5 μM GABA

is added to the aCSF, active GABA uptake within the slice may significantly reduce the ambient concentration to levels close to those measured *in vivo* (0.2–2.5 μM) and that this might provide a way of standardizing tonic GABA measurements (Glykys and Mody, 2006, 2007a).

However, there are some potential confounds to the approach of adding GABA to the aCSF, the most obvious being that it is unclear what the "physiologically-correct" ambient GABA level within the slice "should be." Measurements of extracellular ambient GABA concentrations *in vivo* cover a large range (30 nM–2.5 μM; (Lerma et al., 1986; Tossman et al., 1986; Ding et al., 1998; Bianchi et al., 2003; Xi et al., 2003; de Groote and Linthorst, 2007) and vary according to brain region and behavioral state. For example, GABA levels in the pontine reticular formation and the thalamus are dependent on arousal state, showing variation between wakefulness, rapid-eye-movement (REM) and non-REM sleep (Kekesi et al., 1997; Vanini et al., 2011). Interestingly, in the ventral hippocampus, GABA levels rise to around 800 nM during active exploration (de Groote and Linthorst, 2007).

Microdialysis is the most widespread method used to measure concentrations of various neurotransmitters, including GABA, in the living brain. However, there is some debate as to the origin of the GABA (and glutamate) that is detected (Timmerman and Westerink, 1997; Del Arco et al., 2003; van der Zeyden et al., 2008). Given the questions surrounding the origin of the GABA, the conditions used for measuring dialysate GABA have recently been critically re-examined (van der Zeyden et al., 2008). It was shown that the most widely used chromatographic method for assay of GABA is extremely sensitive to small changes in analytical conditions, particularly pH, and that unless conditions are optimized, measured GABA levels may be artifactually elevated.

Using optimized conditions, these investigators report basal GABA levels of only 2–4 nM in various brain regions, which are significantly lower than in other studies. Since microdialysis probes are also known to create a disrupted zone of tissue that may well influence local neurotransmitter levels, it was suggested that the development of microsensors to assay GABA may be necessary to fully define extracellular GABA concentrations within the brain. This may be compounded by complex tissue topography in the CNS and the potential for GABA concentrations to vary between tissue compartments. Adding GABA may also cause problems since increased GABA levels may recruit additional populations of receptors to contribute to tonic inhibition (Scimemi et al., 2005) as well as desensitizing high affinity δ-GABA$_A$ receptors (Mortensen et al., 2010; Bright et al., 2011). There is also a need to consider how the ambient GABA concentration may influence drug modulation of these receptors (Houston et al., 2012).

Other additions to the aCSF may be made to enhance slice viability, particularly during the slicing process. Common examples of these include sodium pyruvate, ascorbic acid and indomethacin. However, it should be noted that some of these supplementary compounds may also cause variation in the measured tonic current. In particular, it is well established that GABA$_A$ receptors are subject to modulation by redox reagents (Pan et al., 1995; Amato et al., 1999). A recent study has

demonstrated that the tonic current mediated by GABA$_C$ receptors in retinal bipolar cells is significantly enhanced by the endogenous reducing agent, ascorbic acid (Calero et al., 2011). Given that receptors lacking a γ2-subunit are more sensitive to the actions of redox reagents (Amato et al., 1999), extrasynaptic δ-GABA$_A$ receptors may also be significantly modulated by redox compounds. Indeed, it may be that recordings within brain slices underestimate tonic current amplitudes due to wash-out of endogenous reducing agents such as ascorbic acid (Hajos and Mody, 2009).

MEASUREMENT AND ANALYSIS OF TONIC INHIBITION

The standard method to reveal the presence of a tonic GABA current in a whole-cell recording is to apply a saturating concentration of a specific GABA$_A$ receptor antagonist, usually bicuculline, gabazine (SR99531) or picrotoxin (PTX). As well as blocking IPSCs, this treatment will reveal a tonic inhibition by causing a shift in the holding current (outward if E$_{Cl}$ is set to be close to 0 mV as is often the case), and a reduction in the baseline current variance, consistent with a decrease in the number of open GABA$_A$ channels.

PHARMACOLOGICAL BLOCKADE OF GABA$_A$ RECEPTORS

Care should be taken both in the choice of antagonist and its concentration. For instance, bicuculline can either be used as a pure base or as one of a number of charged quaternary salts (methoiodide, methochloride or methobromide). The quaternary salts are used experimentally due to their greater water solubility compared with the base. However, these bicuculline salts are not specific blockers of GABA$_A$ receptors since they will also block some Ca^{2+}-activated K$^+$ channels at similar concentrations (Seutin and Johnson, 1999).

It is also important to note that the different antagonists exhibit different mechanisms of action. Picrotoxin acts as a potent mixed GABA$_A$ receptor antagonist (Smart and Constanti, 1986). The conventional view has been that the action of picrotoxin results from binding to a site within the ion channel, which results in a steric hindrance of ion flow (Takeuchi and Takeuchi, 1969; Inoue and Akaike, 1988; Sedelnikova et al., 2006; Erkkila et al., 2008). However, various studies have shown features of picrotoxin inhibition that are inconsistent with a simple channel-block mechanism and it has therefore been postulated that picrotoxin may also allosterically stabilize one or more nonconducting (i.e., closed or desensitized) states of the channel (Smart and Constanti, 1986; Newland and Cull-Candy, 1992; Krishek et al., 1996; Korshoej et al., 2010). In contrast, bicuculline and gabazine are both competitive antagonists that displace GABA from the agonist binding site and therefore prevent receptor activation (Akaike et al., 1987). Additionally, both these drugs act as negative allosteric inhibitors of channel opening, inhibiting activation of GABA$_A$ receptors by anaesthetic agents, with bicuculline being more effective in this respect (Ueno et al., 1997). This greater inverse agonist activity is likely to explain the ability of bicuculline, but not gabazine, to inhibit spontaneous GABA-independent channel opening in hippocampal pyramidal cells and DGGCs (Bai et al., 2001; Mtchedlishvili and Kapur, 2006; McCartney et al., 2007; Wlodarczyk et al.,

2013). Conversely, the lower inverse agonist activity of gabazine may allow it to selectively block synaptic GABA$_A$ receptors at lower concentrations without affecting the extrasynaptic receptors mediating tonic inhibition (Stell and Mody, 2002; Park et al., 2007; Yamada et al., 2007). Caution should also be exercised when using high concentrations of gabazine ($>100\,\mu$M) since it appears to show some agonist activity (Wlodarczyk et al., 2013).

Some studies have used subtype-selective blockers of GABA$_A$ receptors to identify specific components of the tonic current. The first identified example of a subtype-selective antagonist was furosemide which blocks α6-containing receptors with high affinity (Korpi et al., 1995). Accordingly, concentrations of up to ∼100 μM furosemide that are ineffective at other GABA$_A$ receptors or importantly, at K$^+$Cl$^-$ cotransporters (Hamann et al., 2002) produce a significant blockade of the α6δ-mediated tonic current in cerebellar granule cells (Hamann et al., 2002; Wall, 2002; Bright et al., 2011). At higher concentrations (100–1000 μM), furosemide blocks α4-containing receptors (Wafford et al., 1996) and has been used to inhibit the tonic conductance mediated by these receptors in hippocampal neurons (Mangan et al., 2005; Mtchedlishvili and Kapur, 2006).

Subtype selective inhibition is also provided by the benzodiazepine-site inverse agonist, L-655,708, which blocks α5-containing receptors with at least 50-fold selectivity over receptors containing α1/2/3/6 subunits (Quirk et al., 1996; Casula et al., 2001). This compound has been shown to partially-block tonic currents mediated by α5-receptors in pyramidal cells of both the hippocampus and cortex (Caraiscos et al., 2004; Scimemi et al., 2005; Yamada et al., 2007; Glykys et al., 2008). It should be noted that, whilst radiolabelling with L-655,708 demonstrates that this compound binds with high affinity ($K_D \sim$ 2.5 nM, (Quirk et al., 1996) which correlates with a high potency for block of recombinant α5β3γ2 receptors (IC$_{50}$ = 1.1 nM, Atack et al., 2006), the maximum block achievable is limited (∼17%, Atack et al., 2006). Therefore, L-655,708 is a very weak partial inverse agonist and can only achieve partial blockade of α5-mediated tonic currents.

The cation Zn^{2+} is a useful pharmacological tool to separate components of tonic inhibition since it blocks αβ and αβδ receptors with significantly higher potency (respective IC$_{50}$s 88 nM and 6–16 μM) than receptors containing the γ2 subunit (IC$_{50}$ 300 μM; (Krishek et al., 1998; Hosie et al., 2003). Recordings from cultured hippocampal pyramidal neurons revealed that a component of the tonic current was highly sensitive to inhibition by Zn^{2+}, suggesting the potential contribution (∼10%) of αβ GABA$_A$ receptor isoforms (Mortensen and Smart, 2006). More generally, application of Zn^{2+} has been used to detect the involvement of non-γ2 containing receptors in the generation of tonic currents (Jia et al., 2005; Nani et al., 2013).

Given the sensitivity to Zn^{2+}, it is perhaps not surprising that recently, Cu^{2+}, which shares many characteristics with Zn^{2+} in terms of coordination and is known to inhibit invertebrate GABA receptors (Irving and Williams, 1953; Smart and Constanti, 1982), has been demonstrated to inhibit GABA$_A$ receptors underlying tonic inhibition with high selectivity over synaptic GABA$_A$ receptors (McGee et al., 2013). The trivalent cation, La^{3+}, is also a selective blocker at certain extrasynaptic GABA$_A$ receptors, showing a potent inhibition at α4/6βδ receptors, a weaker inhibition at α4/6βγ2 and α4β receptors, and a potentiation of GABA at α1βδ/γ2 receptors (Im et al., 1992; Saxena et al., 1997; Zhu et al., 1998; Brown et al., 2002; Storustovu and Ebert, 2006). However, La^{3+} has only been used infrequently in studies of tonic conductances (Shen et al., 2005), probably due to its low efficacy of inhibition (Storustovu and Ebert, 2006).

SELECTIVE ACTIVATION/MODULATION OF EXTRASYNAPTIC GABA$_A$ RECEPTORS

Many studies demonstrate relatively small tonic GABA$_A$ current amplitudes under basal conditions in brain slice preparations. It is therefore often advantageous to boost the tonic current by addition of either a selective GABA$_A$ agonist or a positive allosteric modulator. The GABA$_A$ agonist, THIP (4,5,6,7-tetrahydoisoxazolo[5,4-c]pyridin-3(2H)-one) has been extensively characterized as a "super-agonist" at both αβ and αβδ receptors (Adkins et al., 2001; Brown et al., 2002; Storustovu and Ebert, 2006; Mortensen et al., 2010), whilst displaying only partial agonist activity at synaptic αβγ receptors (Ebert et al., 1994; Mortensen et al., 2004). THIP therefore provides a useful selective agonist for activation of the tonic current and has been widely used in this context (e.g., Cope et al., 2005; Maguire et al., 2005; Drasbek and Jensen, 2006).

Selective positive allosteric modulation of extrasynaptic GABA$_A$ receptors may also provide a useful tool for detection and characterization of the tonic current. Endogenous neurosteroids act as potent positive allosteric modulators at all major GABA$_A$ receptor subtypes (Belelli et al., 2002; Belelli and Lambert, 2005) by binding to a defined site on the receptor α subunit (Hosie et al., 2006, 2009). Studies in recombinant systems suggest that receptors containing the δ subunit are more sensitive to neurosteroid-induced potentiation than those containing a γ subunit (Belelli et al., 2002; Brown et al., 2002; Wohlfarth et al., 2002). Consistent with this, in some brain areas the tonic GABA$_A$ current appears to be more sensitive to neurosteroid modulation than synaptic GABA$_A$ currents (e.g., Stell et al., 2003; Maguire et al., 2005; Bright and Smart, 2013). However, caution should be exercised in using neurosteroids since in some cases, synaptic inhibition may be equally, or more sensitive, than the tonic current (Porcello et al., 2003; Cope et al., 2005). Diversity in neurosteroid modulation may arise from variations in ambient GABA level, differences in receptor phosphorylation and the direction of Cl$^-$ flux (reviewed in Herd et al., 2007).

More recently, a novel compound, DS2, has been identified as a selective modulator of δ-containing GABA$_A$Rs (Wafford et al., 2009; Jensen et al., 2013). This compound acts via a new binding site on α4/6βδ receptors to enhance the efficacy of GABA, whilst having little action on αβ or αβγ receptors (Jensen et al., 2013). As expected from the recombinant data, DS2 selectively potentiates the tonic current in thalamic relay neurons with no effect on IPSCs (Wafford et al., 2009; Jensen et al., 2013). Thus, DS2 may represent a more useful tool than neurosteroids for selective modulation of extrasynaptic GABA$_A$ receptor activity.

CHARACTERIZATION OF TONIC CURRENT *in vivo*

Most studies of tonic inhibition have been carried out *in vitro* in brain slice preparations. However, it remains an important question to determine how the tonic GABA current impacts upon neuronal excitability *in vivo*. To date, only a few studies have recorded tonic currents within the living brain. In cerebellar granule cells, a tonic current is manifest upon application of a high concentration (0.5 mM) of the specific antagonist, gabazine (Chadderton et al., 2004; Duguid et al., 2012). Conversely, the tonic current can be selectively activated by application of THIP (Duguid et al., 2012). The tonic inhibition generated by extrasynaptic GABA$_A$ receptor activation appears to be carefully tuned to provide high fidelity transmission of sensory information since either enhancement or blockade leads to a reduction in the signal-to-noise ratio (Duguid et al., 2012). A recent study has also characterized tonic inhibition in granule cells of the olfactory bulb *in vivo* (Labarrera et al., 2013). In these cells, the tonic current was blocked by application of gabazine or furosemide, with, in the case of the latter antagonist, little effect on inhibitory synaptic currents. The selective block of the tonic current with furosemide was used to show that tonic inhibition dominates over the phasic inhibition stimulated by odor presentation and thus provides a key regulator of neuronal excitability. Recording of tonic currents *in vivo* is complicated by the necessity of using high antagonist concentrations to ensure penetration to the site of action and by the fact that drug wash-out is not possible.

MEASUREMENT OF TONIC CURRENT

The magnitude of the tonic current mediated by extrasynaptic GABA$_A$ receptors is typically revealed by measuring the change in the holding current evoked by applying a GABA antagonist. Since most neuronal recordings will also include inhibitory synaptic currents, it is important to exclude these from the tonic current measurement. There are two simple methods that are generally used to measure the holding current shift (**Figure 1**). Firstly, the baseline current can be digitally sampled by averaging over short epochs (1–10 ms) at regular intervals (100–1000 ms). Any baseline points falling on the decay of an IPSC are simply omitted before averaging the remainder to obtain "clean" measures of the holding current before and after antagonist application.

Alternatively, the baseline current can be analyzed by generating an all-points histogram and fitting a Gaussian distribution to the positive side of this histogram, i.e., the side that is uncontaminated by the negative-going IPSCs which will skew the distribution (**Figure 1**; (Nusser and Mody, 2002). Then, the means of the fitted Gaussians are used to define the holding currents before and after antagonist application. In practice, both of these methods for calculating baseline shifts tend to produce nearly identical results. The second method has been refined to allow the simultaneous measurement of tonic and phasic inhibitory currents and to establish any temporal correlation between the two forms of inhibition (Glykys and Mody, 2007b). Tonic currents are often normalized to the cell capacitance (pA/pF) to allow comparison between cells of different sizes and may also be expressed as conductances (pS/pF) to allow comparison with other active channel populations.

FIGURE 1 | Methods of measuring tonic GABA current shifts. (A) Example current trace recorded from a thalamic relay neuron illustrating block of sIPSCs by application of bicuculline (BIC) and the simultaneous block of a tonic GABA current, revealed by the outward shift in the holding current. Epochs used to define the holding current under control and bicuculline conditions are illustrated in blue and red respectively. **(B)** The holding current is sampled by averaging over 5 ms at 100 ms intervals. Any of these baseline points falling on the decay of a sIPSC are omitted, before plotting against time to give the current-time plot shown. Values for the current in control and bicuculline are then calculated by averaging over the 10 s long epochs shown in blue and red respectively—these mean values are shown in the inset. Using this method, the tonic current is 77.1 − 43.3 = 33.8 pA. **(C)** The tonic current can also be defined by generating all-points histograms for the control and bicuculline epochs and then fitting Gaussian curves to the positive side of these histograms. The mean values from the Gaussian fits are then used to define the currents in control and bicuculline. This method thus gives a tonic current of 75.3 − 43.3 = 32 pA.

USE OF CURRENT NOISE

Under conditions where the tonic current amplitude is small, it may be useful to use changes in the current noise as a way of monitoring changes in the activity of tonically open GABA$_A$ receptors

(Mtchedlishvili and Kapur, 2006; Glykys and Mody, 2007a). The current noise may be quantified as either the variance (σ^2) or the RMS noise (the square root of the variance, equivalent to the standard deviation of the signal), defined using the following equations.

$$\sigma^2 = \frac{1}{n} \sum_{x=1}^{n} (I(x) - I)^2$$

where n is the number of samples, $I(x)$ is the current measured for the xth sample and I is the mean current defined by:

$$I = \frac{1}{n} \sum_{x=1}^{n} I(x)$$

If we assume that the channels function independently from each other and are either open or closed (i.e., only two states), then the mean current and variance can also be expressed using the channel open probability, P, and the single channel current, i, as:

$$I = NPi \tag{1}$$

$$\sigma^2 = Ni^2 P(1-P) \tag{2}$$

These equations (see (Hille, 1992) for derivations) can then be combined to give:

$$\sigma^2(I) = iI - \frac{I^2}{N} \tag{3}$$

Equation (3) relates the current I to the variance σ^2. This is a parabolic equation with two roots at $I = 0$ and $I = iN$. Hence, there will be no noise when all of the channels are either fully shut ($P = 0$) or fully open ($P = 1$).

Equation (3) provides the basis for the stationary and non-stationary noise analysis techniques which have been widely used to obtain estimates of i, N, and P for both voltage and ligand gated ion channels (Sigworth, 1980; Traynelis and Jaramillo, 1998; Lingle, 2006; Hartveit and Veruki, 2007). With regard to inhibitory transmission, non-stationary noise analysis has been used in many studies to estimate the underlying single channel conductance and open probability for the synaptic GABA_A receptors that are responsible for fast phasic inhibition (for example, De Koninck and Mody, 1994; Nusser et al., 1997; Houston et al., 2008; Bright et al., 2011). However, noise analysis techniques have only infrequently been applied to extrasynaptic GABA_A receptor activation (Kaneda et al., 1995; Bai et al., 2001; Naylor et al., 2005). We wanted to assess the conditions under which changes to membrane current noise would be a more advantageous measure compared with changes in tonic current for assessing variations in tonic inhibition.

To do this, we have used simulated channel noise generated by the ion channel noise simulation module of WinEDR software (V3.3.8, John Dempster, University of Strathclyde, UK). This uses a simple 2-state model to produce the random fluctuations in current associated with stochastic opening and closing of a population of ion channels. Simulations were configured to mimic our typical conditions for recording tonic currents within brain slice preparations—therefore, we used a sampling interval of 0.05 ms (equivalent to a sampling frequency of 20 kHz) and a background noise variance of 2 pA (measured from room temperature recordings in DGGCs).

Simulation parameters associated with the ion channel population are the single channel current, i, the number of channels, N, the probability of channel opening, P and the open time constant, τ_{open}. Setting of these parameters was guided by values obtained from an earlier study in our lab of agonist-activated single-channel currents in outside-out patches from HEK293 cells expressing recombinant $\alpha 4\beta 3\delta$ GABA_A receptors (Mortensen et al., 2010). Thus, the single channel current i is ~2 pA (equivalent to a conductance of ~30 pS), the maximum open probability P is ~0.1 (for activation by GABA) and the mean open time constant τ_{open} is ~1 ms. The number of channels N was arbitrarily set to be 100.

Tonic inhibition may be modulated by changes in any of the three underlying parameters: single channel current, number of channels and the open probability. Thus, we will consider the impact of changes to these parameters on simulated tonic current and noise.

Changes in open probability

To assess the impact of changes in channel open probability, we simulated 20 s long stretches of channel opening for increasing values of P from 0 to 0.4 in 0.025 increments (**Figure 2**). Baseline current was sampled by averaging over 5 ms epochs every 100 ms and then the mean current was calculated by averaging over 1 s intervals (i.e., 10 baseline points per mean value, 20 mean current points per 20 s long activation). Baseline variance was calculated over 100 ms epochs, before averaging to give a mean variance for each 1 s interval.

Figure 2A shows current segments generated using increasing values of P up to 0.4 for single channels with currents of -1 and -2 pA. The maximum open probability used for these simulations is about 4-fold higher than expected for activation of δ-GABA_A Rs by GABA (Mortensen et al., 2010). Plotting variance vs. mean current plots revealed the expected parabolic curves and these were well fitted by equation (3) (**Figure 2B**). Estimates of N and i obtained from the parabolic fits correlated well with the expected theoretical values ($i = -1$ pA: $N = 101.0$, $i = -0.99$ pA; $i = -2$ pA: $N = 101.0$, $i = -1.98$ pA).

To determine whether the variance σ^2 is a more sensitive indicator of changes in the open probability P than the current I, we plotted both σ^2 and I against P (**Figure 2C**). We then calculated the slopes for the I-P and σ^2-P plots ($\Delta I/\Delta P$ and $\Delta\sigma^2/\Delta P$) and plotted these against P (**Figure 2D**). For a given change in P, the absolute value of the variance will change more than the absolute value of the current if $\Delta\sigma^2/\Delta P$ is greater than $\Delta I/\Delta P$. For example, for the case of a single channel current $i = -1$ pA, $\Delta\sigma^2/\Delta P$ is always less than $\Delta I/\Delta P$ and therefore, the current will be a more sensitive indicator of changes in P. We have quantified this relationship for various values of i (-1, -2, -3, -5, -10, -20, -50 pA) by performing linear regression on the slope plots and finding the P value where each pair of straight

FIGURE 2 | Effects of changing open probability *P* on simulated tonic current and noise. (A) Simulated "noisy" currents generated by using a simple 2-state model in WinEDR. Simulation parameters used for the ion channels were number of channels $N = 100$ and open time constant $\tau = 1$ ms with a background noise of 2 pA. Open probability *P* was increased from 0 to 0.4 in 0.025 increments of which only selected values are shown. Example currents are shown for single channel currents $i = -1$ and -2 pA. **(B)** Plots of the mean current I against variance σ^2 illustrate the expected

parabolic relationship—data points are fitted well with equation (3). **(C)** Plots of mean current I and variance σ^2 against open probability *P* for the two selected single channel currents. **(D)** The slopes of the *I-P* and σ^2-*P* plots were calculated ($\Delta I/\Delta P$ and $\Delta \sigma^2/\Delta P$) and plotted against *P*. Linear regression was performed on the slope plots (solid lines for $\Delta I/\Delta P$ and dashed lines for $\Delta \sigma^2/\Delta P$) and the points of intersection between these lines are indicated by the arrows. The *P* value at these points of intersection, $P_{intercept}$ is plotted against the single channel current (inset).

lines cross ($P_{intercept}$). Thus, we can see that for increasing values of *i*, the range of *P* values over which $\Delta \sigma^2/\Delta P$ is greater than $\Delta I/\Delta P$ increases and $P_{intercept}$ tends toward 0.5 (inset, **Figure 2D**). However, for the low values of *i* expected for physiological activation of δ-GABA$_A$Rs (up to about -2 pA), whether the variance is a more sensitive indicator of changes in open probability will depend upon both *P* and *i*. Thus, for single channel current of -1 pA or less, the current will always be a better indicator of changes in *P*, whilst for -1 pA $< i < -2$ pA, the variance will be more sensitive when *P* is low (less than ~0.25 for $i = -2$ pA).

Changes in number of receptors

The impact of changing the number of channels (*N*) was investigated by again simulating stretches of channel opening with increasing *P* (0–0.4 in 0.025 increments as before with a single channel current $i = -2$ pA) but this time we also varied the value of *N* (5, 10, 20, 50, 100, 200). **Figure 3A** shows example current-variance plots generated for values of $N = 10$, 20, & 50, that are again well fitted by the expected parabolic relationship [equation (3)]. Plotting current and variance against *N* illustrates that both increase linearly and that for values of *P* up to 0.4, the variance is

always greater than the current (**Figure 3B**). Inspection of equations (1) and (2) shows that this linearity is expected since both current and variance are proportional to *N*. Hence, for a given change in *N*, both current and variance will change by the same relative amount e.g., doubling *N* will causes a doubling of both current and variance. However, plotting the slopes of the current and variance with respect to *N* ($\Delta I/\Delta N$ and $\Delta \sigma^2/\Delta N$) reveals that both slopes are constants for a given *P* value (as expected) and that the slope of the variance is always greater than that of the current (**Figure 3C**). Hence, for a given change in *N*, the absolute value of the variance will change more than the absolute value of the current. This simple analysis would suggest that over the range of open probabilities expected for extrasynaptic δ-GABA$_A$R activation by GABA (0–0.1; Mortensen et al., 2010), using changes in variance might be a more sensitive method for detecting changes in receptor number *N*.

Changes in single channel current

Finally, we examined the effect of changing the single channel current *i*. These simulations were also performed for increasing values of *P* (0–0.4 in 0.025 increments) with a fixed number of channels $N = 100$, whilst we varied the value of *i*. Compared with
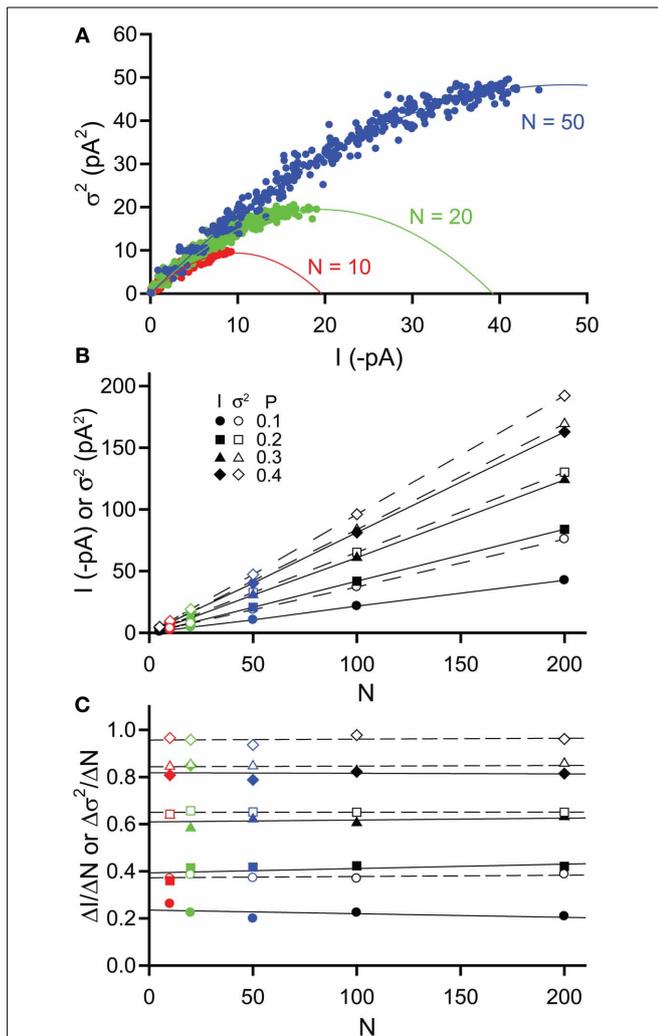
FIGURE 3 | Effects of changing channel number N on simulated tonic current and noise. Simulated currents were generated in WinEDR to investigate the impact of changing channel number N on current and variance. Open probability P was increased from 0 to 0.4 in 0.025 increments **(A)** Plots of the mean current I against variance σ^2 for $N = 10$, 20, & 50 illustrate the expected parabolic relationship—data points are fitted well with equation (3). **(B)** Plots of mean current I (filled symbols, solid lines) and variance σ^2 (open symbols, dashed lines) against channel number N for P values of 0.1, 0.2, 0.3, & 0.4. **(C)** The slopes of the I-N and σ^2-N plots were calculated ($\Delta I/\Delta N$, filled symbols and $\Delta\sigma^2/\Delta N$, open symbols) and plotted against N. Linear regression was performed on the slope plots (solid lines for $\Delta I/\Delta N$ and dashed lines for $\Delta\sigma^2/\Delta N$). The slopes are constant for both current and variance but for a given P value, $\Delta\sigma^2/\Delta N$ is always greater than $\Delta I/\Delta N$.

FIGURE 4 | Effects of changing single channel current i on simulated tonic current and noise. Simulated currents were generated in WinEDR to investigate the impact of changing single channel current i on current and variance. Open probability P was increased from 0 to 0.4 in 0.025 increments with a fixed number of channels $N = 100$. **(A)** Plots of the mean current I against variance σ^2 for $i = -0.5$, -1, & -2 pA illustrate the expected parabolic relationship—data points are fitted well with equation (3). **(B)** Plots of mean current I (filled symbols, solid lines are linear fits to data points) and variance σ^2 (open symbols, dashed lines represent fitting of a quadratic equation to the data points) against single channel current i for P values of 0.1 & 0.2. **(C)** The slopes of the I-i and σ^2-i plots were calculated ($\Delta I/\Delta i$, filled symbols and $\Delta\sigma^2/\Delta i$, open symbols) and plotted against i. Linear regression was performed on the slope plots (solid lines for $\Delta I/\Delta i$ and dashed lines for $\Delta\sigma^2/\Delta i$) and the points of intersection between these lines are indicated by the arrows. The i value at these points of intersection, $i_{intercept}$ is plotted against the open probability P (inset).

the simulations performed in section 2.3.1, we used lower values of i (-0.25, 0.5, -1.0, -1.5, -2.0, and -5.0 pA). Under physiological conditions when the intracellular Cl$^-$ ion concentration is typically low, the driving force for Cl$^-$ ion flux will be small, therefore leading to a reduced single channel current for GABA$_A$ receptors (e.g., for a driving force of 10 mV with a single channel conductance of 30 pS, $i = 0.3$ pA).

Figure 4A shows example current-variance plots generated for $i = -0.5$, -1, & -2 pA that are again well fitted by the expected

parabolic relationship [equation (3)]. If we plot both current and variance against i, then we can see that current increases linearly with i, as expected from equation (1), whilst the variance increases with the square of i, as expected from equation (2) (**Figure 4B**). To establish when the variance is a more sensitive indicator of changes in i than the current, we have plotted the slopes of both

($\Delta I/\Delta i$, $\Delta\sigma^2/\Delta i$) with respect to i (**Figure 4C**). As expected, for a given value of P, the slope of the current is constant whilst that of the variance increases linearly. We have quantified when $\Delta\sigma^2/\Delta i$ is greater than $\Delta I/\Delta i$ by performing linear regression on the slope points and finding the value of i at the point of intersection between these two lines ($i_{intercept}$). Thus, when i is greater than $i_{intercept}$ then the variance should be a more sensitive measure of changes in i. From **Figure 4C**, we can see that $i_{intercept}$ increases with open probability P. For the low values of P expected for the activation of δ-GABA$_A$Rs by GABA ($P < 0.1$), the variance will change more than the current when i is greater than about 0.6 pA. For a 30 pS single channel conductance, this is equivalent to a driving force for Cl$^-$ ions of 20 mV. This indicates that under physiological conditions when the extrasynaptic GABA$_A$ receptor conductance is more shunting than hyperpolarizing, changes in macroscopic current should be a more sensitive indicator.

Summary—when to use current or variance

Using a simple analysis of simulated current noise, we have investigated the conditions under which analysis of the current variance might prove a more sensitive way of determining changes in extrasynaptic receptor activity than monitoring changes in current. Changes in tonic inhibition might result from modulation of receptor open probability P, changes in receptor number N and/or modulation of single channel current i. Of these, perhaps changes in P are most likely, since this will occur when the ambient GABA concentration varies or when activation properties of extrasynaptic receptors are altered (e.g., by phosphorylation). Our analysis would suggest that for the low values of i that are expected when cells are not chloride-loaded, the macroscopic current is a better indicator of changes in P. For higher values of i (-1 pA $< i < -2$ pA), the variance will be a more sensitive monitor, if P remains relatively low (less than ~0.25). Hence, under the usual experimental conditions for recording tonic inhibition (i.e., with high intracellular Cl$^-$ concentration), it might well be better to look for changes in variance.

Changes in tonic inhibition resulting from altered receptor number N might arise if there is an alteration in receptor trafficking. At the moment, very little is known about what controls the surface expression and targeting of extrasynaptic GABA$_A$ receptors compared with their synaptic counterparts (Moss and Smart, 2001; Arancibia-Carcamo and Kittler, 2009; Luscher et al., 2011). We have recently shown that surface expression of δ-GABA$_A$ receptors is reduced by activation of protein kinase C (Bright and Smart, 2013) and it would be surprising if other signaling cascades do not impinge upon extrasynaptic receptor trafficking. Our analysis would suggest that changes in receptor number N lead to a greater change in the variance than the current. Hence, over the range of open probabilities that we have examined (up to $P = 0.4$), the current variance is a more sensitive indicator of changes in N.

Although it is generally accepted that the underlying single channel conductance of GABA$_A$ receptors is not affected by modulators (though c.f. Eghbali et al., 1997), the single channel current will be affected by changes in the ionic driving force. For extrasynaptic GABA$_A$ receptors, changes in single channel current i will occur if the reversal potential for Cl$^-$ ions is altered, under conditions of altered Cl$^-$ homeostasis or if the neuronal membrane potential is changed. Our analysis would indicate that when the single channel current is small (i.e., when the tonic conductance is more shunting than hyperpolarizing), then changes in membrane current represent a more sensitive means of detecting changes in i. However, when the single channel current is greater, (for instance, when GABA$_A$ receptor activity is recorded under conditions of symmetrical intracellular and extracellular Cl$^-$ ion concentration), then the variance will be more sensitive to changes in i.

CONCLUDING REMARKS

Tonic GABA$_A$ receptor-mediated conductances have been recorded under a multiplicity of different experimental conditions, many of which may impact upon the magnitude of the recorded conductance. It would not be possible to recommend one set of "ideal" conditions under which to record tonic inhibition, since experimental methods are determined by many other factors. However, experimenters should be aware of the potential contributions of various experimental conditions, including the age of the animal, how brain slices are stored and perfused, the recording temperature and the composition of the recording solution, in determining the amplitude of the tonic conductance.

Tonic currents are revealed by the application of a specific GABA$_A$ receptor antagonist. Care should be taken with both the choice and concentration of antagonist used since some blockers may not be absolutely selective and different blockers may exhibit distinct pharmacological modes of action. The tonic current is measured by quantifying the current shift upon blockade of GABA$_A$ receptors, taking care to exclude the contribution of synaptic currents. There are two established methods for achieving this (described in section Measurement of Tonic Current), both of which tend to produce nearly identical results.

The use of current noise may provide a complementary approach, both for establishing the presence of a tonic conductance and for monitoring changes in extrasynaptic receptor activity. Our analysis of simulated currents suggests that monitoring changes in current variance may provide a more sensitive way of detecting changes in tonic inhibition under specific conditions than simply measuring changes in current. In particular, it is noteworthy that for the low open probabilities expected for activation of δ-GABA$_A$ receptors, changes in receptor number N, manifest upon altered trafficking, would be more easily detected from changes in the variance than the current. It should be noted that we have used a simple two-state model for generating current activations and that the resulting current-variance relationships are always parabolic. However, it has been shown for synaptic receptor activation that the shape of current-variance relationships can be transformed from parabolic to skewed, depending upon the temporal structure of underlying current fluctuations (Hartveit and Veruki, 2006). At the moment, it is not clear whether physiological extrasynaptic receptor activation generates a parabolic or skewed current-variance relationship. Nevertheless, given that the variance is a "free" signal and is easily measured, it may well represent a useful way of monitoring changes in tonic GABA$_A$ receptor activity.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.