

Glycinergic mIPSCs in mouse and rat brainstem auditory nuclei: modulation by ruthenium red and the role of calcium stores

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Spontaneous miniature inhibitory postsynaptic currents (mIPSCs) recorded in central neurons are usually highly variable in amplitude due to many factors such as intrinsic postsynaptic channel fluctuations at each release site, site-to-site variability between release sites, electrotonic attenuation due to variable dendritic locations of synapses, and the possibility of synchronous multivesicular release. A detailed knowledge of these factors is essential for the interpretation of mIPSC amplitude distributions and mean quantal size. We have studied glycinergic mIPSCs in two auditory brainstem nuclei, the rat anteroventral cochlear nucleus (AVCN) and the mouse medial nucleus of the trapezoid body (MNTB). Our previous results have demonstrated the location of glycinergic synapses on these neurons to be somatic, thus avoiding electrotonic complications. Spontaneous glycinergic mIPSCs were recorded from AVCN and MNTB neurons in brainstem slices, in the presence of TTX to block action potentials, and 6-cyano-7-nitroquinoxaline-2, 3-dione, (\pm)-2-amino-5-phosphopentanoic acid and bicuculline to block glutamatergic and GABAergic synaptic currents. Ruthenium red (RuR), which was used to increase the frequency of mIPSCs, significantly changed the shape of most (90%) mIPSC amplitude distributions by increasing the proportion of large-amplitude mIPSCs. The possibility was investigated (following previous evidence at GABAergic synapses) that large-amplitude glycinergic mIPSCs are due to synchronous multivesicular release initiated by presynaptic calcium sparks from ryanodine-sensitive calcium stores. Interval analysis of mIPSCs indicated that the number of potentially undetected (asynchrony < 0.5 ms) multivesicular mIPSCs was low in comparison with the number of large-amplitude mIPSCs. Ryanodine, thapsigargin and calcium-free perfusate did not reduce the frequency of large-amplitude mIPSCs (> 150 pA), arguing against a significant role for presynaptic calcium stores. Our results support previous evidence suggesting that RuR increases miniature postsynaptic current (mSC) frequency by a mechanism that does not involve presynaptic calcium stores. Our results also indicate that at glycinergic synapses in the AVCN and MNTB, site-to-site variability in mIPSC amplitude, rather than multivesicular release, is a major factor underlying the large range of amplitudes of glycinergic mIPSCs.

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In central neurons, spontaneous miniature postsynaptic currents (mPSCs) are usually highly variable in amplitude and time course, and in many cases mPSC amplitude distributions are substantially skewed towards larger events. There are many factors that may account for this variability, including the intrinsic variability at individual release sites (Bekkers *et al.* 1990; Frerking *et al.* 1995; Liu & Tsien, 1995; Silver *et al.* 1996), differences between release sites (Edwards *et al.* 1990; Borst *et al.* 1994; deKoninck & Mody, 1994; Nusser *et al.* 1997; Singer & Berger, 1999), the effect of dendritic cable filtering on current amplitudes (Hestrin *et al.* 1990; Bekkers, 1994; Jonas & Spruston, 1994; Oleskevich *et al.* 1999) and synchronous multi-

quantal release (Ropert *et al.* 1990; Tong & Jahr, 1994; Llano *et al.* 2000). It is essential that these factors be examined at any synaptic connection under investigation before interpretations can be made of mPSC amplitude distributions and mean amplitude measurements.

A fundamental issue relevant to mIPSC amplitude variability is whether postsynaptic receptors are saturated by the release of a quantum of neurotransmitter (Tang *et al.* 1994; Walmsley *et al.* 1998). If receptors are not saturated following quantal release, then variations in vesicle neurotransmitter content and concentration, as well as stochastic channel properties at each site,

underlie the variability in mIPSC amplitude (Clements, 1996). However, if receptors are subject to saturating concentrations of neurotransmitter, then variability in the number of postsynaptic receptors located at each site will influence the shape of the mIPSC amplitude distribution (Walmsley, 1995; Nusser *et al.* 1997; Lim *et al.* 1999). In this case, the rate of spontaneous release from each site is an important contributor to the shape of the mIPSC amplitude distribution (Walmsley, 1995). For example, if release sites that generate large mIPSC currents also have high rates of spontaneous release, then these sites will skew the mIPSC amplitude distribution towards larger amplitudes.

A major complication in the interpretation of the amplitude and time course variability in mIPSCs is that many neurons in the CNS receive synaptic contacts on their dendrites, which may severely distort and attenuate mIPSCs because of the cable filtering properties of dendrites (Ulrich & Lusher, 1993; Oleskevich *et al.* 1999). To avoid this problem, we have investigated glycinergic mIPSCs in bushy cells of the anteroventral cochlear nucleus (AVCN; Lim *et al.* 1999) and principal cells of the medial nucleus of the trapezoid body (MNTB; Forsythe, 1994). Both neuronal types are simple cells with spherical or ovoid cell bodies, and one or two short, tufted dendrites. These cells receive glycinergic synaptic inputs almost exclusively on the soma, and so electrotonic filtering of synaptic currents is not a complicating factor (AVCN: Lim *et al.* 1999; MNTB: F. J. Alvarez, R. E. W. Fyffe & B. Walmsley, unpublished observations). Previous immunolabelling studies in our laboratory demonstrated a large variability in glycine receptor cluster size for an individual AVCN bushy cell (Lim *et al.* 1999). Our results also showed that glycinergic mIPSCs exhibit considerable variability within an individual neuron (Lim *et al.* 1999). In that study, we recorded glycinergic mIPSCs and measured glycine receptor cluster areas from the same cells to show that there is a significant correlation between mean cluster size and mean mIPSC amplitude (Lim *et al.* 1999). The large variability in glycine receptor cluster size for individual cells therefore suggests that site-to-site difference in the number of available receptors is a major contributor to variability in mIPSC amplitudes. Changes in the rate of spontaneous release may potentially be useful for discriminating between intrinsic variability and site-to-site variability in determining the distribution of quantal amplitudes (Walmsley, 1995; Frerking *et al.* 1997). It would be expected that if intrinsic variability was the major determinant of the amplitude distribution, then changing the rate of release would not alter the amplitude distribution. However, if modulating the release rate significantly changed the distribution of quantal amplitudes, then this would support the proposal of site-to-site variability as a determinant of the mIPSC amplitude distribution (a change in the shape of the mIPSC amplitude distribution would only be evident if

there were differential changes in the rate of release at different sites). In this study we have used ruthenium red (RuR) to change the spontaneous release rate. At the neuromuscular junction, RuR increases the frequency of spontaneous end-plate potentials (EPP) but inhibits the evoked EPP (Rahaminoff & Alnaes, 1973). In the CNS, RuR causes a significant increase in miniature excitatory postsynaptic current (mEPSC) frequency, despite the depletion of intracellular calcium stores (Trudeau *et al.* 1996; Sciancalepore *et al.* 1998). It was proposed that, as a polyvalent cation, RuR does not cross the terminal membrane, but instead interacts with an external binding site (Trudeau *et al.* 1996). RuR has been found to interact with the extracellularly localized sialic acid residues involved in calcium binding, and it has been proposed that RuR induces spontaneous transmitter release by interacting directly with the presynaptic release machinery (Wieraszko, 1986; Trudeau *et al.* 1996). RuR is known to have other effects, including the blocking of voltage-gated calcium channels (Cibulsky & Sather, 1999).

Interpretation of mIPSC amplitude distributions is complicated by the possibility that some spontaneous mIPSCs may be due to synchronous multiquantal release, generated by calcium 'sparks' released from calcium-induced calcium release (CICR) stores in the presynaptic terminal. Llano *et al.* (2000) have shown that large, spontaneous GABAergic mIPSCs are due to calcium release from ryanodine-sensitive calcium stores in the presynaptic terminals of cerebellar neurons. In order to interpret amplitude distributions of glycinergic mIPSCs, it is essential that the possibility of calcium-spark-induced multiquantal release be investigated at glycinergic synapses.

In the study presented here, we have investigated the effects of modulating mIPSC frequency and the possible involvement of calcium sparks in spontaneous mIPSCs in both rat AVCN neurons and mouse MNTB neurons. Our results and conclusions are similar for AVCN and MNTB neurons. No evidence was found to support a significant role for presynaptic calcium stores in spontaneous transmitter release at glycinergic synapses in either the AVCN or MNTB. Our results using RuR support site-to-site variability as a major factor influencing glycinergic mIPSC amplitude distributions.

METHODS

Experiments were performed on slices of rat AVCN and mouse MNTB: the rat AVCN results provide a valuable basis for interpreting our previous studies on glycinergic transmission in the AVCN (Lim *et al.* 1999, 2000), and the mouse MNTB results provide a useful comparison (verification) of the AVCN results, and are essential to ongoing investigations into glycinergic transmission in the auditory brainstem of transgenic and mutant mice. As the results and conclusions were similar for both rat AVCN and mouse MNTB, both studies are presented in a single combined report (although the results for rat AVCN and mouse

MNTB are reported separately for comparison and clarity). For simplicity of presentation, rat AVCN and mouse MNTB are referred to as AVCN and MNTB, respectively.

Wistar rats (12–16 days old) and CBA mice (CBA, 12–14 days old) were anaesthetized with 20 mg kg⁻¹ sodium pentobarbitone, i.p. and decapitated, according to the Australian National University Animal Experimentation Ethics Committee protocol. Parasagittal slices (150 μm) were made of the AVCN in rats, as described previously (Isaacson & Walmsley, 1995a,b; Lim *et al.* 1999). In mice, transverse slices (150 μm) were made of the MNTB (Forsythe, 1994).

Whole-cell patch electrode recordings (at a membrane potential of -60 mV) were made from neurons visualized in slices using infrared differential interference contrast optics. Experiments were performed at room temperature (22–25 °C), and conducted on slices superfused with an artificial cerebrospinal fluid solution containing (mM): 130 NaCl, 3.0 KCl, 1.3 Mg₂SO₄, 2.0 CaCl₂, 1.25 NaH₂PO₄, 26.2 NaHCO₃ and 10 glucose, equilibrated with 95 % O₂ and 5 % CO₂. Patch electrodes (3–5 MΩ resistance) contained (mM): 120 CsCl, 4 NaCl, 4 MgCl₂, 0.001 CaCl₂, 10 Hepes, 3 MgATP, 0.2 GTP-tris and 0.2–10 EGTA (pH 7.2) (Sigma Chemicals, St Louis, MO, USA). Series resistance, which was < 10 MΩ, was routinely compensated by > 80 %. Synaptic currents were recorded and filtered at 10 kHz with an Axopatch 1-D amplifier (Axon Instruments, Inc., Union City, CA, USA) before being digitized at 20 kHz. Data were also recorded on videotape with a videocassette recorder (Vetter) and digitized off-line. Data acquisition and analyses were performed using AxoGraph (Axon Instruments). The amplitudes of spontaneous IPSCs were measured using a semi-automated detection procedure (AxoGraph 4.0, Clements & Bekkers, 1997). This is the same procedure used in our previous studies of glycinergic mIPSCs (Lim *et al.* 1999; Oleskevich *et al.* 1999). The detection technique uses a sliding template that is scaled optimally to fit the trace at each location, and was set in the present experiments to detect all spontaneous mIPSCs with amplitudes of > 4 standard deviations of the background noise. The detection procedure is described in detail in Clements & Bekkers (1997). Records were also checked visually. Results are expressed as the mean ± S.E.M. The significance of results was assessed using a paired *t* test, Spearman non-parametric correlation test, or the Kolmogorov-Smirnov test. The level of statistical significance was set at *P* < 0.05.

Unless indicated otherwise, drugs were added to the perfusate, when required, from stock water-based solutions: 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris; 20 mM stock, dissolved in DMSO), (±)-2-amino-5-phosphonopentanoic acid (APV; RBI), bicuculline methochloride (Tocris), bicuculline methiodide (Sigma), strychnine hydrochloride (Sigma), TTX (Alomone Labs, Ltd, Jerusalem, Israel). Spontaneous glycinergic mIPSCs were isolated using TTX (1 μM), CNQX (10 μM), APV (50 μM) and bicuculline (10 μM). In one series of experiments, following the acquisition of control glycinergic mIPSCs, mIPSCs were recorded during bath application of RuR (100 μM, added to the perfusate). In other experiments to examine intraterminal calcium stores, cadmium (200 μM), BAPTA AM (100 μM; Molecular Probes, Inc., Eugene, OR, USA), ryanodine (Sigma; 100 μM; dissolved in DMSO), thapsigargin (10 μM; Alomone Labs), cyclopiazonic acid (CPA; Sigma; 30 μM) or zero extracellular calcium/EGTA (Sigma; 0.2 mM) were used in conjunction with TTX, CNQX, APV and bicuculline.

RESULTS

Glycinergic mIPSCs were analysed from successful recordings obtained in 25 rat AVCN neurons and 37 mouse MNTB neurons.

RuR increases glycinergic mIPSC frequency and distorts amplitude distributions in AVCN and MNTB neurons

Figure 1 illustrates an AVCN neuron in which RuR induced a significant increase in the frequency of glycinergic mIPSCs. Figure 1 shows a continuous whole-cell recording of glycinergic mIPSCs before (A) and following (B) addition of RuR (100 μM) to the perfusate. The mIPSC frequency increased from 0.58 Hz in control solution to 2.58 Hz in RuR. This cell also displayed a significant increase in the mean mIPSC amplitude from 76 to 180 pA following bath application of RuR (Fig. 1C). This increase in mean mIPSC amplitude was due to an increase in the proportion of larger events following RuR application, as can be seen in Fig. 1D. The distribution of mIPSC amplitudes shows that RuR induced an increase in the frequency of all quantal current amplitudes. However, it is obvious that there is a higher relative proportion of larger events following application of RuR.

AVCN neurons. RuR induced a significant increase in the frequency of glycinergic mIPSCs in all AVCN neurons studied (control = 0.9 Hz; RuR = 2.1 Hz, *P* < 0.01; *n* = 8 cells). The time course of the effect of RuR on mIPSC frequency was determined by plotting a running average of mIPSC frequency. Measurements were made of mIPSCs after reaching a maximal or plateau effect at approximately 10 min following addition of RuR to the perfusate. Amplitude distributions of mIPSCs were significantly different in six out of eight cells (Kolmogorov-Smirnov test, *P* = 0.01). In the six cells exhibiting a difference in mIPSC amplitude distributions, there was a concomitant increase in the mean mIPSC amplitude (control = 80 ± 17 pA; RuR = 120 ± 19 pA; *P* < 0.01, *n* = 6 cells). The remaining two cells exhibited no significant change in mean amplitude following RuR application, despite increases in mIPSC frequency of 202 % and 288 %. Consistent with the somatic location of glycinergic synapses, RuR did not alter the rise time of mIPSCs (control = 0.46 ms ± 0.05; RuR = 0.52 ms ± 0.04; *P* > 0.05, *n* = 6 cells).

MNTB neurons. Glycinergic mIPSC frequency in MNTB neurons was increased in RuR (100 μM), from 0.10 ± 0.02 Hz to 0.9 ± 0.2 Hz, *P* < 0.0001, *n* = 11 cells). RuR also increased glycinergic mIPSC amplitude, from a mean of 107 ± 19 pA to a mean of 150 ± 19 pA (*n* = 11 cells, *P* < 0.005), but did not significantly alter the rise times (control = 0.60 ± 0.03 ms; RuR = 0.40 ± 0.02 ms, *n* = 11 cells). There was a significant change in the shape of mIPSC amplitude distributions following application of RuR, in all 11 cells analysed (Kolmogorov-Smirnov test).

Are large glycinergic mIPSCs multiquantal?

Our results show that modulation of the rate of spontaneous release in AVCN and MNTB neurons can significantly alter the distribution of mIPSC amplitudes, consistent with the proposal that a difference in the number of receptors between sites is a major source of variability in mIPSC amplitude. However, it is possible that large quantal events may be due to synchronous

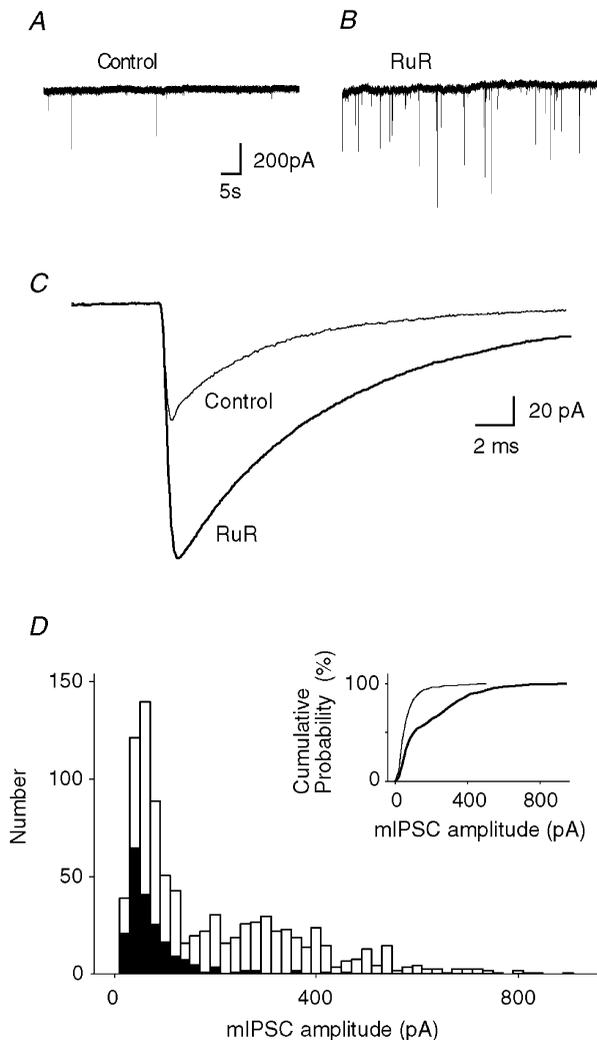


Figure 1. Ruthenium red (RuR) increases the frequency and mean peak amplitude of glycinergic miniature inhibitory postsynaptic currents (mIPSCs)

Rat anteroventral cochlear nucleus (AVCN) data. *A* and *B*, continuous whole-cell recordings of glycinergic mIPSCs in a rat AVCN neuron before and after the application of RuR ($100 \mu\text{M}$). There was a significant increase in the frequency of mIPSCs following application of RuR. The increase in frequency was maximal or reached a plateau 10 min after adding RuR to the perfusion solution. *C*, RuR induced a significant increase in the mean amplitude of glycinergic mIPSCs (bold trace). *D*, histograms of glycinergic mIPSC peak amplitudes in control (shaded) and in the presence of RuR (open) reveal an increase in the number of large quantal events following application of RuR, which is also illustrated by the shift to the right (RuR; bold line) of the cumulative probability distribution (inset).

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multiquantal release of transmitter from the same or adjacent sites (Tong & Jahr, 1994; Frerking *et al.* 1997; Llano *et al.* 2000). If multiquantal release is generated by intraterminal calcium sparks from calcium stores (Llano *et al.* 2000), then the amount of asynchrony of quantal release in this process is likely to be similar to (or greater than) the asynchrony of nerve-evoked (calcium-mediated) quantal release (Isaacson & Walmsley, 1995a). Closely separated multiple quantal events may only be detected in mIPSC recordings if the release asynchrony is relatively large compared with the rise time of the mIPSCs. At very short intervals, multiquantal mIPSCs will have the appearance of large single quantal mIPSCs, albeit with a slightly slower time course due to the asynchrony of release (Isaacson & Walmsley, 1995a). However, no change was observed in the mean rise time of mIPSCs following a RuR-mediated increase in the proportion of large-amplitude mIPSCs (see above). In addition, the rise times of large mIPSCs ($> 150 \text{ pA}$) are not significantly different to the rise times of small mIPSCs (data not shown).

To further study the possibility of multivesicular release, we have used interval measurements to obtain an estimate

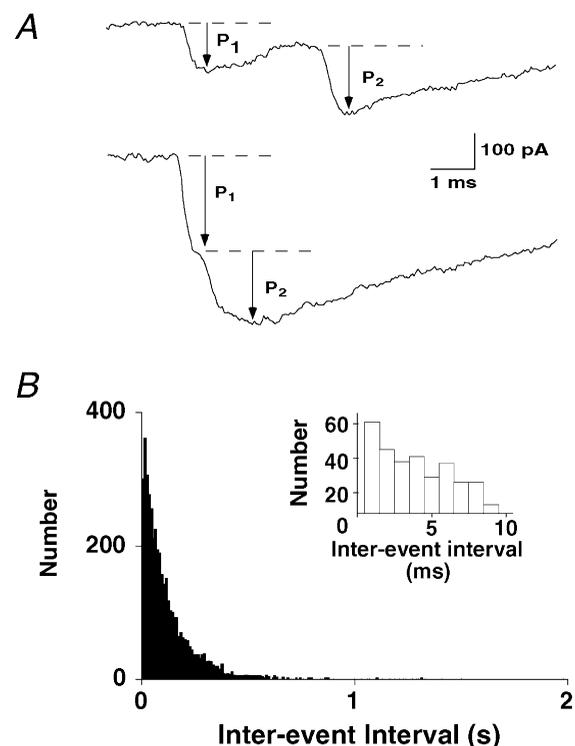


Figure 2. Interval analysis of mIPSCs does not indicate multiquantal release

Rat AVCN data. *A*, two pairs of closely spaced quantal events (P1 and P2) in the presence of RuR. The interevent interval was measured as the time between the peak amplitudes of the two closely spaced events. *B*, histogram of interevent intervals between all mIPSCs recorded in the presence of RuR. The inset shows the distribution of interevent intervals of mIPSCs that occurred within 0.5 and 10 ms of each other (bin size = 1 ms).

of the number of mIPSCs that may be multiquantal but appear as single events. Figure 2A illustrates doublets of mIPSCs with different interevent intervals. The peak amplitudes of the doublets are clearly discernable even at very short intervals (~ 0.5 ms). Figure 2B illustrates the interevent intervals of mIPSCs recorded in an AVCN neuron in the presence of RuR. The distribution of interevent intervals could not be well-fitted by a single exponential, as would be expected from a random Poisson process (Tang *et al.* 1994; Auger *et al.* 1998). This suggests that there is a larger number of closely spaced events than would be expected from a random process (Thompson *et al.* 1997). To gain insight into the possibility of near-synchronous multiquantal release, we have plotted the number of mIPSC pairs with interevent intervals between 0.5 and 10 ms, displayed in 1.0 ms bins (Fig. 2B, inset). An estimate of the number of undetected mIPSC pairs that may have occurred with a separation of 0–0.5 ms, was obtained by two means. Firstly, a regression line fitted to the distribution of interevent intervals was extrapolated to estimate that 29 such events may have occurred with an interval of 0–0.5 ms. Secondly, if multiquantal release asynchrony is similar to nerve-evoked quantal release, then from our previous measurements at central synapses (nerve-evoked quantal release at room temperature; Isaacson & Walmsley, 1995a), approximately one-half of the quanta would be released within 0–0.5 ms of each other, and the remaining half released between 0.5 and 1.0 ms. If it is assumed that all (in the extreme case) of the mIPSC pairs with a separation of 0.5–1.0 ms are generated by multiquantal release at the same or adjacent sites, then this provides an upper limit to the number of undetected multiquantal mIPSCs (i.e. with separations between 0.0 and 0.5 ms). It would be expected for this cell (Fig. 2), that

only 30 mIPSCs may have been undetected multiquantal events (similar to the extrapolated number of 29), which represents only 0.6% of the total number (4294) of mIPSCs. The same conclusion was reached for a further two AVCN neurons analysed in detail, with estimates of 0.3% and 0.6% of mIPSCs representing potential undetected multiquantal mIPSCs. Although indirect, these results indicate that if multiquantal release occurs, it must be extraordinarily well synchronized (to less than several hundred microseconds, see Discussion). One possible candidate for such close synchronization is the generation of presynaptic calcium sparks in glycinergic terminals, as suggested for GABAergic terminals by Llano *et al.* (2000).

Presynaptic voltage-activated calcium channels, RuR and transmitter release

Presynaptic voltage-activated calcium channels provide a potential source of calcium to initiate transmitter release directly or to trigger calcium release from intraterminal stores. Calcium channels also provide a potential mechanism for the action of RuR in increasing spontaneous transmitter release. These possibilities were investigated in MNTB neurons by recording mIPSCs under control conditions (in the presence of TTX) and after the addition of cadmium ($200 \mu\text{M}$) to the perfusion solution to block presynaptic voltage-activated calcium channels, and subsequently adding RuR ($100 \mu\text{M}$). These experiments required long-term stability in recording conditions, since the resting frequency of mIPSCs in MNTB neurons is very low. Complete sequences of recordings were obtained in four MNTB neurons. Figure 3 illustrates the results for one of these MNTB neurons. The addition of cadmium ($200 \mu\text{M}$) did not alter the amplitude

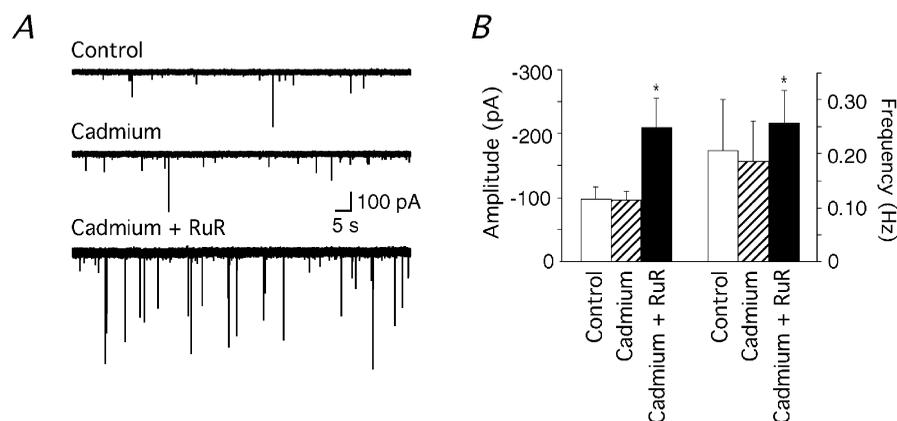


Figure 3. RuR does not act via voltage-activated calcium channels to increase mIPSC frequency

Mouse medial nucleus of the trapezoid body (MNTB) data. *A*, sample continuous recordings of glycinergic mIPSCs from a mouse MNTB neuron in the presence of TTX, under control conditions, with added cadmium ($200 \mu\text{M}$) and with added cadmium + RuR. A minimum of 10 min was allowed following application of each solution before measurements were made of mIPSCs. *B*, summary data for mIPSC amplitude and frequency ($n = 4$ cells) shows no difference between control and cadmium conditions, but a significant increase in both the frequency and amplitude of mIPSCs following addition of RuR.

or frequency of mIPSCs (control amplitude = 97 ± 20 pA, cadmium = 95 ± 15 pA; control frequency = 0.21 ± 0.06 Hz, cadmium = 0.19 ± 0.09 Hz; $n = 4$ cells). The addition of RuR ($100 \mu\text{M}$) in the presence of cadmium, increased the frequency and mean amplitude of mIPSCs (cadmium + RuR: frequency = 0.26 ± 0.06 Hz; amplitude = 209 ± 47 pA, $P < 0.002$). These results indicate that voltage-activated calcium channels are not involved in determining the resting frequency of mIPSCs, and that blocking these calcium channels does not eliminate the action of RuR to increase spontaneous mIPSC frequency. The latter result is not surprising, since RuR alone has been shown to block expressed P/Q-, N- and L-type calcium channels at different concentrations of less than $100 \mu\text{M}$ (Cibulsky & Sather, 1999).

BAPTA AM does not affect mIPSC amplitude distributions

If some glycinergic mIPSCs are generated by intraterminal calcium sparks, then it may be possible to reduce the effectiveness of the store-released calcium by increasing calcium buffering in presynaptic terminals. Nerve-evoked glycinergic IPSCs were generated in MNTB neurons by placing a stimulating electrode nearby. Control experiments verified that the addition of $100 \mu\text{M}$ BAPTA-AM for 5–10 min is sufficient to block (by more than 95%) nerve-evoked IPSCs in MNTB slices ($P < 0.025$, $n = 3$, data not shown). RuR was used to increase the

frequency of mIPSCs, including large-amplitude mIPSCs (Fig. 4A). Subsequent addition of BAPTA AM did not significantly affect the mean amplitude or frequency of glycinergic mIPSCs (Fig. 4C, $n = 6$ cells). RuR increased mIPSC amplitude from 122 ± 31 pA (control) to 175 ± 29 pA (RuR, $P < 0.05$, $n = 6$ cells), a level that was unchanged by further addition of BAPTA AM (169 ± 32 pA, $P > 0.45$, $n = 6$ cells). RuR also increased the frequency from 0.1 ± 0.4 Hz (control) to 0.8 ± 0.3 Hz (RuR, $P < 0.02$, $n = 6$ cells), and this was not changed by BAPTA AM (1.3 ± 0.3 Hz, $P > 0.15$, $n = 6$ cells). RuR and BAPTA AM did not significantly affect the rise time (control = 0.5 ± 0.04 ms; RuR = 0.6 ± 0.04 ms, $n = 6$ cells) of mIPSCs. The lack of effect of BAPTA AM, at a concentration known to block nerve-evoked release, argues against a significant role for calcium sparks in generating spontaneous glycinergic mIPSCs.

mIPSCs and presynaptic calcium stores

Llano *et al.* (2000) showed that both ryanodine ($100 \mu\text{M}$) and zero extracellular calcium concentration significantly decreased the proportion of large-amplitude GABAergic mIPSCs. We have investigated this possibility for glycinergic mIPSCs in both AVCN and MNTB neurons. Experiments were carried out in the presence of RuR ($100 \mu\text{M}$) to enhance the frequency of mIPSCs, and in particular, large-amplitude mIPSCs. As illustrated in

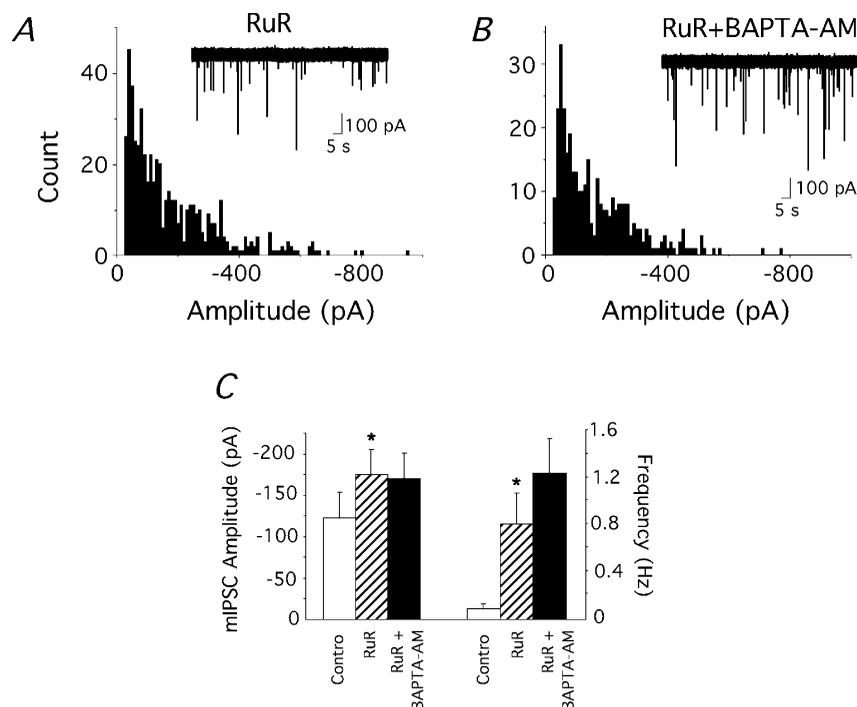


Figure 4. BAPTA AM does not affect mIPSC amplitude and frequency

Mouse MNTB data. A, amplitude histogram of mIPSCs ($n = 551$) in the presence of RuR ($100 \mu\text{M}$). B, amplitude histogram of mIPSCs ($n = 431$) for the same cell as A, following the addition of BAPTA AM ($100 \mu\text{M}$). C, summary data ($n = 6$ cells) showing a significant increase in mIPSC frequency and amplitude between control and following addition (> 10 min after solution change) of RuR ($100 \mu\text{M}$), but no significant difference between RuR and RuR + BAPTA AM ($100 \mu\text{M}$).

Fig. 5A, C and D, the addition of ryanodine ($100 \mu\text{M}$) did not affect significantly the amplitude distribution (Kolmogorov-Smirnov test), mean amplitude or frequency of mIPSCs in MNTB neurons (control amplitude = $123 \pm 3 \text{ pA}$, ryanodine = $121 \pm 3 \text{ pA}$; control frequency = $1.0 \pm 0.1 \text{ Hz}$, ryanodine = $1.1 \pm 0.1 \text{ Hz}$; $n = 10$ cells). Experiments on AVCN neurons demonstrated a similar lack of effect of ryanodine on glycinergic mIPSCs ($n = 8$ cells). In both the MNTB ($n = 10$ cells) and AVCN ($n = 8$ cells), there was no significant difference in the proportion of large-amplitude mIPSCs ($> 150 \text{ pA}$) between control and ryanodine conditions.

Experiments were also carried out on MNTB neurons using thapsigargin ($10 \mu\text{M}$) to deplete calcium stores. As shown in Fig. 5B, C and D, thapsigargin did not affect the amplitude distribution, mean amplitude or frequency of mIPSCs ($n = 6$ cells). There was no significant difference in the proportion of large-amplitude ($> 150 \text{ pA}$) mIPSCs between control and thapsigargin conditions ($n = 6$ cells).

Application of cyclopiazonic acid (CPA, $30 \mu\text{M}$), which has been used previously to block intracellular calcium stores and affect spontaneous release (Emptage *et al.* 2001), in the presence of RuR ($100 \mu\text{M}$), did not cause a significant change in the amplitude or frequency of glycinergic mIPSCs in MNTB neurons (RuR: amplitude = $143 \pm 10 \text{ pA}$, frequency = $0.79 \pm 0.14 \text{ Hz}$; RuR + CPA: amplitude = $15 \pm 17 \text{ pA}$, frequency = $1.3 \pm 0.28 \text{ Hz}$, $n = 5$ cells; data not shown).

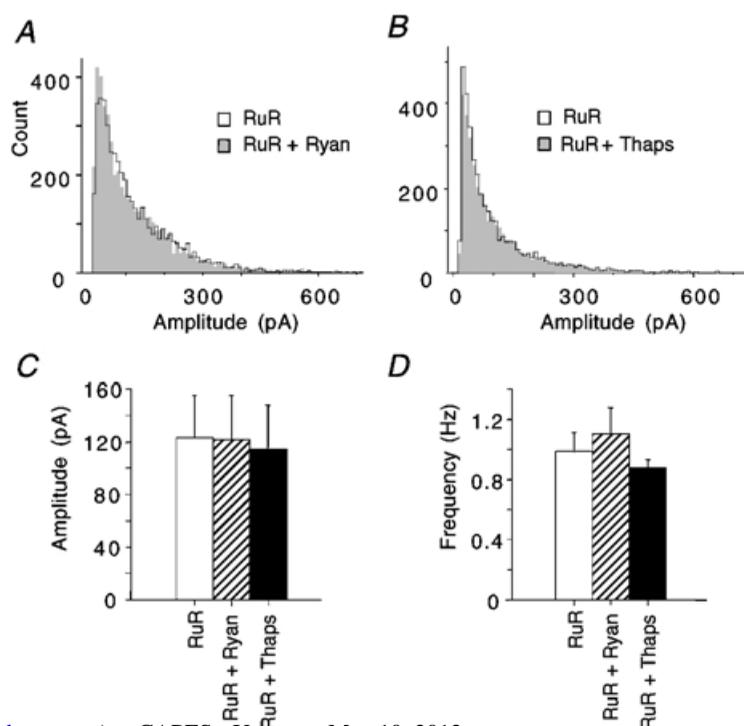
Additional experiments on AVCN neurons demonstrated that zero extracellular calcium did not alter mIPSC amplitude or frequency ($n = 4$ cells, data not shown).

DISCUSSION

We have shown previously that there is a significant correlation between mean glycine receptor cluster area and mean glycinergic mIPSC amplitude in rat AVCN bushy cells (Lim *et al.* 1999). It was also shown that the size of the postsynaptic glycine receptor cluster area varies considerably within an individual AVCN bushy cell (Lim *et al.* 1999). This suggests that there may be significant site-to-site differences in mIPSC amplitude within the same cell. In the present study, we have investigated variability in the size of the spontaneous glycinergic currents by modulating the rate of spontaneous release. If the variability in quantal current amplitudes is due to differences between release sites, then modulation of the spontaneous release rate may alter the distribution of mIPSCs (Walmsley, 1995; Frerking *et al.* 1997). Our results in both the rat AVCN and mouse MNTB show that in most (90%) cells exposed to RuR, there is a significant change in the distribution of mIPSC amplitudes, thus supporting the proposal of significant site-to-site variability. This is in agreement with the results of Auger & Marty (1997), which demonstrated site-to-site variability in quantal size at GABAergic synapses. In previous studies in the hippocampus, despite causing a substantial increase in mPSC frequency, RuR was found to have no effect on the amplitude distributions of glutamatergic mEPSCs (Trudeau *et al.* 1996) and GABAergic mIPSCs (Sciancalepore *et al.* 1998). These results are difficult to interpret, since RuR may have caused a relatively uniform increase in spontaneous frequency at all synapses, or there may be relatively little site-to-site variability in quantal size between synapses at these connections. Our results

Figure 5. Ryanodine and thapsigargin do not affect mIPSC amplitude or frequency

Mouse MNTB data. A, summed mIPSC amplitude histograms (RuR, $n = 4870$ mIPSCs; RuR + ryanodine (Ryan), $n = 4790$ mIPSCs; 10 cells), showing a lack of effect of ryanodine ($100 \mu\text{M}$) on the distribution of mIPSC amplitudes. B, summed mIPSC amplitude histograms (RuR, $n = 3654$ mIPSCs; RuR + thapsigargin (Thaps), $n = 3498$ mIPSCs; six cells), showing a lack of effect of thapsigargin ($10 \mu\text{M}$) on the distribution of mIPSC amplitudes. C, summary data showing a lack of effect of ryanodine ($100 \mu\text{M}$) and thapsigargin ($10 \mu\text{M}$) on mIPSC mean amplitude (C, $n = 10$ cells) and mean frequency (D, $n = 6$ cells) in the presence of RuR ($100 \mu\text{M}$).



demonstrated that the response to RuR was invariably an increase in frequency of mIPSCs of all amplitudes, and usually an increase in the proportion of large mIPSCs (six out of eight AVCN neurons, 11 out of 11 MNTB neurons). If RuR was randomly affecting different release sites, then it could be expected that the proportion of large mIPSCs may decrease, not change, or increase in different cells. However, not all release sites are the same, and those synapses with larger amplitude mIPSCs may be associated with larger postsynaptic densities, larger active zones and larger presynaptic terminals. Larger active zones (potentially generating larger mIPSCs) and larger boutons may present a larger target per synapse for the binding or action of modulators of release, such as RuR. In fact, previous neuroanatomical electron microscope studies have demonstrated that there are correlations between a number of the anatomical features of synapses, including active zone size and bouton size (Yeow & Peterson, 1991; Pierce & Lewin, 1994). However, the possibility of intrinsic differences, apart from structural features, in the sensitivity of different synapses to modulators of release cannot be ruled out. The mechanism of action of RuR to increase the frequency of mPSPs remains unknown, but our results agree with previous studies that RuR appears not to be acting directly on presynaptic calcium stores (Trudeau *et al.* 1996; Sciancalepore *et al.* 1998). Since RuR was applied to the whole slice, another possibility that must be considered is that global release of other agents as a result of RuR may have produced some indirect effect on spontaneous release.

It is possible that large events contributing to the positive skew of the glycinergic mIPSC distributions are not unquantal, but are due to synchronous multiquantal release (Tong & Jahr, 1994; Frerking *et al.* 1997; Auger *et al.* 1998; Llano *et al.* 2000). However, our mIPSC interval analysis results in the AVCN suggest that the number of synchronous events that occur within a 0–0.5 ms time period and which may be undetected multiquantal events, represents only a small fraction of the number of observed large events. In a previous study, Llano *et al.* (2000) showed that the proportion of large-amplitude GABAergic mIPSCs was greatly decreased by application of ryanodine or zero extracellular calcium concentration. We have repeated these experiments for glycinergic mIPSCs in AVCN and MNTB neurons, and found no effect. If multivesicular release is due to calcium sparks, then the rise in calcium concentration at release site(s) must be very rapid, otherwise asynchrony in release would be evident. We reasoned that the addition of a calcium buffer such as BAPTA AM at concentrations sufficient to severely reduce or block nerve-evoked transmitter release should also interfere with calcium sparks and decrease synchronous multiquantal release. Our results in the MNTB showed no significant effects of BAPTA AM. Therefore, we propose that large glycinergic mIPSCs are

uniquantal, and are generated at synapses with large receptor clusters. However, we cannot rule out the possibility that large mIPSCs may be due to other mechanisms, such as the fusion of vesicles prior to release, which would synchronize release to a much greater degree than even nerve-evoked quantal release.

In summary, our results support site-to-site variability between synapses as a major influence on the distribution of glycinergic mIPSC amplitudes. Importantly, for the interpretation of glycinergic mIPSC distributions, no evidence was found to support the possibility that large glycinergic mIPSCs are due to synchronous multiquantal release generated by spontaneous calcium sparks in presynaptic terminals. It should be emphasized that the results of Llano *et al.* (2000), showing substantial calcium store involvement in spontaneous transmitter release, were obtained at GABAergic synapses in the cerebellum, and there are likely to be differences between these GABAergic synapses and glycinergic synapses in the auditory brainstem nuclei. Our results suggest that caution must be used when interpreting a change in mean mIPSC amplitude, as this may reflect a presynaptic change in the rate of release at different sites with different mean mIPSC amplitudes. Our results also support previous studies proposing RuR as a useful tool in the study of transmitter release at central synapses (Trudeau *et al.* 1996; Sciancalepore *et al.* 1998)

REFERENCES

- Auger C, Kondo S & Marty A (1998). Multivesicular release at single functional synaptic sites in cerebellar stellate and basket cells. *J Neurosci* **18**, 4532–4547.
- Auger C & Marty A (1997). Heterogeneity of functional synaptic parameters among single release sites. *Neuron* **19**, 139–150.
- Bekkers JM (1994). Quantal analysis of synaptic transmission in the central nervous system. *Curr Biol* **4**, 360–365.
- Bekkers JM, Richerson GB & Stevens CF (1990). Origin of variability in quantal size in cultured hippocampal neurons and hippocampal slices. *Proc Natl Acad Sci U S A* **87**, 5359–5362.
- Borst JGG, Lodder JC & Kits KS (1994). Large amplitude variability of GABAergic IPSCs in melanotropes from *Xenopus laevis*: evidence that quantal size differs between synapses. *J Neurophysiol* **71**, 639–655.
- Cibulsky SM & Sather WA (1999). Block by ruthenium red of cloned neuronal voltage-gated calcium channels. *J Pharmacol Exp Ther* **289**, 1447–1453.
- Clements JD (1996). Transmitter time course in the synaptic cleft: its role in central synaptic function? *Trends Neurosci* **19**, 163–171.
- Clements JD & Bekkers JM (1997). Detection of spontaneous synaptic events with an optimally scaled template. *Biophys J* **73**, 220–229.
- De Koninck Y & Mody I (1994). Noise analysis of miniature IPSCs in adult rat brain slices: properties and modulation of synaptic GABA_A receptor channels. *J Neurophysiol* **71**, 1318–1335.
- Edwards FA, Konnerth A & Sakmann B (1990). Quantal analysis of inhibitory synaptic transmission in the dentate gyrus of rat hippocampal slices: a patch clamp study. *J Physiol* **430**, 213–249.

- Emptage NJ, Reid CA & Fine A (2001). Calcium stores in hippocampal synaptic boutons mediate short-term plasticity, store-operated calcium, and spontaneous transmitter release. *Neuron* **29**, 197–208.
- Forsythe ID (1994). Direct patch recording from identified presynaptic terminals mediating glutamatergic EPSCs in the rat CNS, *in vitro*. *J Physiol* **479**, 381–387.
- Frerking M, Borges S & Wilson M (1995). Variation in GABA mini amplitude is the consequence of variation in transmitter concentration. *Neuron* **15**, 885–895.
- Frerking M, Borges S & Wilson M (1997). Are some minis multiquantal? *J Neurophysiol* **78**, 1293–1304.
- Hestrin S, Sah P & Nicoll RA (1990). Mechanisms generating the time course of dual component excitatory synaptic currents recorded in hippocampal slices. *Neuron* **5**, 247–253.
- Isaacson JS & Walmsley B (1995a). Counting quanta: direct measurements of transmitter release at a central synapse. *Neuron* **15**, 875–884.
- Isaacson JS & Walmsley B (1995b). Receptors underlying excitatory synaptic transmission in slices of the rat anteroventral cochlear nucleus. *J Neurophysiol* **73**, 964–973.
- Jonas P & Spruston N (1994). Mechanisms shaping glutamate-mediated excitatory postsynaptic currents in the CNS. *Curr Biol* **4**, 366–372.
- Lim R, Alvarez FJ & Walmsley B (1999). Quantal size is correlated with receptor cluster area at glycinergic synapses in the rat brainstem. *J Physiol* **516**, 505–512.
- Lim R, Alvarez FJ & Walmsley B (2000). GABA mediates presynaptic inhibition at glycinergic synapses in a rat auditory brainstem nucleus. *J Physiol* **525**, 447–459.
- Liu G & Tsien RW (1995). Properties of synaptic transmission at single hippocampal synaptic boutons. *Nature* **375**, 404–408.
- Llano I, Gonzales J, Caputo C, Lai FA, Blaney LM, Tan YP & Marty A (2000). Presynaptic calcium stores underlie large-amplitude miniature IPSCs and spontaneous calcium transients. *Nat Neurosci* **3**, 1256–1265.
- Nusser Z, Cull-Candy S & Farrant M (1997). Differences in synaptic GABA_A receptor number underlie variation in GABA mini amplitude. *Neuron* **19**, 697–709.
- Oleskevich S, Alvarez FJ & Walmsley B (1999). Glycinergic miniature synaptic currents and receptor cluster sizes differ between spinal cord interneurons. *J Neurophysiol* **82**, 312–319.
- Pierce JP & Lewin GR (1994). An ultrastructural size principle. *Neuroscience* **58**, 441–446.
- Rahamimoff R & Alnaes E (1973). Inhibitory action of ruthenium red on neuromuscular transmission. *Proc Natl Acad Sci USA* **70**, 3613–3616.
- Ropert N, Miles R & Korn H (1990). Characteristics of miniature inhibitory postsynaptic currents in CA1 pyramidal neurones of rat hippocampus. *J Physiol* **428**, 707–722.
- Sciancalepore M, Savic N, Gyori J & Cherubini E (1998). Facilitation of miniature GABAergic currents by ruthenium red in neonatal rat hippocampal neurons. *J Neurophysiol* **80**, 2316–2322.
- Silver RA, Cull-Candy S & Takahashi T (1996). Non-NMDA glutamate receptor occupancy and open probability at a rat cerebellar synapse with single and multiple release sites. *J Physiol* **494**, 231–250.
- Singer JH & Berger AJ (1999). Contribution of single-channel properties to the time course and amplitude variance of quantal glycine currents recorded in rat motoneurons. *J Neurophysiol* **81**, 1608–1616.
- Tang C, Margulis M, Shi Q & Fielding A (1994). Saturation of postsynaptic glutamate receptors after quantal release of transmitter. *Neuron* **13**, 1385–1393.
- Thompson SM, Poncer J, Capogna M & Gähwiler BH (1997). Properties of spontaneous miniature GABA_A receptor mediated synaptic currents in area CA3 of rat hippocampal slice cultures. *Can J Physiol Pharmacol* **75**, 495–499.
- Tong G & Jahr CE (1994). Multivesicular release from excitatory synapses of cultured hippocampal neurons. *Neuron* **12**, 51–59.
- Trudeau LE, Doyle RT, Emery DG & Haydon PG (1996). Calcium-independent activation of the secretory apparatus by ruthenium red in hippocampal neurons: a new tool to assess modulation of presynaptic function. *J Neurosci* **16**, 46–54.
- Ulrich D & Lusher H (1993). Miniature excitatory synaptic currents corrected for dendritic cable properties reveal quantal size and variance. *J Neurophysiol* **69**, 1769–1773.
- Walmsley B (1995). Interpretation of ‘quantal’ peaks in distributions of evoked synaptic transmission at central synapses. *Proc R Soc Lond B Biol Sci* **261**, 245–250.
- Walmsley B, Alvarez FJ & Fyffe REW (1998). Diversity of structure and function at mammalian central synapses. *Trends Neurosci*, **21**, 81–88.
- Wieraszko A (1986). Evidence that ruthenium red disturbs the synaptic transmission in the rat hippocampal slices through interacting with sialic acid residues. *Brain Research* **378**, 120–126.
- Yeow MB & Peterson EH (1991). Active zone organization and vesicle content scale with bouton size at a vertebrate central synapse. *J Comp Neurol* **307**, 475–486.

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