
Figures and figure supplements

Spontaneous and evoked neurotransmission are partially segregated at inhibitory synapses

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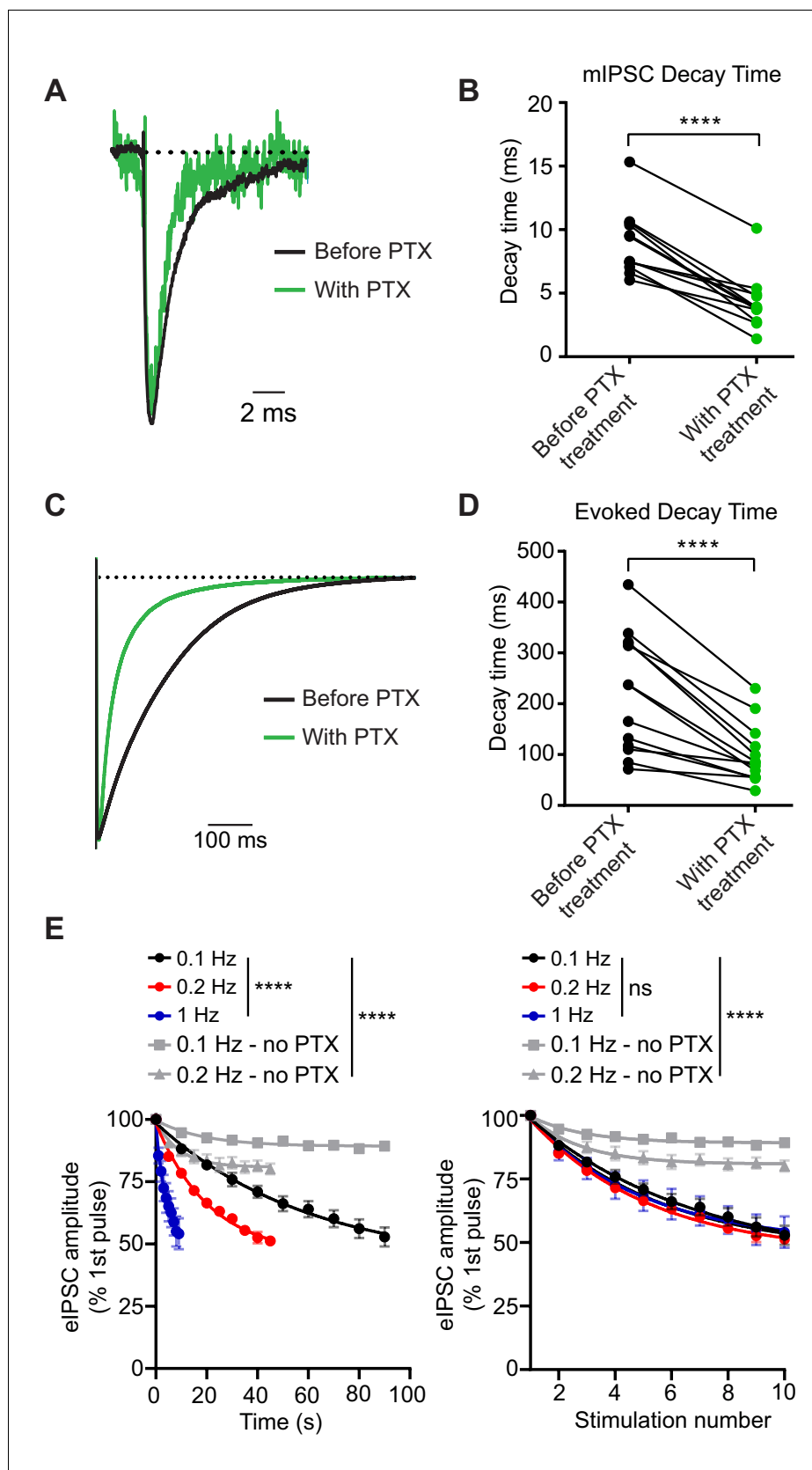


Figure 1. PTX blocks GABA_ARs in a use-dependent manner consistent with open-channel block. (A) Scaled example traces of mIPSCs before and after PTX addition. (B) Quantification showing average event decay times

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Figure 1 continued

obtained from the same cell before and after (8 min) treatment with 50 μ M PTX. mIPSC decay time is decreased following PTX treatment (paired t-test $t_{(11)} = 9.055$, $p < 0.0001$, $n = 12$). (C) Scaled example traces of evoked responses to 0.1 Hz stimulation before and after PTX addition. Average trace taken from the 10th response to stimulation in PTX following 8 min of PTX application at rest (no stimulation). (D) Quantification showing average evoked response decay time obtained from the same cell before and after (8 min) treatment with 50 μ M PTX. Evoked response decay time is decreased following PTX treatment (paired t-test $t_{(12)} = 6.097$, $p < 0.0001$, $n = 13$). (E) (Left) PTX block of evoked response plotted by total treatment time. (Right) PTX block of evoked response plotted by stimulation number. PTX blocks evoked response as a function of stimulation number, rather than time, indicating it is a use-dependent blocker (non-linear regression single exponential fit for conditions with PTX; Time: Sum-of-Squares F test $F_{(6, 141)} = 38.16$, $p < 0.0001$ that is one curve does not fit all datasets; Stimulation number: Sum-of-Squares F test $F_{(6, 141)} = 1.005$, $p = 0.4243$ that is one curve does fit all datasets, $n = 5$ all groups). Decay of the eIPSC response without PTX is significantly less than with PTX, indicating that rundown is not responsible for the decrease in response. (non-linear regression single exponential fit for all conditions; Time: Sum-of-Squares F test $F_{(12, 705)} = 115.9$, $p < 0.0001$ that is one curve does not fit all datasets; Stimulation number: Sum-of-Squares F test $F_{(12, 705)} = 101.9$, $p < 0.0001$ that is one curve does not fit all datasets, 0.1 Hz - no PTX $n = 46$, 0.2 Hz - no PTX $n = 11$) Graphs are mean \pm SEM. **** indicates $p < 0.0001$.

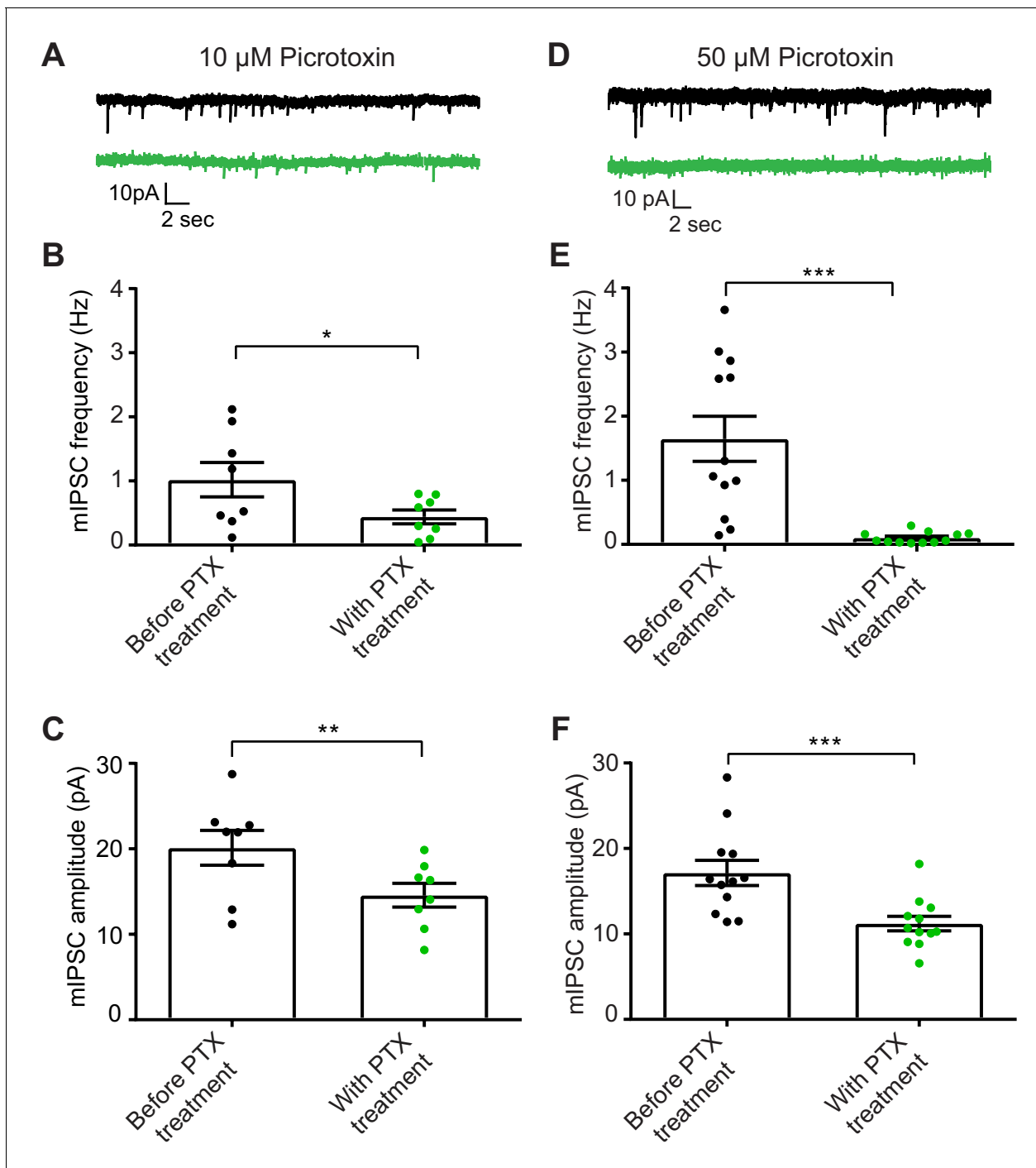


Figure 1—figure supplement 1. Examination of different doses of PTX. (A) Example traces of mIPSCs before (above) and after (below) treatment with 10 μ M of PTX. (B) Quantification of mIPSC frequency before and after (8 min) treatment with 10 μ M of PTX. Frequency is decreased following 10 μ M PTX treatment (paired t-test $t_{(7)} = 3.213$, $p = 0.0148$, $n = 8$). (C) Quantification of mIPSC amplitude after (8 min) treatment with 10 μ M of PTX. Amplitude is slightly decreased following 10 μ M PTX treatment (paired t-test $t_{(7)} = 4.591$, $p = 0.0025$, $n = 8$). (D) Example traces of mIPSCs before (above) and after (below) treatment with 50 μ M of PTX. (E) Quantification of mIPSC frequency before and after (8 min) treatment with 50 μ M of PTX. Frequency is drastically decreased following 50 μ M PTX treatment (paired t-test $t_{(11)} = 4.685$, $p = 0.0007$, $n = 12$). (F) Quantification of mIPSC amplitude after (8 min) treatment with 50 μ M of PTX. For the few remaining events, amplitude is slightly decreased following 50 μ M PTX treatment (paired t-test $t_{(11)} = 4.675$, $p = 0.0007$, $n = 12$). 50 μ M of PTX blocks mIPSCs more fully than 10 μ M of PTX. Graphs are mean \pm SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

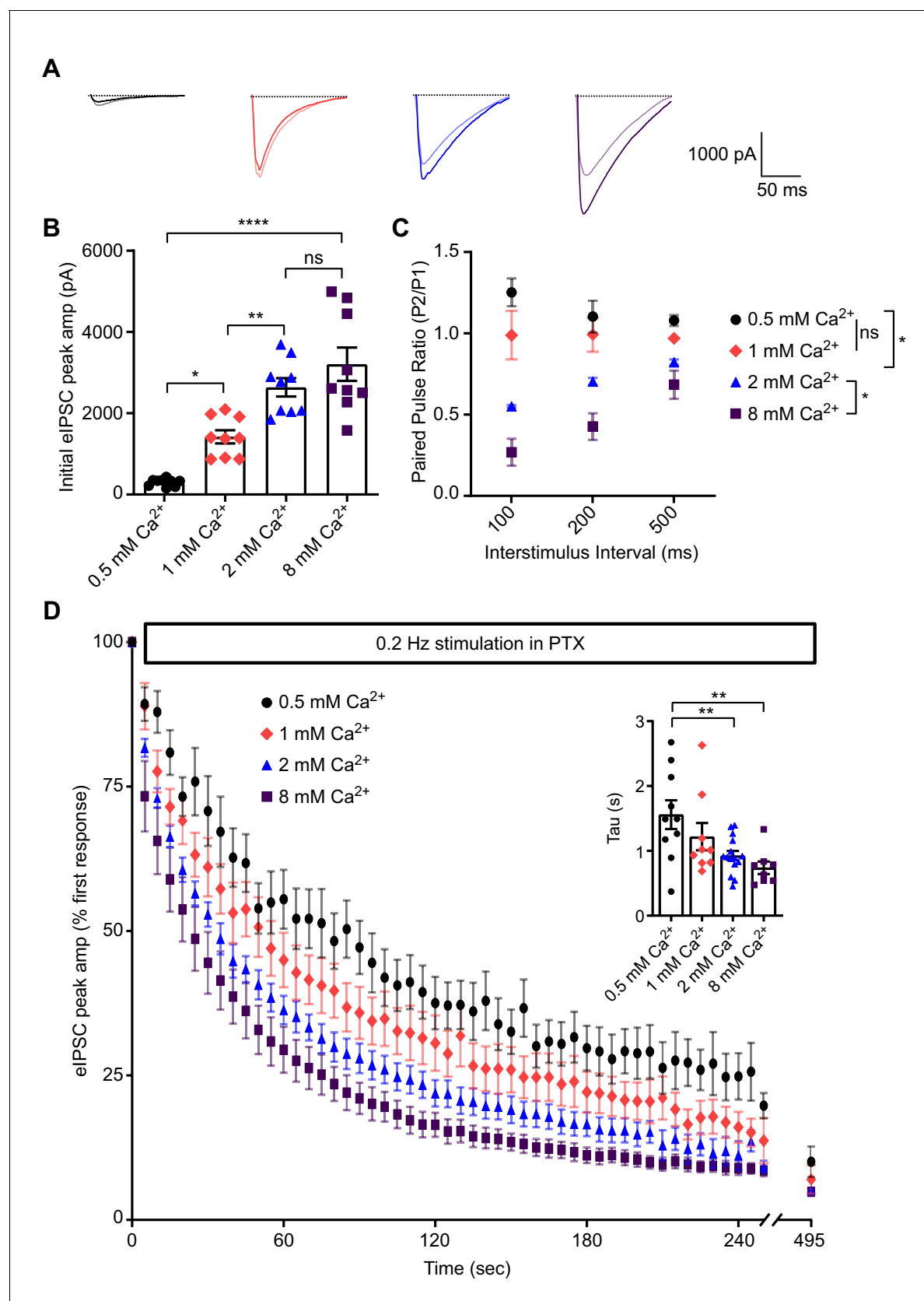


Figure 2. PTX can be used to compare release probability of inhibitory synapses. (A) Example traces of paired pulse responses at an interstimulus interval of 100 ms in 0.5 mM Ca^{2+} (pulse one black, pulse two gray), 1 mM Ca^{2+} (pulse one pink, pulse two light pink), 2 mM Ca^{2+} (pulse one blue, pulse two light blue), 8 mM Ca^{2+} (pulse one purple, pulse two light purple). Figure 2 continued on next page

Figure 2 continued

two light blue) or 8 mM Ca^{2+} (pulse one purple, pulse two light purple). (B) Quantification of initial peak amplitude of eIPSC in different Ca^{2+} concentrations. Increasing Ca^{2+} concentration increases the initial peak eIPSC amplitude, consistent with increased release probability (one-way ANOVA $F_{(3,32)} = 27.24$, $p < 0.0001$, Tukey's post-hoc testing 0.5 mM Ca^{2+} vs 1 mM Ca^{2+} $p = 0.0160$, 1 mM Ca^{2+} vs 2 mM Ca^{2+} $p = 0.0079$, 2 mM Ca^{2+} vs 8 mM Ca^{2+} $p = 0.3821$, 0.5 mM Ca^{2+} vs 8 mM Ca^{2+} $p < 0.0001$, $n = 9$ all groups). (C) Paired pulse ratio recorded from cells in different Ca^{2+} concentrations. Increasing Ca^{2+} concentration decreased paired pulse ratio, consistent with increased release probability (two-way ANOVA interaction $F_{(6,26)} = 2.801$, $p = 0.0308$, interevent interval factor $F_{(2,26)} = 2.220$, $p = 0.1287$, Ca^{2+} concentration factor $F_{(3,26)} = 43.55$, $p < 0.0001$, Tukey's post-hoc testing 0.5 mM Ca^{2+} vs 1 mM Ca^{2+} $p = 0.1202$, 1 mM Ca^{2+} vs 2 mM Ca^{2+} $p = 0.0024$, 2 mM Ca^{2+} vs 8 mM Ca^{2+} $p = 0.0107$, 0.5 mM Ca^{2+} vs 8 mM Ca^{2+} $p < 0.0001$, $n = 3$ for 0.5 mM Ca^{2+} ; $n = 3$ for 1 mM Ca^{2+} ; $n = 3$ for 2 mM Ca^{2+} ; $n = 4$ for 8 mM Ca^{2+}). (D) eIPSC peak amplitude over successive 0.2 Hz stimulations in the presence of PTX. Increasing Ca^{2+} concentration increased the rate of eIPSC block. (Inset) Individual time constants of single exponentials fitted to each experiment. Increasing Ca^{2+} concentration decreased the time constant, consistent with an increased rate of block, demonstrating the utility of PTX to estimate release probability (one-way ANOVA $F_{(3,38)} = 5.125$, $p = 0.0045$, Tukey's post-hoc testing 0.5 mM Ca^{2+} vs 1 mM Ca^{2+} $p = 0.4468$, 0.5 mM Ca^{2+} vs 2 mM Ca^{2+} $p = 0.0162$, 0.5 mM Ca^{2+} vs 8 mM Ca^{2+} $p = 0.0062$, 2 mM Ca^{2+} vs 8 mM Ca^{2+} $p = 0.8203$, $n = 10$ for 0.5 mM Ca^{2+} ; $n = 8$ for 1 mM Ca^{2+} ; $n = 14$ for 2 mM Ca^{2+} ; $n = 9$ for 8 mM Ca^{2+}). Graphs are mean \pm SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.0001$, ns indicates not significant.

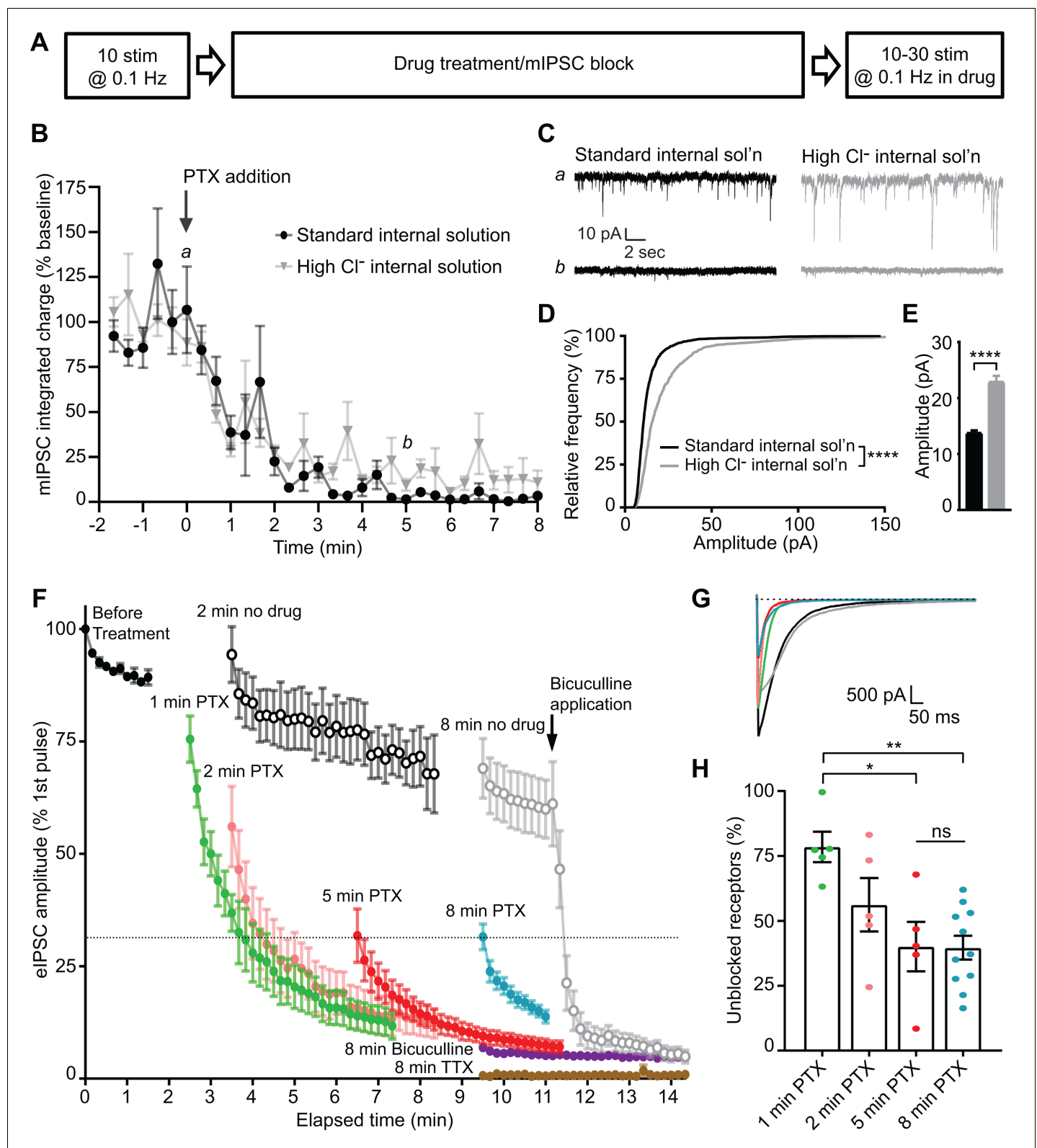


Figure 3. Evoked and spontaneous neurotransmission are partly segregated at inhibitory synapses. (A) Schematic showing experiment design. (B) Time course indicating mIPSC block following the addition of PTX measured using standard internal solution (black) or high Cl⁻ internal solution (gray). Integrated charge is binned in 20 s intervals. PTX diminished mIPSC frequency within 5 min. This time course is unchanged when measured using a high Cl⁻ internal solution. (C) Example traces of mIPSC recordings from indicated time points in B. (D) Cumulative histogram of spontaneous event Figure 3 continued on next page

Figure 3 continued

amplitudes in standard and high Cl^- internal solutions. High Cl^- internal solution shifted the distribution of mIPSCs toward higher amplitudes (Kolmogorov-Smirnov test $D = 0.3350$, $p < 0.0001$, $n = 1200$ events from 12 standard internal solution recordings and 600 events from six high Cl^- internal solution recordings, 100 events randomly selected per recording). (E) Average of spontaneous event amplitudes in standard and high Cl^- internal solutions. High Cl^- internal solution increased the average amplitude of mIPSC events (unpaired t-test $t_{(1798)} = 10.96$, $p < 0.0001$, $n = 1200$ events from 12 standard internal solution recordings and 600 events from six high Cl^- internal solution recordings, 100 events randomly selected per recording). (F) Evoked inhibitory response to stimulation before drug treatment and following: no drug (open symbols, $n = 6$ for 2 min, $n = 7$ for 8 min), 1–8 min PTX ($n = 5$ for 1 min, $n = 5$ for 2 min, $n = 5$ for 5 min, $n = 11$ for 8 min), 8 min bicuculline ($n = 4$), or 8 min TTX treatment ($n = 3$). Treatment of the 8 min no drug condition with bicuculline after the 10th stimulation drastically reduced the response amplitude down to the level of 8 min bicuculline treatment, indicating that the measured response is mediated through GABA_A Rs. Treatment with PTX for increasing amounts of time decreased the initial evoked response to stimulation, which continued to decay upon successive stimulations in all cases. However, initial evoked response was not further decreased after a 5 min treatment with PTX. (G) Example traces of initial evoked response after PTX treatment or rest (black = 2 min no PTX, gray = 8 min no PTX, green = 1 min PTX, pink = 2 min PTX, red = 5 min PTX, blue = 8 min PTX). (H) Quantification of the percent of the initial evoked response that is mediated by GABA_A Rs which are unblocked following PTX treatment. Values are adjusted for bicuculline baseline and no drug treatment maximum response. After 5 min in PTX, when all receptors activated by mIPSCs are blocked, the unblocked evoked response is $40.1 \pm 9.6\%$ of the maximum response. This response is not further decreased following an 8 min treatment with PTX ($39.7 \pm 4.6\%$; one-way ANOVA $F(3,22) = 6.228$, $p = 0.0032$, Tukey's post-hoc testing 1 min vs 2 minutes $p = 0.2260$, 1 min vs 5 minutes $p = 0.0124$, 1 min vs 8 minutes $p = 0.0028$, 5 min vs 8 minutes $p > 0.9999$, $n = 5$ for 1 min, $n = 5$ for 2 min, $n = 5$ for 5 min, $n = 11$ for 8 min). Graphs are mean \pm SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$, **** indicates $p < 0.0001$, ns indicates not significant.

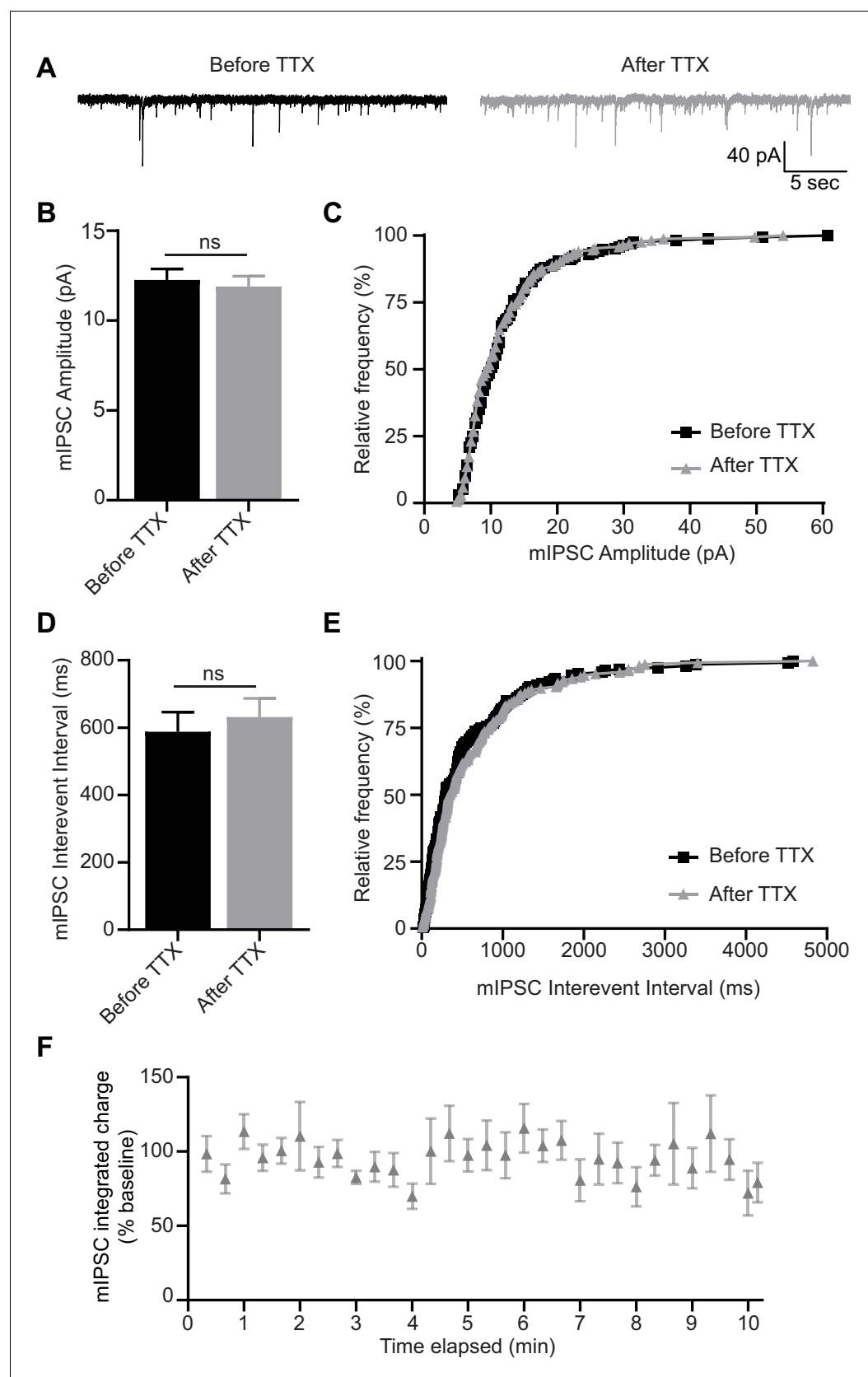


Figure 3—figure supplement 1. Detected spontaneous events are unaffected by TTX application. (A) Example traces of spontaneous events before (left, black) and after (gray, right) TTX application. (B) Average amplitude of detected spontaneous inhibitory events (i.e. in the absence of stimulation) Figure 3—figure supplement 1 continued on next page

Figure 3—figure supplement 1 continued

before and 10 min after TTX application. Amplitude of detected events is unaltered by TTX addition (unpaired t-test $t_{(334)} = 0.4353$, $p=0.6636$, $n = 168$ events from seven recordings, 21 events randomly selected per recording). (C) Cumulative histogram plot of amplitudes of detected spontaneous inhibitory events before and after TTX. (D) Average interevent interval of detected spontaneous inhibitory events (i.e. in the absence of stimulation) before and 10 min after TTX. Interevent interval is unaltered by TTX addition (unpaired t-test $t_{(334)} = 0.5429$, $p=0.5876$, $n = 168$ events from seven recordings, 21 intervals randomly selected per recording). (E) Cumulative histogram plot of interevent intervals of detected spontaneous inhibitory events before and after TTX. TTX application did not affect spontaneous event amplitudes or frequencies, indicating spontaneously recorded events are bona fide mIPSCs. (F) Time course of mIPSC events following application of TTX. mIPSCs are unaffected by metabolic rundown seen in evoked recordings. Graphs are mean \pm SEM. ns indicates not significant.

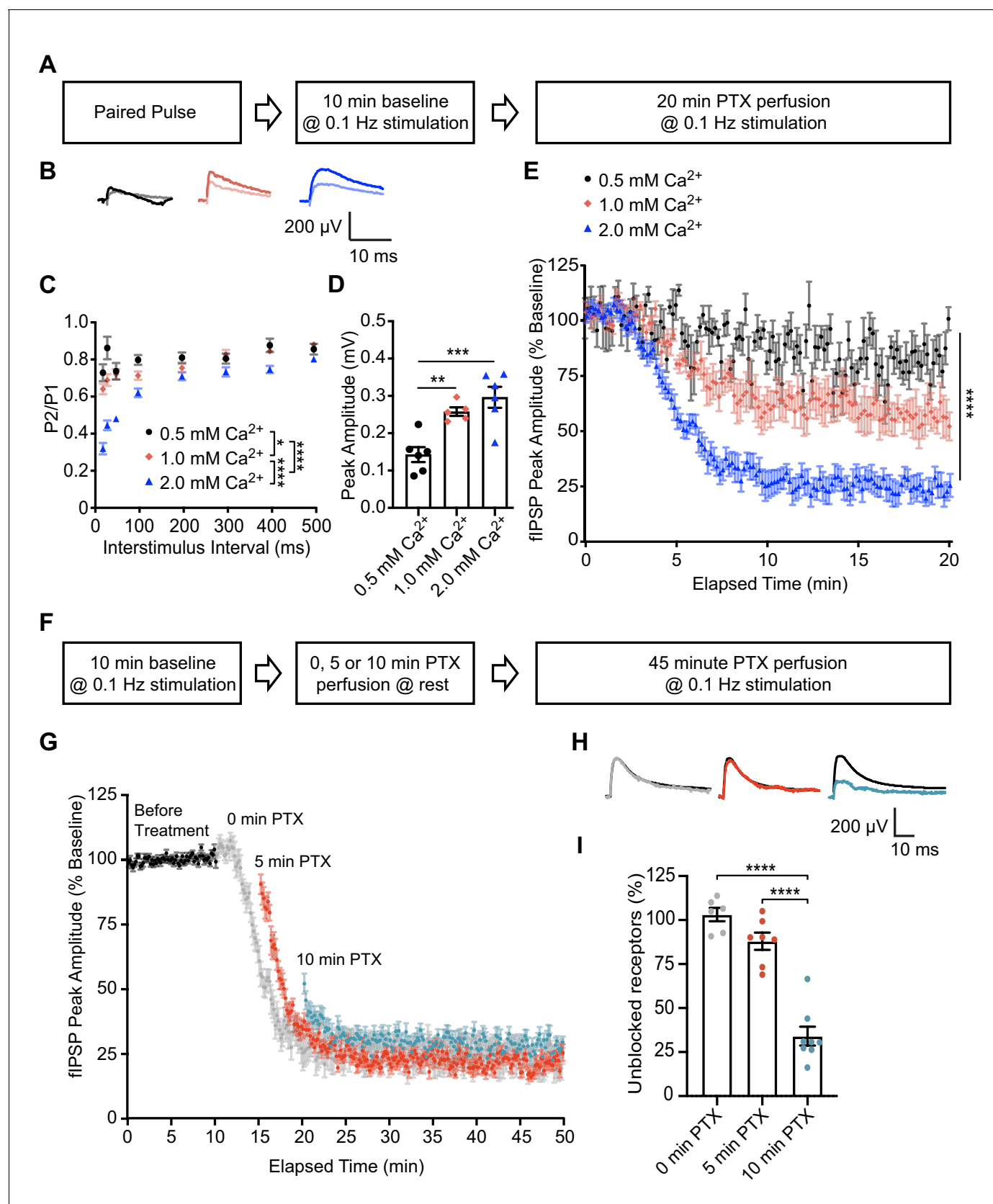


Figure 4. PTX exhibits use-dependency in hippocampal slices and demonstrates partial segregation of evoked and spontaneous neurotransmission at inhibitory synapses. (A) Schematic showing experimental design in B-E. (B) Averaged fIPSP paired pulse representative traces at an interstimulus interval

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Figure 4 continued

of 100 ms in 0.5 mM Ca^{2+} (pulse one black, pulse two gray), 1 mM Ca^{2+} (pulse one pink, pulse two light pink) or 2 mM Ca^{2+} (pulse one blue, pulse two light blue). (C) Paired pulse ratio (PPR) (P2/P1) was lower in 2 mM extracellular Ca^{2+} than in 0.5 mM Ca^{2+} or 1 mM Ca^{2+} and lower in 1 mM Ca^{2+} than 0.5 mM Ca^{2+} , indicating that extracellular Ca^{2+} concentration is positively associated with presynaptic release probability (repeated measures two-way ANOVA $F_{(2,48)} = 45.96$, $p < 0.0001$, Tukey's post hoc testing 0.5 mM Ca^{2+} vs 1 mM Ca^{2+} $p = 0.0145$, 0.5 mM Ca^{2+} vs 2 mM Ca^{2+} $p < 0.0001$, 1 mM Ca^{2+} vs 2 mM Ca^{2+} $p < 0.0001$, $n = 6$ for 0.5 mM Ca^{2+} , $n = 5$ for 1 mM Ca^{2+} , $n = 6$ for 2 mM Ca^{2+}). (D) Quantification of baseline peak amplitudes confirming that greater extracellular Ca^{2+} concentration increases presynaptic release probability and is associated with greater peak amplitude of fIPSPs (one-way ANOVA $F_{(2,14)} = 13.85$, $p = 0.0005$, Tukey's post-hoc testing 0.5 mM Ca^{2+} vs 1 mM Ca^{2+} $p = 0.0072$, 0.5 mM Ca^{2+} vs 2 mM Ca^{2+} $p = 0.0005$, $n = 6$ for 0.5 mM Ca^{2+} , $n = 5$ for 1 mM Ca^{2+} , $n = 6$ for 2 mM Ca^{2+}). (E) Time course showing block of 0.1 Hz evoked fIPSPs following PTX application in 0.5 mM Ca^{2+} (black), 1 mM Ca^{2+} (pink) or 2 mM Ca^{2+} (blue). Greater presynaptic release probability via increased extracellular Ca^{2+} is associated with faster block of GABA_ARs (non-linear regression single exponential fit, Sum-of-Squares F test $F_{(6, 2048)} = 571.3$, $p < 0.0001$, $n = 6$ for 0.5 mM Ca^{2+} , $n = 5$ for 1 mM Ca^{2+} , $n = 6$ for 2 mM Ca^{2+}). (F) Schematic showing experimental design in G-I. (G) Time course showing evoked fIPSP response to 0.1 Hz stimulation before (black) and following application of PTX at rest for 0 min (gray), 5 min (red) or 10 min (blue). Treatment of PTX for increasing amounts of time resulted in a lesser remaining response upon continuation of 0.1 Hz stimulation ($n = 6$ for 0 min, $n = 7$ for 5 min, $n = 8$ for 10 min). (H) Averaged fIPSP representative traces at baseline (black) and first fIPSP response following 0 min (gray), 5 min (red) or 10 min (blue) of PTX administration at rest. (I) Quantification of unblocked GABA_AR mediating response remaining after 0, 5 or 10 min perfusion of PTX at rest, adjusted for upper and lower boundaries of fIPSP response. Following PTX administration at rest, the unblocked evoked response is $103.12 \pm 3.82\%$ of baseline after 0 min (gray), $88.0 \pm 4.89\%$ of baseline after 5 min (red) and $34.05 \pm 5.37\%$ of baseline after 10 min (blue) (one-way ANOVA $F_{(2,18)} = 56.30$, $p < 0.0001$, Tukey's post-hoc testing 0 min vs 10 min $p < 0.0001$, 5 min vs 10 min $p < 0.0001$, $n = 6$ for 0 min, $n = 7$ for 5 min, $n = 8$ for 10 min). Thus a response to evoked stimulation remains following 10 min of GABA_AR block by spontaneous neurotransmission, indicating partial segregation. Graphs are mean \pm SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$.

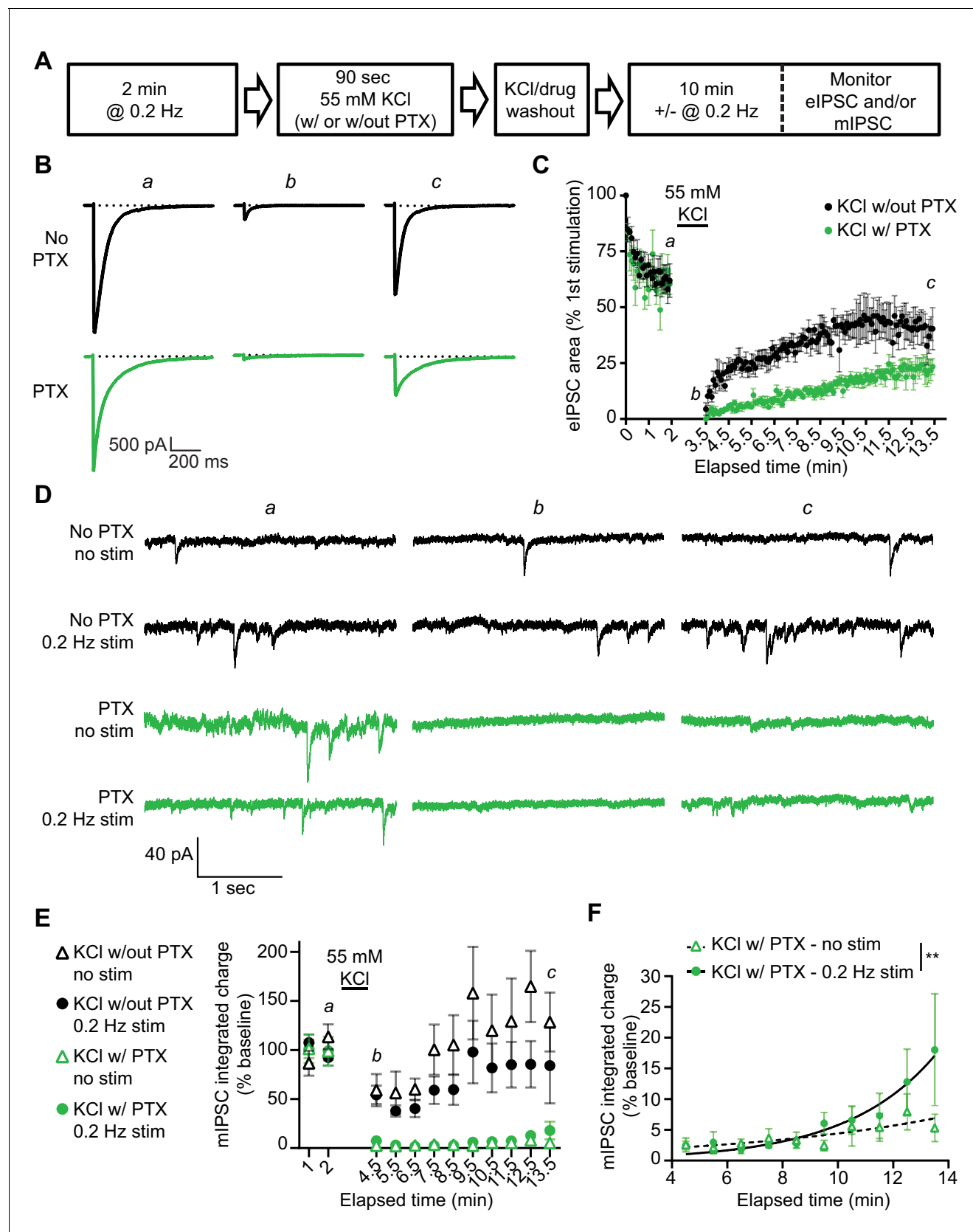


Figure 5. Recovery from PTX block of receptors activated by spontaneous GABA release is enhanced by the administration of evoked stimulation. (A) Schematic showing experiment design. (B) Example traces of eIPSC responses from time points indicated in C. Stimulus artifacts were removed for Figure 5 continued on next page

Figure 5 continued

clarity. (C) Quantification of eIPSC recovery following KCl treatment with and without PTX. KCl treatment initially depresses the eIPSC response. The evoked response is recovered after KCl treatment without PTX, but recovery after KCl treatment with PTX proceeds over a longer time course indicating GABA_ARs which were activated by KCl treatment and blocked by PTX are being unblocked with successive stimulations. (D) Example traces of mIPSCs from the timepoints indicated in E recorded in the presence or absence of stimulation before and after KCl treatment with and without PTX. (E) Quantification of mIPSC recovery (1 min bins) in the presence or absence of stimulation following KCl treatment with and without PTX. KCl treatment without PTX initially depresses mIPSCs, but does not fully block them. KCl treatment with PTX blocks mIPSCs, after which they recover slowly (KCl w/out PTX – no stim, $n = 6$; KCl w/out PTX – 0.2 Hz stim, $n = 5$; KCl w/PTX – no stim, $n = 6$; KCl w/PTX – 0.2 Hz stim, $n = 6$). (F) Analysis of mIPSC recovery (1 min bins) in PTX treated samples in the absence (open triangles) and presence (closed circles) of 0.2 Hz stimulation. Data were fitted with non-linear regression exponential model (no stimulation, dashed curve, $n = 6$; 0.2 Hz stimulation, solid curve, $n = 6$) which were significantly different from each other (Sum-of-squares F test $F_{(2,109)} = 6.976$, $p = 0.0014$). These data indicate that stimulation increases the recovery of mIPSCs, consistent with partial overlap of receptors activated by evoked and spontaneous signaling at inhibitory synapses. Graphs are mean \pm SEM. ** indicates $p < 0.01$.

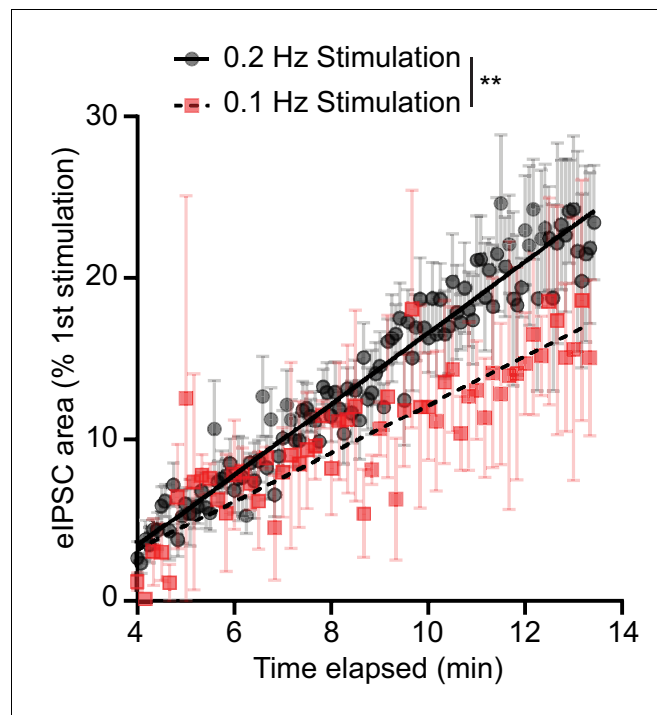


Figure 5—figure supplement 1. Rate of unblock following PTX application is use-dependent. Quantification of eIPSC recovery following KCl treatment with PTX. The eIPSC response recovers more slowly with lower stimulation frequency indicating that unblock of GABA_ARs from PTX is at least partially use-dependent. Data were fitted with linear regressions (0.2 Hz stimulation, solid line, $n = 6$; 0.1 Hz stimulation, dashed line, $n = 4$) which were significantly different from each other (Sum-of-squares F test $F_{(1,873)} = 18.57$, $p < 0.0001$). Graph is mean \pm SEM. ** indicates $p < 0.01$.

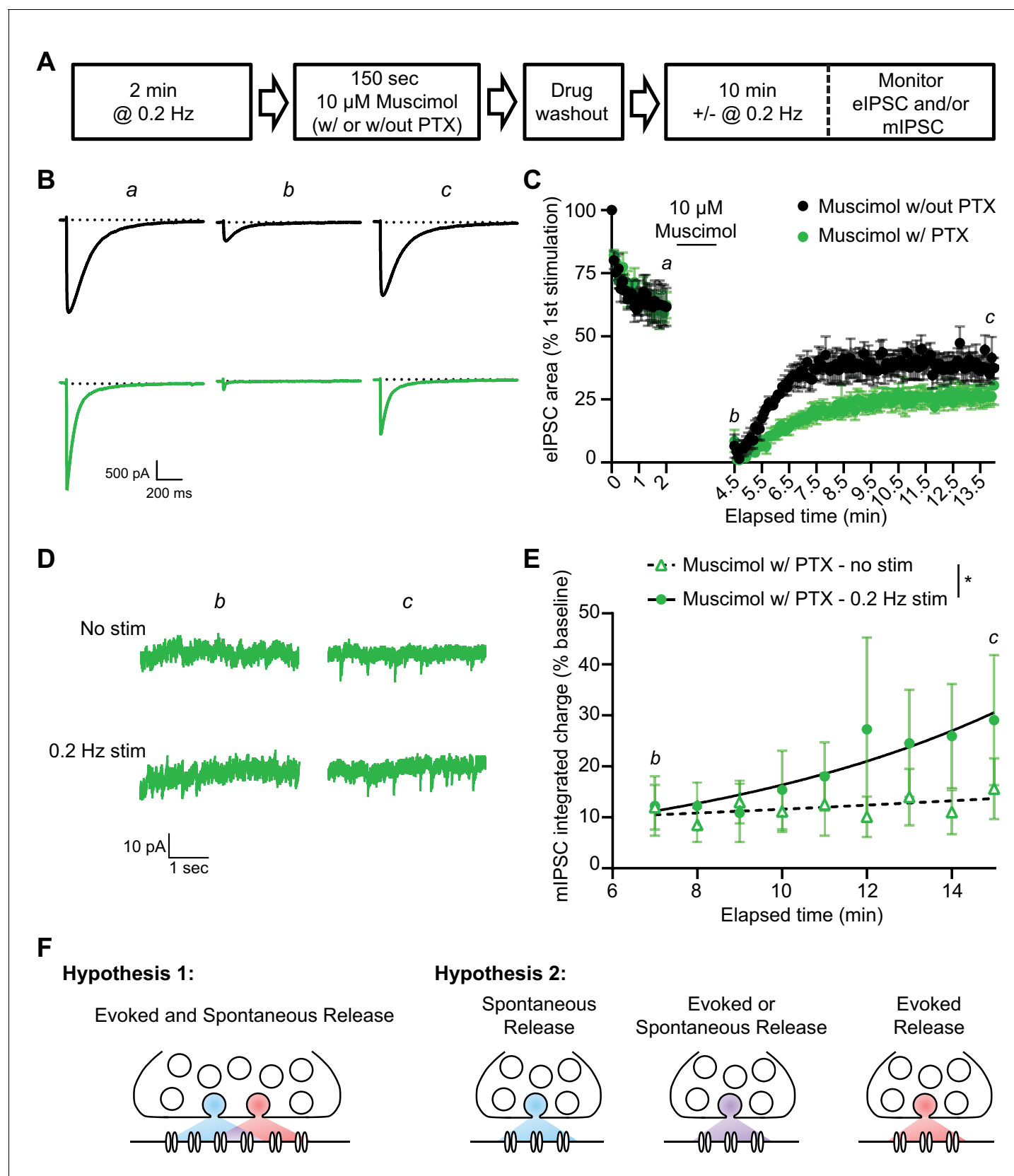


Figure 6. A similar pattern of mIPSC recovery from block is detected when using muscimol to activate GABA_ARs during PTX block. (A) Schematic showing experiment design. (B) Example traces of eIPSC responses from time points indicated in C. Stimulus artifacts removed for clarity. (C)

Figure 6 continued on next page

Figure 6 continued

Quantification of eIPSC recovery following muscimol treatment with and without PTX ($n = 4$, all groups). Muscimol treatment initially depresses the eIPSC response. The evoked response is recovered after muscimol treatment without PTX, but recovery after muscimol treatment with PTX proceeds over a longer time course indicating GABA_ARs which were activated by muscimol treatment and blocked by PTX are being unblocked with successive stimulations. (D) Example traces of mIPSCs from the timepoints indicated in E. (E) Analysis of mIPSC recovery (1 min bins) in PTX treated samples in the absence (open triangles) and presence (closed circles) of 0.2 Hz stimulation. Data were fitted with non-linear regression exponential models (no stimulation, dashed curve, $n = 6$; 0.2 Hz stimulation, solid curve, $n = 3$) which were significantly different from each other (Sum-of-squares F test $F_{(2,74)} = 4.832$, $p=0.0107$). These data indicate that stimulation increased the recovery of mIPSCs, consistent with partial overlap of receptors activated by evoked and spontaneous signaling at inhibitory synapses. (F) Graphic summary of findings indicating a partial overlap of receptors activated by spontaneous and evoked GABA release. Partial overlap could be achieved either through spatial segregation of evoked and spontaneous presynaptic release and postsynaptic receptors within the same synapse, or through specialization of synapses for either spontaneous release, evoked release, or both. Graphs are mean \pm SEM. * indicates $p<0.05$.