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Inhibition of synaptic transmission and G protein modulation by synthetic $Ca_V2.2$ Ca^{2+} channel peptides

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Non-technical summary Nerve cells (neurones) in the body communicate with each other by releasing chemicals (neurotransmitters) which act on proteins called receptors. An important group of receptors (called G protein coupled receptors, GPCRs) regulate the release of neurotransmitters by an action on the ion channels that let calcium into the cell. Here, we show for the first time that small peptides based on specific regions of calcium ion channels involved in GPCR signalling can themselves inhibit nerve cell communication. We show that these peptides act directly on calcium channels to make them more difficult to open and thus reduce calcium influx into native neurones. These peptides also reduce GPCR-mediated signalling. This work is important in increasing our knowledge about modulation of the calcium ion channel protein; such knowledge may help in the development of drugs to prevent signalling in pathways such as those involved in pain perception.

Abstract Modulation of presynaptic voltage-dependent Ca^{2+} channels is a major means of controlling neurotransmitter release. The $Ca_V2.2$ Ca^{2+} channel subunit contains several inhibitory interaction sites for G$\beta\gamma$ subunits, including the amino terminal (NT) and I–II loop. The NT and I–II loop have also been proposed to undergo a G protein-gated inhibitory interaction, whilst the NT itself has also been proposed to suppress Ca_V2 channel activity. Here, we investigate the effects of an amino terminal ($Ca_V2.2$[45–55]) 'NT peptide' and a I–II loop alpha interaction domain ($Ca_V2.2$[377–393]) 'AID peptide' on synaptic transmission, Ca^{2+} channel activity and G protein modulation in superior cervical ganglion neurones (SCGNs). Presynaptic injection of NT or AID peptide into SCGN synapses inhibited synaptic transmission and also attenuated noradrenaline-induced G protein modulation. In isolated SCGNs, NT and AID peptides reduced whole-cell Ca^{2+} current amplitude, modified voltage dependence of Ca^{2+} channel activation and attenuated noradrenaline-induced G protein modulation. Co-application of NT and AID peptide negated inhibitory actions. Together, these data favour direct peptide interaction with presynaptic Ca^{2+} channels, with effects on current amplitude and gating representing likely mechanisms responsible for inhibition of synaptic transmission. Mutations to residues reported as determinants of Ca^{2+} channel function within the NT peptide negated inhibitory effects on synaptic transmission, Ca^{2+} current amplitude and gating and G protein modulation. A mutation within the proposed QXXER motif for G protein modulation did not abolish inhibitory effects of the AID peptide. This study suggests that the $Ca_V2.2$ amino terminal and I–II loop contribute molecular determinants for Ca^{2+} channel function; the data favour a direct interaction of peptides with Ca^{2+} channels to inhibit synaptic transmission and attenuate G protein modulation.

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Abbreviations AID, alpha interaction domain; GPCR, G protein-coupled receptor; NA, noradrenaline; NT, amino terminal; PP, prepulse; PPF, prepulse facilitation; SCG, superior cervical ganglion; SCGN, SCG neurone.

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Introduction

Presynaptic inhibition of neurotransmitter release is a fundamental process at central synapses and may be controlled via the modulation of voltage-dependent Ca^{2+} channel activity. The presynaptic Ca_V2 Ca^{2+} channel family (comprising $Ca_V2.1$, $Ca_V2.2$ and $Ca_V2.3$ isoforms) encode the major, pore-forming $\alpha1$ subunit, which associates with auxiliary $Ca_V\beta$, $\alpha2$-δ and, sometimes, γ subunits (Ertel *et al.* 2000; Catterall *et al.* 2005). A dominant form of feedback inhibition of neurotransmitter release from presynaptic terminals is mediated by the interaction between $G\beta\gamma$ subunits, liberated by activation of G protein-coupled receptors (GPCRs), and Ca_V2 $\alpha1$ subunits (Ikeda, 1996; Herlitze *et al.* 1996; Kajikawa *et al.* 2001; Stephens & Mochida, 2005; Stephens, 2009). Ca_V2 $\alpha1$ subunits contain distinct regions which interact with $G\beta\gamma$ subunits (Dolphin, 2003; De Waard *et al.* 2005; Tedford & Zamponi, 2006). The major interaction regions are: (i) the amino terminal (NT) (Page *et al.* 1998; Stephens *et al.* 1998*b*; Simen & Miller, 1998, 2000; Cantí *et al.* 1999); (ii) sites on the intracellular loop connecting domains I and II (I–II loop), the major site partially overlapping with a $Ca_V\beta$ binding site and termed the alpha interaction domain (AID) (De Waard *et al.* 1997; Herlitze *et al.* 1997; Zamponi *et al.* 1997); and (iii) the carboxyl terminal (Qin *et al.* 1997; Li *et al.* 2004). Overall, the carboxyl terminal is suggested to play only a minor role (Stephens *et al.* 1998*b*; Agler *et al.* 2005), but may act to increase $G\beta\gamma$ affinity for the channel (Li *et al.* 2004). In addition to these studies, Agler *et al.* (2005) have proposed a physical interaction between the NT and the I–II loop to form a G protein-gated inhibitory module and cause a 'constitutive' suppression of Ca^{2+} current. The co-expression of the NT domain has also been shown to cause a dominant-negative suppression of Ca_V2 channels (Raghib *et al.* 2001; Page *et al.* 2004, 2010).

The studies described above have been performed in recombinant cells and the situation in native neurones is less clear. Moreover, despite the identification and extensive molecular mapping of Ca^{2+} channel/$G\beta\gamma$ interaction sites, the relative *functional* contributions of different Ca_V2 sites to presynaptic inhibition of neurotransmitter release remain unknown. Superior cervical ganglion (SCG) neurones (SCGNs) expressing $Ca_V2.2$ channels represent a well-characterized system for investigating synaptic transmission and $G\beta\gamma$ modulation (Ikeda, 1991, 1996; Stephens & Mochida, 2005). Therefore, we examined the effects of synthetic $Ca_V2.2$ peptides based on the NT and AID region in SCGNs. We demonstrate, for the first time, that $Ca_V2.2$ peptides can act directly to inhibit synaptic transmission; we present evidence that peptide mechanism of action involves a reduction in Ca^{2+} current amplitude and also a modification of activation gating properties. These effects are accompanied by an attenuation of G protein modulation. Together, these data may explain previous findings for peptides based on the AID and NT regions. We also use mutagenesis to identify $Ca_V2.2$ NT residues important for inhibitory effects, suggesting that the NT is a crucial determinant of Ca^{2+} channel function, including inhibition of transmitter release and G protein modulation.

Methods

Tissue culture

SCGNs for microelectrode recording. Cells were prepared as previously described (Mochida *et al.* 1994). Briefly, Wistar rats were decapitated on postnatal day 7 under diethylether anaesthesia according to the guidelines of the Physiological Society of Japan. SCGNs were isolated and maintained in culture for 6–7 weeks in a growth medium of 84% Eagle's minimal essential medium, 10% fetal calf serum, 5% horse serum, 1% penicillin–streptomycin (all Gibco Invitrogen Corporation, NY, USA) and 25 ng ml^{-1} nerve growth factor (2.5 S, grade II; Alomone, Jerusalem, Israel).

SCGNs for patch clamp recordings. Neurones were acutely dissociated from the SCG of 3- to 6-week-old Wistar rats according to the guidance of the UK Animals (Scientific Procedures) Act 1986. Animals were anaesthetized with isoflurane (BDH, Loughborough, UK) and decapitated. Ganglia were dissociated and transferred to L-15 medium (Sigma Aldrich, Poole, UK) containing 5% penicillin–streptomycin (Gibco Invitrogen, Paisley, UK). After centrifugation at 800 r.p.m. (= 72 g) for 2 min at room temperature, L-15 medium was replaced by an enzymatic solution of 0.5 mg ml^{-1} trypsin, 1 mg ml^{-1} collagenase with 3.6 mg ml^{-1} glucose (all Sigma Aldrich). Ganglia were maintained in the enzymatic solution for 30–40 min at 37°C and dissociated by gentle trituration every 10 min with fire-polished Pasteur pipettes. Enzymatic digestion was arrested by addition of growth medium comprising: 84% Eagle's minimal essential medium, 10% fetal calf serum, 5% horse serum (all Lonza, Wokingham, UK) and 1% penicillin–streptomycin (Gibco Invitrogen). After centrifugation at 800 r.p.m. (= 72 g) for 4 min at room temperature (20–22°C), pellets were re-suspended in growth medium and plated onto coverslips coated with poly-L-ornithine (Sigma Aldrich) and incubated overnight at 37°C (5% CO_2) prior to electrophysiological recordings.

Electrophysiology

Synaptic transmission between SCGNs. Conventional paired intracellular recordings were made from two proximal neurones using microelectrodes filled with 1 M

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potassium acetate (70–90 MΩ) as described previously (Mochida *et al.* 2003). Briefly, SCGNs were superfused with modified Krebs solution containing (in mM): NaCl 136, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 11, Na-Hepes, 3; pH 7.4 (adjusted with NaOH). Action potentials were generated in a neurone by current injection (at 0.1 Hz) through the intracellular recording electrode and excitatory postsynaptic potentials (EPSPs) recorded from the other neurone. Peptides were dissolved to a final concentration of 1 mM in a solution containing (in mM): potassium acetate 150, Mg-ATP 5, Hepes 10; pH 7.3, and introduced for 3–4 min into the cell body of SCGNs from a glass pipette (16–18 MΩ) along with fast green FCS (Sigma Aldrich) to visualize successful injection (Mochida *et al.* 1996). Data were analysed using software written by the late Ladislav Tauc (CNRS, France). Statistical significance was determined using Student's *t* test.

Whole-cell patch clamp recordings. Whole-cell recording from isolated SCGNs was performed as previously described (Stephens & Mochida, 2005) using an intracellular solution containing (in mM): CsCl 140, Hepes 10, $CaCl_2$ 0.1, $MgCl_2$ 1.0, Mg-ATP 4, EGTA 1.0; pH 7.3 (adjusted with CsOH) and an extracellular solution containing (in mM): TEA-Br 160, Hepes 10, KCl 3, $MgCl_2$ 1, glucose 4, $NaHCO_3$ 1.0 with 10 mM Ba^{2+} as charge carrier; pH 7.4 (adjusted with Sigma 7–9 base). Data were obtained at room temperature. Membrane currents were acquired with an Axopatch 200B patch-clamp amplifier. Linear components of capacitive and leak currents were subtracted using the standard *P*/4 protocol. Series resistance compensation of >70% was typically employed. For GPCR-mediated inhibition studies, noradrenaline (10 μM final concentration) was bath-applied. NT, AID or mutated peptides were freshly dissolved in intracellular solution to a final concentration of 1 mM in the patch electrode immediately prior to use. Data were analysed offline using WinWCP v4.0.7 (John Dempster, University of Strathclyde), OriginPro 7.0 (Microcal, Northampton, MA, USA) and MATLAB (The MathWorks, Natick, MA, USA) software. Values reported are means ± SEM. Statistical significance was determined using Mann–Whitney *U* tests or Student's *t* test as appropriate.

Pharmacology. The following agents were used: ω-conotoxin GVIA (Alomone Labs, Jerusalem, Israel), noradrenaline (Sigma Aldrich), pertussis toxin (PTX) (Tocris Cookson, Bristol, UK). Synthetic peptides were purchased from Peptide Specialty Laboratories GmbH (Heidelberg, Germany) and had the following sequences: NT peptide (rat $Ca_V2.2$[45–55]), YKQSIAQRART; mutant NT peptide, YKQSIAQAAAT; AID peptide (rat $Ca_V2.2$[377–393]), RQQQIERELNGYLEWIF; mutant AID peptide, RQQQLERELNGYLEWIF.

The authors have read, and the experiments comply with, the policies and regulations of *The Journal of Physiology* described by Drummond (2009).

Results

Effects of AID and NT peptides on synaptic transmission and G protein modulation

In model SCGN synapses. We first investigated the effects of presynaptic injection of an amino terminal ($Ca_V2.2$[45–55]) 'NT peptide' and a I–II loop alpha interaction domain ($Ca_V2.2$[377–393]) 'AID peptide' in long-term SCGN cultures, an appropriate model for the study of fast synaptic transmission and modulation of Ca^{2+} channels (Stephens & Mochida, 2005; Ma & Mochida, 2007); for all experiments in model SCGN synapses, $Ca_V2.2$ peptides were injected into the presynaptic cell at 1 mM. Presynaptic injection of the NT peptide caused a significant decrease in EPSP amplitude recorded in synaptically coupled cells (25.3 ± 5.5%, $n = 6$, $P < 0.05$; Fig. 1*Aa* and *d*). Similarly, AID peptide caused a significant decrease in EPSP amplitude (36.5 ± 11.0%, $n = 6$, $P < 0.05$; Fig. 1*Ab* and *d*). A scrambled 17mer AID peptide had no significant effect on EPSP amplitude (4.6 ± 4.2%, $n = 8$, $P > 0.05$; Fig. 1*Ac* and *d*). These data are consistent with synthetic $Ca_V2.2$ peptides causing direct inhibition of cholinergic transmission in model SCGN synapses. We next investigated the effects of the NT and AID peptide on G protein modulation. Consistent with our previous studies (Stephens & Mochida, 2005), the GPCR agonist noradrenaline (NA; 10 μM) caused a clear reduction in EPSP amplitude (40.4 ± 6.8%, $n = 5$; Fig. 1*Ba* and *d*). This NA-induced decrease in EPSP amplitude was significantly reduced in the presence of 1 mM NT peptide (20.8 ± 6.2%, $n = 5$, $P < 0.05$; Fig. 1*Bb* and *d*) and virtually abolished in the presence of 1 mM AID peptide (1.0 ± 6.1%, $n = 5$, $P < 0.05$; Fig. 1*Bc* and *d*). Here, NA effects were normalized to inhibition caused by pre-injection of the NT or AID peptide; therefore, residual NA effects in the presence of the NT peptide most likely reflect differences in magnitude of inhibition between AID and NT peptides (see Fig. 1*Ad*). These data demonstrate that $Ca_V2.2$ NT and AID peptides inhibit synaptic transmission in SCGN synapses and that these actions are accompanied by an attenuation of G protein modulation. It has been reported that similar AID-based peptides (Herlitze *et al.* 1997; Zamponi *et al.* 1997) and a peptide based on the entire NT[1–95] domain (Agler *et al.* 2005) attenuate G protein modulation in recombinant cells; however, it has been unclear if peptides simply chelate free G$\beta\gamma$ (Zamponi *et al.* 1997) or, alternatively, bind the channel itself and the resultant complex is now insensitive

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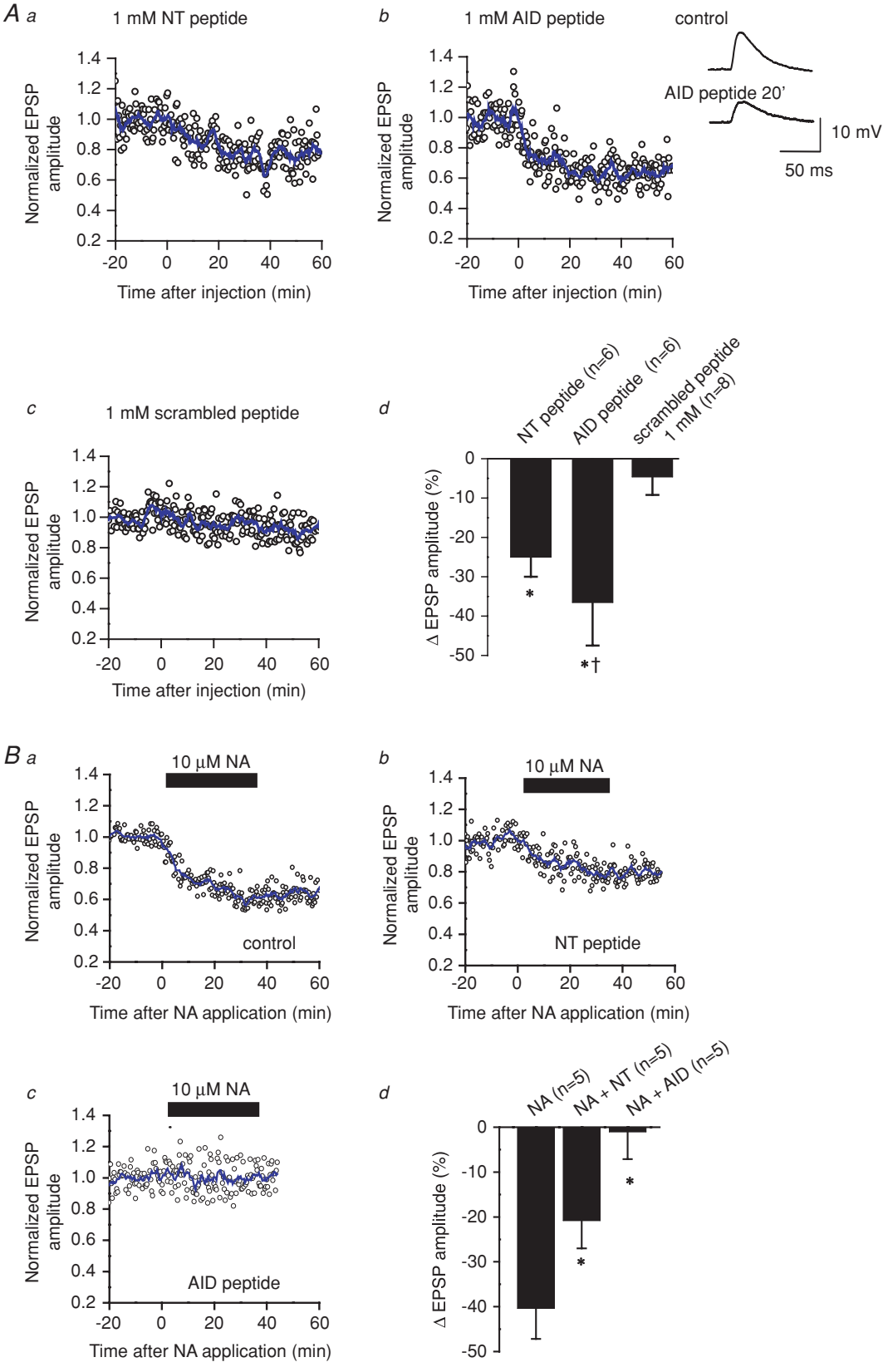

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to G protein modulation (Agler *et al.* 2005). Our data on synaptic transmission initially favour the latter scenario, as $G\beta\gamma$ chelation would be expected to remove G protein inhibition and increase synaptic transmission.

Effects of AID and NT peptides on Ca^{2+} channels and G protein modulation in isolated SCGNs

In order to explore potential mechanisms of peptide action, we examined the effects of the NT and AID peptide on whole-cell Ca^{2+} current (measured as I_{Ba}) in isolated SCGNs as the best available model of the heterogeneous Ca^{2+} channel population most likely present at the presynapse; for all experiments in isolated SCGNs, $Ca_V2.2$ peptides were included in the intracellular solution at 1 mM. We first noted that NT and AID peptides had effects on the voltage dependence of Ca^{2+} channel activation. In control cells, activation curves were seen to display symmetry about their midpoint and, therefore, were best fitted by a single Boltzmann distribution ($96 \pm 1\%$, $n = 10$; Fig. 2*Aa*, Table 1). By contrast, in the presence of either $Ca_V2.2$ peptide, activation curves could be well described only by the sum of two Boltzmann distributions. Thus, a lower, more hyperpolarized first component of midpoint of voltage dependence of activation $V_{0.5(1)}$ component (NT peptide: $62 \pm 3\%$, $n = 11$; AID peptide: $56 \pm 3\%$, $n = 8$) and a higher, more depolarized second component of midpoint of voltage dependence of activation $V_{0.5(2)}$ component (NT peptide: $38 \pm 3\%$, $n = 11$; AID peptide: $44 \pm 3\%$, $n = 8$) were required to best fit the data (Fig. 2*Ab* and *c*, Table 1). The second component was also associated with a shallow slope factor (Table 1).

We have previously demonstrated that NA inhibits synaptic transmission in SCGNs via $G\beta\gamma$-mediated inhibition of I_{Ba} (Stephens & Mochida, 2005). NA (10 μM) significantly reduced I_{Ba} by $55.4 \pm 4.8\%$, $n = 11$ ($P < 0.05$ *vs.* pre-drug I_{Ba}) (Fig. 2*Ba* and *d*). NA inhibition of Ca^{2+} current was accompanied by a significant, characteristic depolarizing shift in $V_{0.5}$ of $\sim$+12 mV (Fig. 2*Ca*, Table 1); to best estimate this effect, fully $G\beta\gamma$-modulated channels were compared with channels following a conditioning prepulse (PP) to +120 mV to maximally relieve any endogenous G protein modulation (Stephens *et al.* 1998*a*). Pre-incubation (20 h) with 500 ng ml^{-1} pertussis toxin (PTX) abolished the NA-induced shift in $V_{0.5}$ (Table 1). NA-induced inhibition of I_{Ba} was subject to clear prepulse facilitation (PPF), as measured by a significant increase in facilitation ratio (P2:P1) from 1.44 ± 0.14 to 1.88 ± 0.24 at -10 mV ($n = 10$, $P < 0.05$; Fig. 3*Aa* and *Ba*). PPF also reversed the NA-induced shift in $V_{0.5}$ (to 2.8 ± 3.1 mV, $n = 10$, $P < 0.05$; Fig. 3*Ca*, Table 1). Effects of prepulses on NA-mediated inhibition of I_{Ba} were also prevented by PTX pre-treatment, as evidenced by abolition of the NA-induced shift in $V_{0.5}$ (Table 1). Together, these data confirm a robust GPCR-mediated inhibition of Ca^{2+} channels and the isolation of appropriate voltage-dependent pathways to study $Ca_V2.2$ peptide effects in native SCGNs.

We next investigated the effects of free cytosolic NT or AID peptide on G protein modulation of Ca^{2+} channels. NA-induced inhibition of I_{Ba} was significantly attenuated in the presence of the NT ($32.4 \pm 3.5\%$, $n = 11$, $P < 0.05$; Fig. 2*Bb* and *d*) and the AID ($35.6 \pm 2.8\%$, $n = 10$, $P < 0.01$; Fig. 2*Bc* and *d*) peptide. PPF of the NA-inhibited I_{Ba} was significantly reduced in the presence of the NT (P2:P1: 1.21 ± 0.03, $n = 11$; Fig. 3*Ab* and *Bb*) and the AID (P2:P1: 1.09 ± 0.05, $n = 8$; Fig. 3*Ac* and *Bc*) peptide (measured at -10 mV, both $P < 0.01$ *vs.* control facilitation ratio shown in Fig. 3*Ba*). Whilst large depolarizing prepulses readily reversed the NA-induced shift in $V_{0.5}$ in control conditions (Fig. 3*Ca*, Table 1), no PPF was evident in the presence of either the NT (Fig. 3*Cb*, Table 1) or the AID (Fig. 3*Cc*, Table 1) peptide. These data demonstrate that $Ca_V2.2$ peptides had inhibitory effects on voltage-dependent pathways under these conditions. In the absence of NA-mediated inhibition, a small amount of basal PPF was seen in control conditions (Fig. 3*Ba*). This basal PPF was also seen in the presence of the NT (P2:P1: 1.23 ± 0.03, $n = 11$; Fig. 3*Bb*) and the AID (P2:P1: 1.24 ± 0.05, $n = 8$; Fig. 3*Bc*) peptide

Figure 1. Presynaptic injection of synthetic $Ca_V2.2$ peptides inhibits synaptic transmission and attenuates subsequent G protein modulation in SCGN synapses
A, effect of NT (1 mM) (*a*), AID (1 mM) (*b*; inset shows representative EPSP traces for effects of AID peptide, prior to (control) and 20 min after peptide injection (AID peptide 20′)) and scrambled 17mer AID (1 mM) (*c*) peptides on EPSP amplitude. *Ad*, bar graph summarizing effects of synthetic $Ca_V2.2$ peptides on mean EPSP amplitudes (at 20 min after peptide injection), $^*P < 0.05$ *vs.* control EPSP amplitude prior to peptide injection or †$P < 0.05$ for AID peptide (only) *vs.* decrease in EPSP amplitude caused by scrambled AID peptide (Student's *t* test). In this figure and Figs 4–6, EPSPs were evoked at a frequency of 0.1 Hz and were normalized to amplitude prior to peptide injection into the presynapse at time = 0 min; continuous line shows smoothed values of the EPSP amplitudes. *B*, effect of 10 μM NA on EPSP amplitude in control (*a*) and in the presence of NT peptide (1 mM) (*b*) and AID peptide (1 mM) (*c*); NA was bath-applied 30 min after injection of the peptide. EPSPs were normalized to amplitude prior to NA application at time = 0 min. *Bd*, bar graph summarizing NA effects, $^*P < 0.05$ *vs.* NA effects in controls (Student's *t* test).

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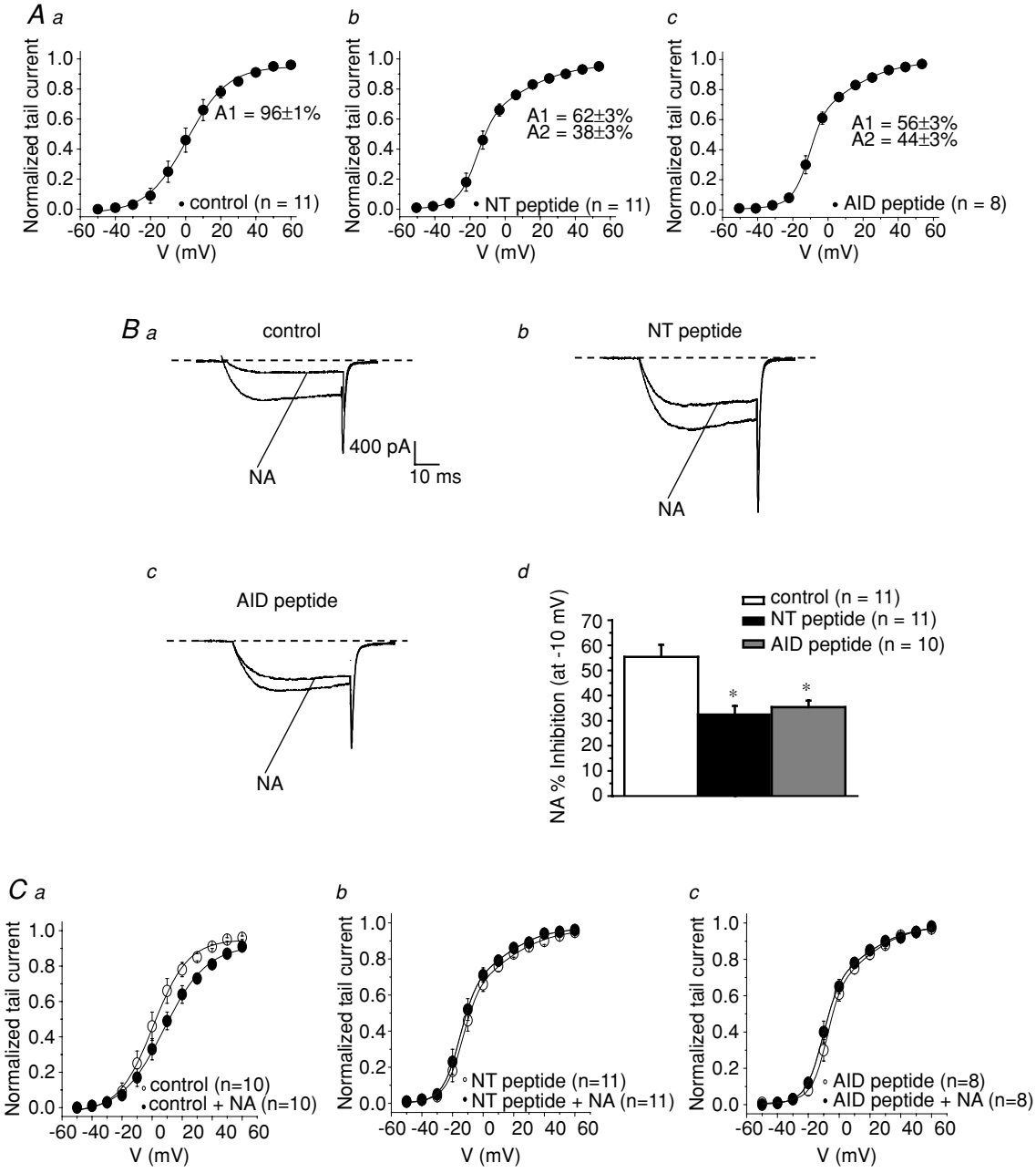

Figure 2. Synthetic $Ca_V2.2$ peptides attenuate NA-induced inhibition of I_{Ba}
A, voltage dependence of activation curves from tail current amplitude data in control (*a*) and in the presence of NT peptide (1 mM) (*b*) and AID peptide (1 mM) (*c*). In this and subsequent figures, tail current amplitude at −120 mV was normalized to the maximum current and plotted against test pulse potential. Control tail current activation curve data (*Aa*) were described by one Boltzmann distribution; data recorded in the presence of the NT (*Ab*) and the AID (*Ac*) peptide were well fitted only by two Boltzmann distributions (mean ± SEM values derived from individual cells are shown in Table 1). *B*, representative traces showing NA (10 μM)-induced inhibition of I_{Ba} in control (*a*), and in the presence of NT peptide (1 mM) (*b*) and AID peptide (1 mM) (*c*). *Bd*, bar graph summarizing percentage I_{Ba} inhibition by NA (10 μM) showing pooled data for controls (white bar), and in the presence of NT peptide (black bar) and AID peptide (grey bar); $^*P < 0.01$ *vs.* control (Mann–Whitney *U* test). In this and subsequent figures, effects were measured at −10 mV (as G protein modulation is most pronounced at moderate depolarizing steps, Bean, 1989); holding current = −70 mV, scale bars in *Ba* apply to each trace *Ba–c*. *C*, effects of 10 μM NA on voltage dependence of activation curves from tail current amplitude data in control (*a*) and in the presence of NT peptide (1 mM) (*b*) and AID peptide (1 mM) (*c*). Control tail current activation curve data (*Ca*) were described by one Boltzmann distribution; data recorded in the presence of the NT (*Cb*) and AID (*Cc*) peptide (± NA) were well fitted only by two Boltzmann distributions (mean ± SEM values derived from individual cells are shown in Table 1).

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Table 1. Electrophysiological characteristics of SCGN whole-cell Ca^{2+} current

	$V_{0.5(1)}$ (mV)	k_1 (mV)	$V_{0.5(2)}$ (mV)	k_2 (mV)	A1	A2	*n*
CTL	3.6 ± 3.4	8.0 ± 0.8	—	—	0.96 ± 0.01	—	10
CTL+PP	−0.1 ± 3.1	6.2 ± 0.9	—	—	0.96 ± 0.01	—	
CTL+NA	11.9 ± 3.2$^{*\delta}$	12.0 ± 0.7$^{**\delta\delta}$	—	—	0.96 ± 0.01	—	
CTL+NA+PP	2.8 ± 3.1†	8.2 ± 0.8††	—	—	0.95 ± 0.01	—	
NT	−12.1 ± 1.8	4.0 ± 0.6	18.0 ± 5.2	16.3 ± 1.5	0.62 ± 0.03	0.38 ± 0.01	11
NT+PP	−14.0 ± 1.5	3.2 ± 0.5	15.1 ± 5.6	16.7 ± 2.7	0.67 ± 0.04	0.33 ± 0.04	
NT+NA	−14.4 ± 1.7	3.6 ± 0.6	11.2 ± 6.2	14.5 ± 1.2	0.55 ± 0.05	0.44 ± 0.05	
NT+NA+PP	−15.8 ± 1.7	3.7 ± 0.7	14.9 ± 6.0	15.0 ± 1.5	0.72 ± 0.04	0.27 ± 0.04	
AID	−6.9 ± 2.0	3.6 ± 0.6	16.2 ± 4.0	15.6 ± 1.4	0.56 ± 0.03	0.44 ± 0.03	8
AID+PP	−8.7 ± 1.9	3.6 ± 0.4	14.2 ± 5.3	12.5 ± 1.4	0.64 ± 0.05	0.38 ± 0.05	
AID+NA	−9.2 ± 2.1	4.6 ± 1.1	21.7 ± 6.8	14.9 ± 2.3	0.64 ± 0.07	0.36 ± 0.07	
AID+NA+PP	−11.8 ± 1.5	4.2 ± 0.4	23.8 ± 7.4	13.4 ± 1.8	0.65 ± 0.05	0.35 ± 0.05	
Mutant AID	−7.5 ± 1.9	3.6 ± 0.7	19.3 ± 3.1	16.1 ± 1.4	0.56 ± 0.04	0.45 ± 0.05	7
Mutant AID+PP	−10.5 ± 1.9	3.5 ± 0.3	16.2 ± 2.1	14.7 ± 2.2	0.58 ± 0.05	0.43 ± 0.05	
Mutant AID+NA	−7.4 ± 3.3	5.0 ± 0.3	21.2 ± 9.1	17.1 ± 3.2	0.62 ± 0.05	0.40 ± 0.05	
Mutant AID+NA+PP	−7.3 ± 3.5	4.3 ± 1.0	19.4 ± 8.8	13.9 ± 3.4	0.60 ± 0.10	0.40 ± 0.10	
Mutant NT	−3.8 ± 3.5	8.3 ± 1.6	—	—	0.96 ± 0.01	—	8
Mutant NT+PP	−8.4 ± 2.9	6.6 ± 1.4	—	—	0.94 ± 0.01	—	
Mutant NT+NA	−1.4 ± 2.6*	9.7 ± 0.9**	—	—	0.94 ± 0.01	—	
Mutant NT+NA+PP	−5.0 ± 3.0	8.4 ± 1.5	—	—	0.95 ± 0.01	—	
NT+AID	−3.5 ± 1.1	9.4 ± 0.9	—	—	0.97 ± 0.01	—	10
NT+AID+PP	−8.7 ± 0.7	7.9 ± 0.6	—	—	0.97 ± 0.01	—	
NT+AID+NA	5.0 ± 1.7$^{**\delta}$	15.3 ± 1.3$^{**\delta\delta}$	—	—	0.96 ± 0.02	—	
NT+AID+NA+PP	−4.0 ± 1.3†	10.7 ± 1.2††	—	—	0.94 ± 0.01	—	
PTX	−7.2 ± 2.2	7.9 ± 1.0	—	—	0.96 ± 0.01	—	6
PTX+PP	−9.0 ± 1.8	7.2 ± 1.5	—	—	0.96 ± 0.01	—	
PTX+NA	−7.2 ± 1.8	9.0 ± 1.0	—	—	0.94 ± 0.01	—	
PTX+NA+PP	−10.2 ± 0.9	7.7 ± 1.2	—	—	0.96 ± 0.01	—	

Normalized tail current amplitude data were plotted against voltage and fitted with either a single or dual Boltzmann as appropriate. CTL, control; NA, noradrenaline (10 μM); PP, prepulse (to +120 mV). A1 = amplitude of first component of Boltzmann function; A2 = amplitude of second component of Boltzmann function. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control + PP; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ *vs.* control + NA; $^{\delta}P < 0.05$, $^{\delta\delta}P < 0.01$ *vs.* control; Mann–Whitney *U* tests.

at levels not significantly different to control (measured at −10 mV, both $P > 0.05$). Whilst $Ca_V2.2$ dominate somatic whole-cell Ca^{2+} current in SCGNs, there is a small contribution from Ca_V1 channels (Jones & Jacobs, 1990). Although Ca_V1 channels do not contribute to NA effects here (see Supplemental Fig. 1, available online only) or support transmitter release (Mochida *et al.* 2003), basal PPF was significantly reduced by 10 μM nifedipine (Supplemental Fig. 1), consistent with an alternative, Ca_V1-mediated component of basal voltage-dependent PPF (Lee *et al.* 2006) in SCGNs. The lack of difference in basal PPF between control and $Ca_V2.2$ peptides further suggests that peptides do not interact with Ca_V1 channels here.

As seen in controls above, under conditions of NA-induced inhibition and subsequent application of a conditioning prepulse, Ca^{2+} channel activation followed a single Boltzmann distribution in control cells (Figs 2*Ca* and 3*Ca*; Table 1), but again was best fitted by a dual Boltzmann distribution for the NT (Figs 2*Cb* and 3*Cb*; Table 1) and the AID (Fig. 2*Cc* and 3*Cc*; Table 1) peptide under both conditions. There were no significant effects on either $V_{0.5(1)}$ or $V_{0.5(2)}$ components in the response to NA in the presence of the NT (Fig. 2*Cb*, Table 1) and the AID (Fig. 2*Cc*, Table 1) peptide or for the effects of depolarizing PP in the presence of the NT (Fig. 3*Cb*, Table 1) and the AID (Fig. 3*Cc*, Table 1) peptide. Given these effects of $Ca_V2.2$ peptides on activation gating, we next investigated the effects of $Ca_V2.2$ peptides on Ca^{2+} current amplitude as a potential mechanism underlying the inhibition of synaptic transmission reported above. Following a conditioning prepulse to +120 mV to maximally relieve any endogenous G protein modulation, the NT and the AID peptide both caused a significant decrease in Ca^{2+} current density (Fig. 3*D*, Table 2).

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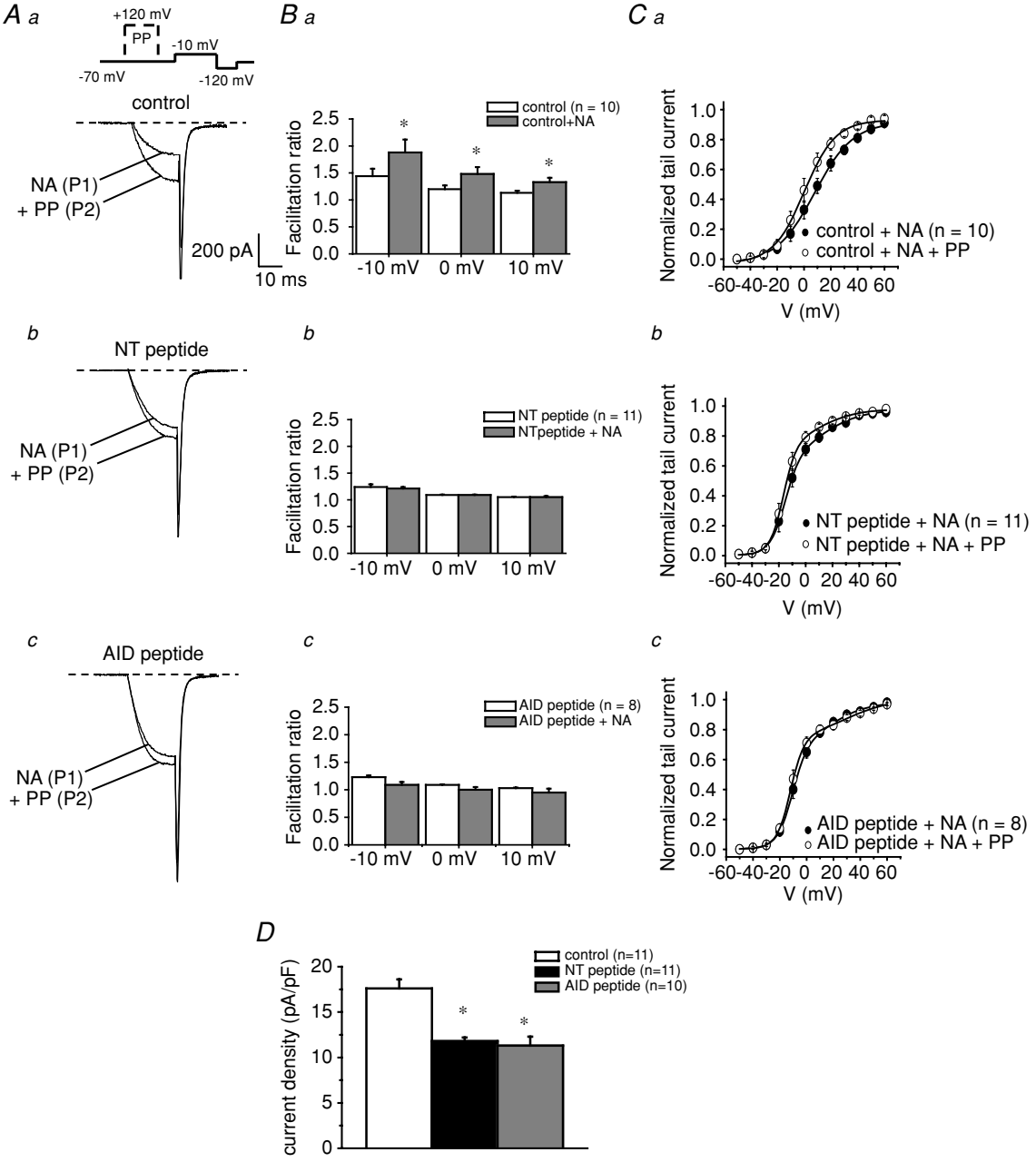

Figure 3. Synthetic $Ca_V2.2$ peptides abolish prepulse facilitation of NA-induced inhibition of I_{Ba}
A, superimposed representative traces showing NA-induced inhibition of I_{Ba} (P1) and effects of a depolarizing prepulse to +120 mV (P2) in control (*a*), and in the presence of NT peptide (1 mM) (*b*) and AID peptide (1 mM) (*c*); test potential −10 mV; scale bars in *Aa* apply to each trace *Aa*–*c*. *B*, bar graphs summarizing facilitation ratio (P2:P1) in the absence (white bars) and in the presence (grey bars) of NA (10 μM) in control (*a*), and in the presence of NT peptide (1 mM) (*b*) and AID peptide (1 mM) (*c*); $^*P < 0.01$ (paired Student's *t* test). *C*, voltage dependence of activation curves from tail current amplitude data in control (*a*), and in the presence of NT peptide (1 mM) (*b*) and AID peptide (1 mM) (*c*). Filled circles represent tail current in the presence of 10 μM NA; open circles represent tail current following a conditioning prepulse to +120 mV in the continued presence of NA. Control tail current activation curve data (*Ca*) were described by one Boltzmann distribution; data recorded in the presence of the NT (*Cb*) and AID (*Cc*) peptides (± PP) were well fitted only by two Boltzmann distributions (mean ± SEM values derived from individual cells are shown in Table 1). *D*, bar graph summarizing inhibition of Ca^{2+} current density by NT peptide (black bar) and AID peptide (grey bar) (mean ± SEM values derived from individual cells and conditions are shown in Table 2); $^*P < 0.01$ *vs.* control (Mann–Whitney *U* test).

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Table 2. Effects of $Ca_V2.2$ peptides on Ca^{2+} current density

Condition	Current density (pA pF^{-1})
Control ($n = 11$)	17.6 ± 1.0
NT peptide ($n = 11$)	11.8 ± 0.4*
AID peptide ($n = 7$)	11.3 ± 1.0*
Mutant NT peptide ($n = 6$)	15.2 ± 0.9
Mutant AID peptide ($n = 5$)	7.8 ± 0.7*
NT + AID peptide ($n = 5$)	17.5 ± 0.6

Current density was measured at least 15 min after formation of whole-cell configuration following a conditioning prepulse to +120 mV to maximally relieve any endogenous G protein modulation (P2 at −10 mV, Δt from P1 = 20 ms). Peptides that reduced EPSP amplitude in SCGN synapses (NT, AID and mutant AID) all caused a significant decrease in current density. By contrast, peptides that had no effect on EPSP amplitude in SCG synapses (mutant NT) had no effect on current density. *$P < 0.05$ *vs.* control (Mann–Whitney *U* test). *$P < 0.05$ *vs.* control; Mann–Whitney *U* tests.

Overall, $Ca_V2.2$ peptides had an important inhibitory effect on somatic Ca^{2+} channels in isolated SCGNs causing a modification in channel gating and a reduction in current amplitude. These effects were also accompanied by a reduced sensitivity to NA-induced G protein inhibition and an inability to exhibit prepulse facilitation. Such inhibitory peptide effects at presynaptic Ca^{2+} channels are consistent with the reduction in synaptic transmission and attenuation of G protein modulation demonstrated in model SCGN synapses.

Effects of mutations to residues implicated in Ca^{2+} channel function in NT and AID peptides

Previous studies have used site-directed mutagenesis of full-length $Ca_V2.2$ subunits to implicate specific amino acids within the NT and I–II loop as molecular determinants of Ca^{2+} channel function (Dolphin, 2003; De Waard *et al.* 2005; Tedford & Zamponi, 2006). With these points in mind, we next examined the effect of a $Ca_V2.2$[377–393] peptide containing an isoleucine to leucine mutation in the third position within the QXXER motif (I381L) ('mutant AID peptide'). This isoleucine residue has been reported to be a crucial determinant for G protein modulation (Herlitze *et al.* 1997). In the presence of the mutant AID peptide, activation curves were asymmetrical about the midpoint and required the sum of two Boltzmann distributions to be adequately fitted (Fig. 4*A*), similar to the situation for the parent AID peptide. The mutant AID peptide had an intermediate effect on NA-induced inhibition of I_{Ba} (42.0 ± 5.0%, $n = 9$; Fig. 4*Ba* and *b*). This degree of NA-mediated inhibition was not significantly different either from control or that seen with the unmodified AID peptide; there was also no significant NA-induced shift in $V_{0.5}$ with mutant AID (Table 1). As also seen with the unmodified AID peptide, the mutant AID peptide abolished PPF of NA-induced inhibition of I_{Ba} (P2:P1: 1.37 ± 0.15 at −10 mV, $n = 7$, $P > 0.05$; Fig. 4*Ca* and *b*) and negated any PP-induced shift in voltage dependence of activation (Fig. 4*Cc*, Table 1). Mutant AID peptide also caused a significant reduction in Ca^{2+} current density (Fig. 4*D*, Table 2). Consistent with these actions, the mutant AID peptide caused a clear inhibition in cholinergic transmission in model SCGN synapses (18 ± 3% decrease in EPSP amplitude, $n = 6$, $P < 0.05$; Fig. 4*E*). Overall, a $Ca_V2.2$ I381L mutation within the AID peptide sequence did not abolish the inhibitory actions of the unmodified AID peptide on synaptic transmission or on Ca^{2+} channels in isolated SCGNs.

We also investigated the effects of a $Ca_V2.2$[45–55] peptide with two arginine residues replaced by alanine ('mutant NT peptide'). A full-length $Ca_V2.2$ R52A,R54A channel is insensitive both to G protein modulation (Cantí *et al.* 1999) and to dominant-negative suppression by the NT terminal (Page *et al.* 2010). By contrast to the unmodified NT peptide described above, in the presence of the mutant NT peptide, tail current data were best fitted by a single, major Boltzmann distribution (Fig. 5*A*). The mutant NT peptide had negligible effects on NA-induced inhibition, 10 μM NA caused a robust inhibition of I_{Ba} in isolated SCGNs (59.8 ± 5.5%, $n = 9$; Fig. 5*Ba* and *b*), comparable to inhibition in controls and significantly increased compared to the unmodified NT peptide; NA also induced a significant shift in $V_{0.5}$ of ∼+7 mV (Table 1). PPF of NA-induced inhibition was still prominent with the mutant NT peptide (P2:P1: 1.70 ± 0.17 at −10 mV, $n = 8$, $P < 0.05$; Fig. 5*Ca* and *b*). PPF also caused a partial reversal of the NA-induced shift in $V_{0.5}$ of ∼−4 mV (Fig. 5*Cc*, Table 1); however, this was outside the significance range ($P = 0.16$). Ca^{2+} current density was unaffected by the mutant NT peptide (Fig. 5*D*, Table 2). In model SCGN synapses, the mutant NT peptide had no effect on synaptic transmission (1.0 ± 3.5% decrease in EPSP amplitude, $n = 5$; Fig. 5*E*). These data implicate arginine residues within the $Ca_V2.2$[45–55] NT peptide as being essential molecular determinants for effects on Ca^{2+} channels and inhibition of synaptic transmission in these systems.

Effects of AID and NT peptide co-application

Finally, we investigated the effects of co-application of the NT peptide and the AID peptide to determine any additive, antagonistic or supplemental effects in these systems. In the joint presence of NT and AID peptides, tail current data were best fitted by a single, major Boltzmann distribution (Fig. 6*A*). Under joint NT/AID peptide application, 10 μM

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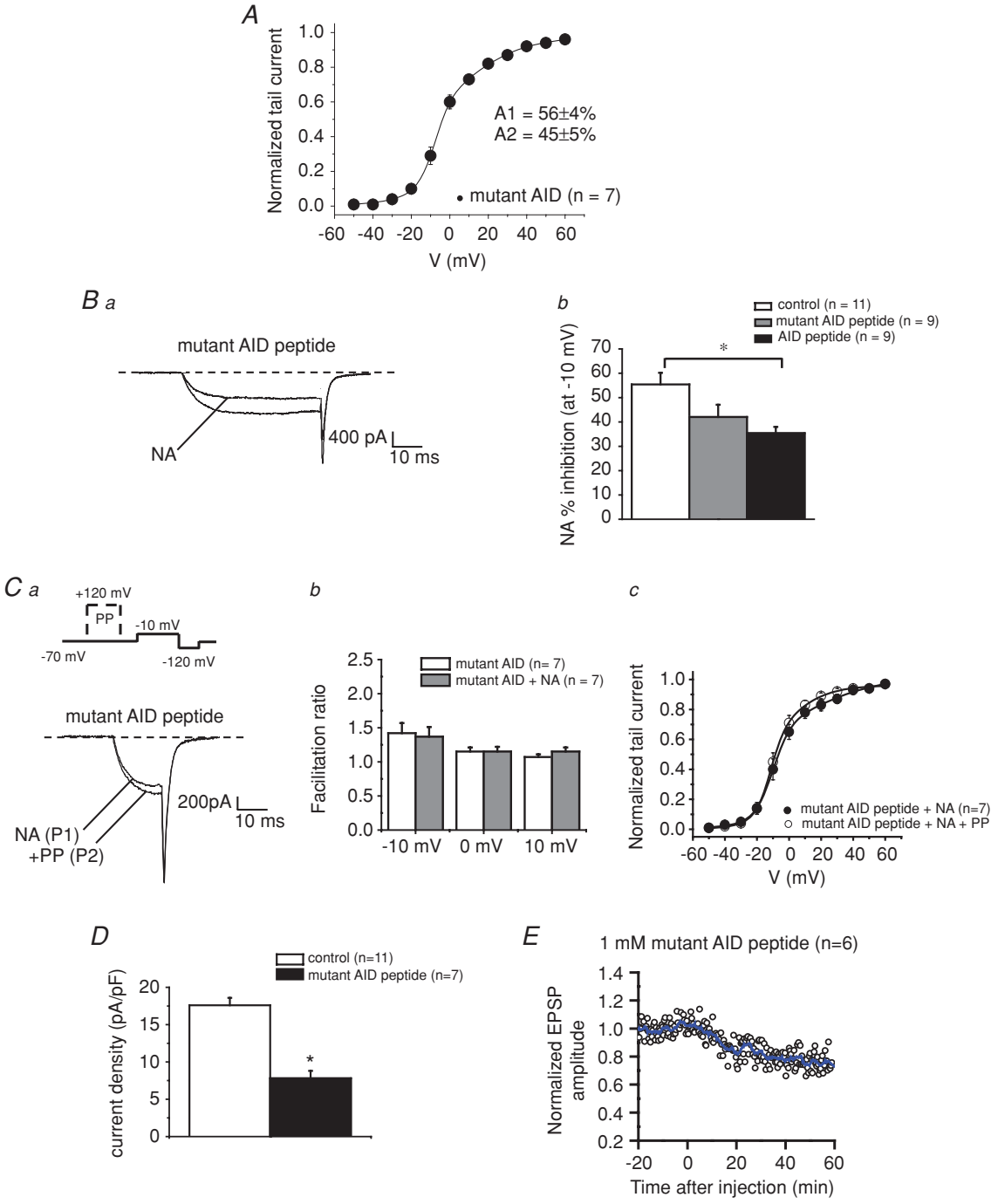

Figure 4. A mutant AID peptide retains inhibitory effects on G protein modulation and synaptic transmission

A, voltage dependence of activation curves from tail current amplitude data in the presence of the mutant AID peptide (containing a $Ca_V2.2$ I381L mutation) (1 mM). Data recorded in the presence of the mutant AID peptide were best fitted by two Boltzmann distributions (mean ± SEM values derived from individual cells are shown in Table 1). *Ba*, representative traces showing 10 μM NA-induced inhibition of I_{Ba} in the presence of mutant AID peptide (1 mM). *Bb*, bar graph summarizing percentage I_{Ba} inhibition by NA showing pooled data for controls (white bar) and in the presence of mutant AID peptide (grey bar) and unmodified AID peptide (black bar); $^*P < 0.01$ (Mann–Whitney *U* test). *Ca*, superimposed representative traces showing NA-induced inhibition of I_{Ba} (P1) and effects of a prepulse (P2) in the presence of mutant AID peptide (1 mM). *Cb*, bar graph summarizing facilitation ratio (P2:P1) for mutant AID peptide in the absence (white bars) and presence (grey bars) of NA (10 μM). *Cc*, voltage dependence of activation curves for mutant AID peptide (1 mM) in the presence of NA (10 μM, filled circles) and effect of conditioning prepulses in the continued presence of NA (open circles). In both

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NA caused a robust inhibition of I_{Ba} in isolated SCGNs (54.2 ± 7.0%, $n = 10$; Fig. 6*Ba* and *b*), comparable to inhibition in controls, and NA induced a significant shift in $V_{0.5}$ of ∼+14 mV (Table 1). PPF of NA-induced inhibition was still prominent with NT/AID peptides (P2:P1: 1.80 ± 0.30 at −10 mV, $n = 10$, $P < 0.05$; Fig. 6*Ca* and *b*); PPF also caused a reversal of the NA-induced shift in $V_{0.5}$ of ∼−9 mV (Fig. 6*Cc*, Table 1; $P < 0.05$). Ca^{2+} current density was unaffected by co-application of NT/AID peptides (Fig. 6*D*, Table 2). In model SCGN synapses, combined injection of NT/AID peptides had no effect on synaptic transmission (7.0 ± 5.7% decrease in EPSP amplitude, $n = 5$; Fig. 6*E*). These data favour a scenario whereby $Ca_V2.2$[45–55] and $Ca_V2.2$[377–393] peptides act in an antagonistic manner for effects on Ca^{2+} channels and inhibition of synaptic transmission in these systems.

Discussion

We demonstrate that synthetic Ca^{2+} channel peptides based on the $Ca_V2.2$ NT or AID region inhibit synaptic transmission and attenuate G protein modulation in model SCGN synapses. We provide evidence that the peptide mechanism of action in native SCGNs involves effects on Ca^{2+} current amplitude and channel gating, actions consistent with presynaptic inhibition of neurotransmitter release. Furthermore, co-application of NT and AID peptides negated effects of individual peptides, consistent with a scenario whereby the $Ca_V2.2$ NT[45–55] and AID[377–393] sequences may interact.

Synthetic $Ca_V2.2$ peptides inhibit synaptic transmission and attenuate G protein modulation via an inhibitory effect on Ca^{2+} channels

A major advance here is the demonstration that small 11mer $Ca_V2.2$ NT[45–55] and 17mer $Ca_V2.2$ AID[377–393] peptides act to functionally inhibit neurotransmission in SCGN synapses, an appropriate model for studying presynaptic modulation of fast cholinergic transmission (Mochida *et al.* 1994, 2003). Such effects are accompanied by an attenuation of G protein modulation. In agreement with our previous study describing a $G\beta\gamma$-mediated $\alpha 2$-adrenoceptor pathway in SCGNs (Stephens & Mochida, 2005), we report that NA caused a significant decrease in EPSP amplitude. The AID peptide fully blocked NA inhibition, whilst the NT peptide caused a significant reduction in NA effects. The latter data were fully consistent with the level of inhibition seen for individual peptides in the absence of NA and suggest a maximum EPSP amplitude inhibition of ∼40% for this pathway.

To investigate peptide mechanisms of action, we made somatic recordings from isolated SCGNs. At SCGN presynapses, transmitter release is supported predominantly by $Ca_V2.2$ channels (Mochida *et al.* 2003). Ca_V expression is associated with heterogeneous $Ca_V\beta$ and $G\beta\gamma$ subunit expression, both of which modulate Ca_V activity (De Waard *et al.* 2005; Tedford & Zamponi, 2006); thus, we tested our peptides in the same native SCG neuronal preparation as an appropriate model of heterogeneous Ca^{2+} channels/auxiliary subunits at the presynapse. NA inhibition of $Ca_V2.2$ channels occurred via voltage-dependent pathways (and not voltage-independent pathways, which are also prevalent in SCGNs. Hille, 1994; Suh *et al.* 2010), as confirmed by blockade of the prepulse relief of NA-induced inhibition by PTX. Peptides that inhibited synaptic transmission also caused a decrease in Ca^{2+} current amplitude (by contrast, peptides lacking effects on synaptic transmission were without effect on current amplitude). There was some discrepancy here, in that the AID peptide had a stronger inhibitory effect on EPSP amplitude than the NT peptide, whilst a similar reduction in somatic Ca^{2+} current was seen; this may reflect differences in peptide access to Ca^{2+} channels between whole-cell recordings at the soma and in SCGN model synapses, in which peptides are injected into the presynaptic partner and must diffuse into presynaptic terminals in order to act (Mochida *et al.* 1996). Here, we cannot fully rule out that NT and AID peptide are transported differentially to the presynapse. Overall, reductions in Ca^{2+} current amplitude favour a major mechanism whereby NT and AID peptides interact with presynaptic Ca^{2+} channels in SCGN synapses, such that fewer channels are activated in response to depolarizing action potentials and, consequently, less acetylcholine is released.

NT and AID peptides were found to have complex inhibitory effects on the voltage dependence of activation. Control activation was best described by a single Boltzmann component; by contrast, in the presence of NT or AID peptide, a double Boltzmann fit was required. Dual Boltzmann distributions have been correlated with a

cases, tail current activation curve data recorded in the presence of the mutant AID peptide were well fitted only by two Boltzmann distributions (mean ± SEM values derived from individual cells are shown in Table 1). *D*, bar graph summarizing inhibition of Ca^{2+} current density by mutant AID peptide (black bar) (mean ± SEM values derived from individual cells and conditions are shown in Table 2); $^*P < 0.01$ *vs.* control (Mann–Whitney *U* test). *E*, effect of mutant AID peptide (1 mM in the pipette) on EPSP amplitude in model SCGN synapses. Note that $Ca_V2.2$ I381L mutant peptide retained the inhibitory effects seen for unmodified AID peptide.

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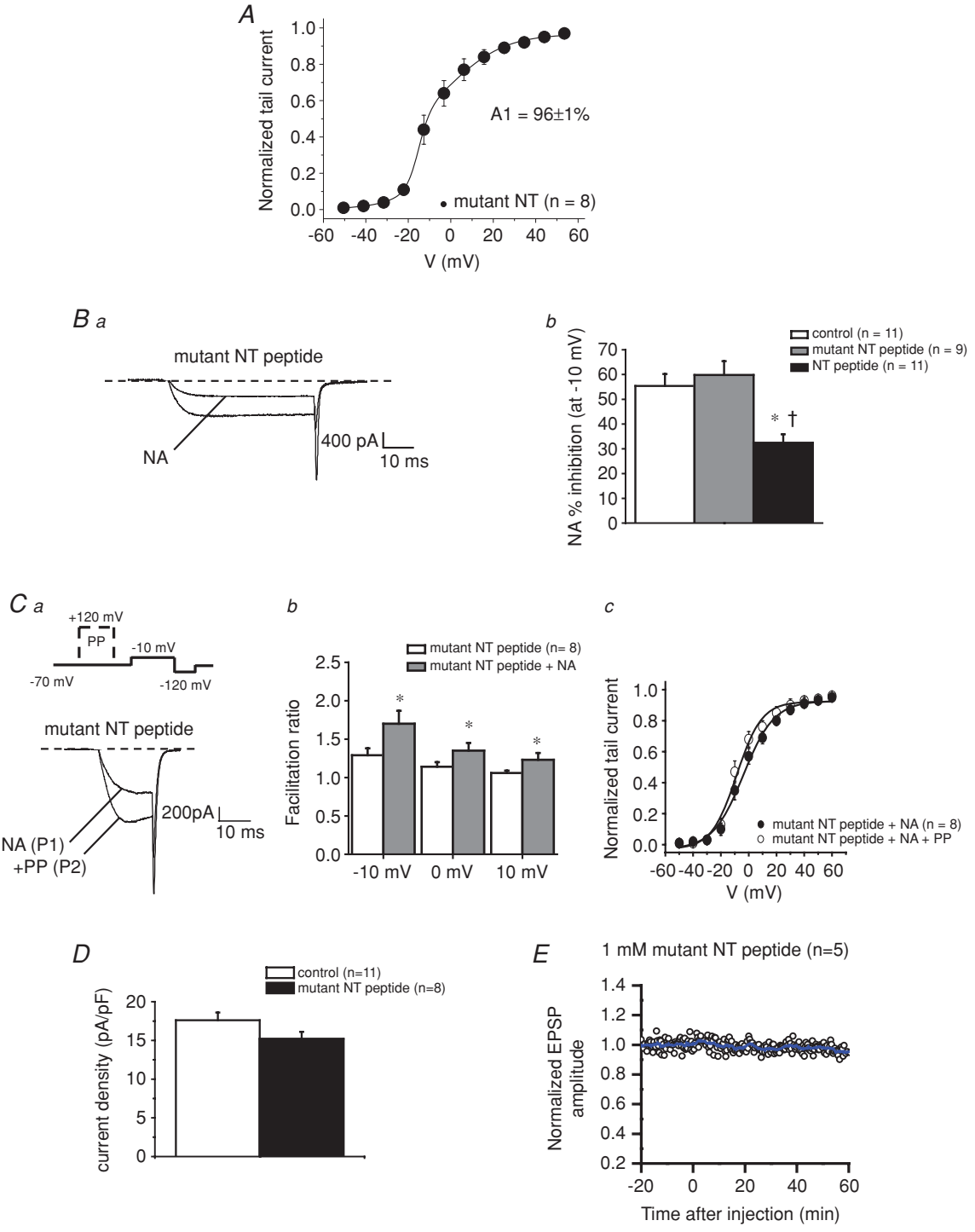

Figure 5. A mutant NT peptide lacks inhibitory effects on G protein modulation and synaptic transmission

A, voltage dependence of activation curves from tail current amplitude data in the presence of the mutant NT peptide (containing $Ca_V2.2$ R52A,R54A mutations) (1 mM). Mutant NT peptide tail current activation curve data were best described by a single Boltzmann distribution (mean ± SEM values derived from individual cells are shown in Table 1). *Ba*, representative traces showing 10 μM NA-induced inhibition of I_{Ba} in the presence of mutant NT peptide (1 mM). *Bb*, bar graph summarizing percentage I_{Ba} inhibition by NA showing pooled data for controls (white bar) and in the presence of mutant NT peptide (grey bar) and unmodified NT peptide (black bar); $^*P < 0.01$ *vs.* control, †$P < 0.01$ *vs.* mutant NT peptide (Mann–Whitney *U* tests). *Ca*, superimposed representative traces showing NA-induced inhibition of I_{Ba} (P1) and effects of a prepulse (P2) in the presence of mutant NT peptide

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lower, G$\beta\gamma$-unbound 'willing' and a higher, G$\beta\gamma$-bound 'reluctant' activation state (Bean, 1989; Ikeda, 1991). Moreover, Agler *et al.* (2003) have developed a compound gating model to describe G protein inhibition whereby, in the absence of G$\beta\gamma$, channels are willing to open following transition through five closed states (C1–C5); the binding of G$\beta\gamma$ specifies reluctant opening by promoting entry into five corresponding deep closed states (C1*–C5*). Here, NT and AID peptides may promote transitions into reluctant C1*–C5* states, as substantiated by the switch from a single, willing population to one also containing a second, reluctant component. If we apply dual Boltzmann fits to the control data, significantly more channels are described by the lower 'willing' component ($85 \pm 2\%$), and significantly fewer channels are described by the higher 'reluctant' component ($13 \pm 2\%$) than the corresponding components with either NT or AID peptide (each $P < 0.05$, Mann–Whitney U tests); such differences are consistent with more channels being 'reluctant' to open in the presence of these inhibitory peptides. Overall, our functional data demonstrate that the $Ca_V2.2$ peptide-induced changes to activation gating were accompanied by an reduction in Ca^{2+} current amplitude; such actions at presynaptic Ca^{2+} channels are consistent with inhibition of synaptic transmission. The demonstration that $Ca_V2.2$ peptides alone inhibit synaptic transmission is consistent with an action independent from previously reported effects for AID-based peptides on G protein modulation (Herlitze *et al.* 1997; Zamponi *et al.* 1997). Previous studies have shown that the co-expression of the NT[1–95] domain suppresses $Ca_V2.2$ Ca^{2+} current (Raghib *et al.* 2001; Page *et al.* 2004, 2010; Agler *et al.* 2005). Here, we extend these reports to show that a synthetic peptide minimally consisting of the $Ca_V2.2$[45–55] N-terminal can modulate Ca^{2+} channels and inhibit synaptic transmission.

Accompanying their effects on Ca^{2+} current amplitude and channel gating, NT and AID peptides also disrupted hallmark properties of GPCR-mediated inhibition in SCGNs (Stephens & Mochida, 2005). Thus, NT and AID peptides attenuated (i) NA-induced inhibition of EPSP amplitude in SCGN synapses, (ii) NA-induced inhibition of I_{Ba} amplitude, (iii) NA-induced shift in voltage dependence of activation, and (iv) prepulse relief of NA-induced inhibition in isolated SCGNs. Modulation of G protein inhibition could be explained by peptides binding and chelating free G$\beta\gamma$ (Zamponi *et al.* 1997), or by peptides interacting with the Ca^{2+} channel itself (Agler *et al.* 2005). Here, several observations argue against sequestration of G$\beta\gamma$ as an exclusive mechanism. Thus, in the presence of peptides: (i) synaptic transmission in SCGN synapses was inhibited, a simple chelation of G$\beta\gamma$ subunits would be expected to remove tonic inhibition; (ii) there were no clear changes in endogenous G protein modulation (including basal facilitation ratio); (iii) voltage dependence of activation was best described by a dual, and not a single, component; for the latter, G$\beta\gamma$ sequestration by peptides would be expected to favour dominance by a single, uninhibited channel population; (iv) there was no decrease in G protein reinhibition kinetics following a depolarizing prepulse (Supplemental Fig. 2).

Overall, our data favour a scenario in which inhibitory effects of the NT and the AID peptide are due to interaction with Ca^{2+} channels; thus, these peptides may bind the $Ca_V2.2$ subunit to cause inhibition. Experiments with co-application of NT and AID peptides showed an antagonistic effect on synaptic transmission, Ca^{2+} channel action and G protein modulation, such that the two peptides may bind each other to negate the effects of the parent peptide; such data strengthen the hypothesis of a physical interaction between $Ca_V2.2$ NT[45–55] and AID[377–393] sequences. We propose that peptide/$Ca_V2.2$ channel interactions (whether at a NT-I–II loop or alternative binding site) result in changes to gating and reduced current amplitude, and a consequent inhibition of synaptic transmission. Under these conditions, the peptide/Ca^{2+} channel complex is unresponsive to G protein modulation, as reported by other groups (Herlitze *et al.* 1997; Zamponi *et al.* 1997; Agler *et al.* 2005; Page *et al.* 2010). Agler *et al.* (2005) have demonstrated binding between the NT[1–95] domain and the first third of the I–II loop ($Ca_V2.2$[357–393]) in yeast two-hybrid assays and proposed that this promotes 'constitutively reluctant' Ca^{2+} channel gating. By contrast, no interaction between NT[1–95] and the I–II loop were

(1 mM). *Cb*, bar graph summarizing facilitation ratio (P2:P1) for mutant NT peptide in the absence (white bars) and presence (grey bars) of NA (10 μM); *$P < 0.01$ (paired Student's t test). *Cc*, voltage dependence of activation curves for mutant NT peptide (1 mM) in the presence of NA (10 μM, filled circles) and effect of conditioning prepulses in the continued presence of NA (open circles). In both cases, tail current activation curve data recorded in the presence of the mutant NT peptide were well fitted by a single Boltzmann distribution (mean ± SEM values derived from individual cells are shown in Table 1). *D*, bar graph summarizing effect of mutant NT peptide (black bar) on Ca^{2+} current density (mean ± SEM values derived from individual cells and conditions are shown in Table 2). *E*, effect of mutant NT peptide (1 mM in the pipette) on EPSP amplitude in model SCGN synapses. Note that $Ca_V2.2$ R52A,R54A mutant peptide had no inhibitory effects on G protein modulation or synaptic transmission, by contrast to effects seen with unmodified NT peptide.

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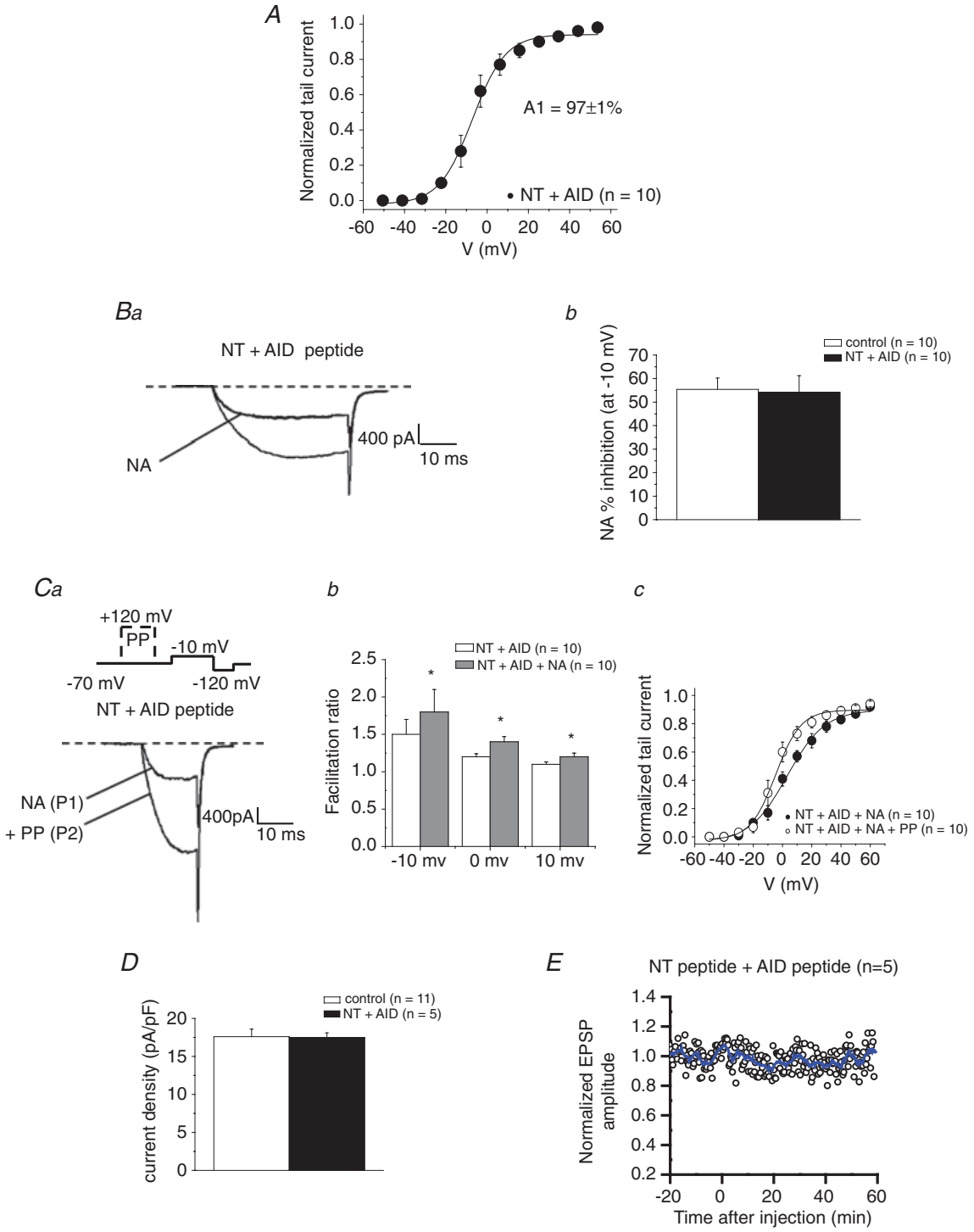

Figure 6. Co-application of NT and AID peptides negates inhibitory effects on G protein modulation and synaptic transmission

A, voltage dependence of activation curves from tail current amplitude data for combined application of the NT and the AID peptide (both 1 mM). NT/AID peptide tail current activation curve data were best described by a single Boltzmann distribution (mean ± SEM values derived from individual cells are shown in Table 1). *Ba*, representative traces showing 10 μM NA-induced inhibition of I_{Ba} in the presence of NT/AID peptide (both 1 mM). *Bb*, bar graph summarizing percentage I_{Ba} inhibition by NA showing pooled data for controls (white bar) and in the presence of NT/AID peptide (grey bar). *Ca*, superimposed representative traces showing NA-induced inhibition of I_{Ba} (P1) and effects of a prepulse (P2) in the presence of NT/AID peptide (1 mM). *Cb*, bar graph summarizing facilitation

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found in similar assays (Page *et al.* 2010), who instead proposed that the NT may interact with a distant binding pocket to cause functional effects. Additionally, Page *et al.* (2004) have proposed that expression of the NT causes activation of the endoplasmic reticulum resident RNA-dependent kinase, PERK, to suppress Ca^{2+} channel translation; however, such a mechanism is likely to be more longer term than the acute effects reported in the present study. Importantly, previously studies using $Ca_V2.2$ peptides have been performed exclusively in recombinant cells; here, we extend studies to native neurones.

Mutations to synthetic $Ca_V2.2$ peptides reveal an important role for the NT in Ca^{2+} channel regulation

As discussed above, we propose that the $Ca_V2.2$ peptides used here interact with the Ca^{2+} channel to affect function. Therefore, mutations to residues shown to be determinants of Ca^{2+} channel function may affect actions of NT or AID peptides. In this regard, NT residues arginine 52 and arginine 54 are important molecular determinants for G protein regulation (Page *et al.* 1998; Stephens *et al.* 1998*b*; Cantí *et al.* 1999) and for dominant-negative suppression of Ca_V2 channels (Page *et al.* 2010). Most significantly here, when the NT peptide was modified to contain equivalent $Ca_V2.2$ R52A,R54A mutations, the resultant peptide was unable to inhibit synaptic transmission and lacked the inhibitory effects on Ca^{2+} channels seen with the unmodified NT peptide. With regard to G protein modulation, Agler *et al.* (2005) have proposed that the inhibitory interaction between NT and AID is dependent on the presence of $G\beta\gamma$; our data lend support to this concept, as mutations that prevent G protein modulation also ablate the inhibitory effects of the NT peptide on synaptic transmission and G protein modulation.

By contrast, our data do not provide support a role for isoleucine 381 as a crucial determinant for the Ca^{2+} channel functions investigated here. However, it is important to point out that isolated peptides lack the tertiary structure of the channel in the native environment and that mutation of isoleucine to leucine within the proposed QXXER binding motif has been shown to affect G protein modulation in the full-length channel (Herlitze *et al.* 1997). Moreover, we cannot exclude the involvement of alternative residues within the AID region in Ca^{2+} channel functions such as G protein modulation (De Waard *et al.* 1997; Zamponi *et al.* 1997, Tedford *et al.* 2010).

It also remains to be fully determined how peptide effects on other elements may also contribute. For example, it is possible that disrupting $Ca_V2(\alpha1)/Ca_V\beta$ interactions, crucial to Ca^{2+} channel function (Dolphin, 2003), may also contribute to effects reported here. $Ca_V\beta$ binds the AID region (Richards *et al.* 2004) and NT[44–55] contributes to $Ca_V\beta$ modulation of inactivation (Stephens *et al.* 2000). The $Ca_V\beta$ subunit has been proposed to be indispensable for $G\beta\gamma$ inhibition (Meir *et al.* 2000; Herlitze *et al.* 2001; Hümmer *et al.* 2003). $Ca_V\beta$ has been shown to modulate the IS6-AID linker region responsible for $\alpha1$ subunit activation gating (Opatowsky *et al.* 2004; Van Petegem *et al.* 2004) and disruption of this region prevents $G\beta\gamma$ inhibition (Zhang *et al.* 2008). Interestingly, a $Ca_V2.2$[353–371] peptide, encompassing the IS6-AID linker, was shown to block G protein modulation (Zamponi *et al.* 1997). Overall, our data add to accumulating evidence suggesting that modulation of Ca^{2+} channels is dependent on interaction between multiple intracellular elements of presynaptic Ca_V2 channels; our data support a dominant role for the NT and, in particular R52 and R54, as important determinants of Ca^{2+} channel function.

Potential therapeutic significance

$Ca_V2.2$ channels are key determinants of hyperexcitability disorders associated with increased or ectopic neuronal firing, for example, in neuropathic pain (Chaplan *et al.* 1994; Matthews & Dickenson, 2001; Yang & Stephens, 2009). $Ca_V2.2$ channels are therapeutically targeted by GPCR ligands such as opioids, but remain a significant focus for novel drug development (Altier & Zamponi, 2004). The search for improved analgesics generated the $Ca_V2.2$ blocker ziconotide; although providing a useful proof-of-concept, this compound has a narrow

ratio (P2:P1) for NT/AID peptide in the absence (white bars) and presence (grey bars) of NA (10 μM); *$P < 0.01$ (paired Student's *t* test). *Cc*, voltage dependence of activation curves for NT/AID peptide (1 mM) in the presence of NA (10 μM, filled circles) and effect of conditioning prepulses in the continued presence of NA (open circles). In both cases, tail current activation curve data recorded in the presence of NT/AID peptide were well fitted by a single Boltzmann distribution (mean ± SEM values derived from individual cells are shown in Table 1). *D*, bar graph summarizing effect of NT/AID peptide (black bar) on Ca^{2+} current density (mean ± SEM values derived from individual cells and conditions are shown in Table 2). *E*, effect of NT/AID peptide (1 mM in the pipette) on EPSP amplitude in model SCGN synapses. Note that co-application of NT/AID peptide had no inhibitory effects on G protein modulation or synaptic transmission, by contrast to effects seen with unmodified parent peptides.

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therapeutic window (Williams *et al.* 2008). We show that small, synthetic $Ca_V2.2$ peptides can inhibit transmitter release; knowledge of mechanisms of presynaptic inhibition may serve as a starting point to develop new therapeutic agents.

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Author contributions

Experiments were performed at the University of Reading (G.B., G.J.S.) and Tokyo Medical University (S.M., G.J.S.). All authors contributed to the conception and design of the experiments, the collection, analysis and interpretation of data, and drafting the article or revising it critically for important intellectual content. All authors have approved the final version of the manuscript.

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