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Target-specific control of nicotinic receptor expression at developing interneuronal synapses in chick

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Abstract

Neuronal differentiation and development of synaptic specializations are strongly influenced by cellular interactions. We compared the effects of interaction with distinct autonomic targets on the molecular and biophysical differentiation of ‘upstream’ neuron–neuron synapses. Contact with cardiac tissue induced expression of nicotinic receptor channels (nAChRs) distinct from those induced by renal tissue in presynaptic autonomic neurons. The kinetics of cholinergic currents at interneuronal synapses are dictated by the peripheral target contacted. Analysis of the nAChR channel subtypes and subunits in individual neurons demonstrated that the profile of transmitter receptors expressed at mature neuron–neuron synapses develops from the convergent influences of input-derived (anterograde) and target-specific (retrograde) signals.

The formation, maintenance and remodeling of synapses involves regulated expression and targeting of specific transmitter receptors to sites of cellular contact. Presynaptic input and input-derived signals are potentially important regulators of receptor expression at neuron–neuron synapses. In particular, expression of specific subtypes of neuronal ionotropic glutamate and acetylcholine receptors are controlled by synaptic activity and/or neuregulin-type growth factors^{1,2}.

When chick sympathetic neurons establish contact with their pre- and postsynaptic partners during embryonic development, the magnitude of inward current responses to acetylcholine (ACh) increases, the number, localization and biophysical profile of nAChR channels are altered, and mRNA levels for the predominant nAChR subunits ($\alpha 3$, $\alpha 5$, $\alpha 7$, $\beta 4$) are upregulated^{3,4}. Despite the general enhancement of nicotinic responses evident with ganglionic development *in vivo*, sympathetic neurons are heterogeneous in the levels and subtypes of nAChRs expressed^{3,4}. Differences in receptor expression may be due to distinct, target-derived influences^{5,6}, as neurons within individual ganglia innervate a variety of peripheral targets. *In-vivo* studies reveal aberrant receptor expression by autonomic neurons deprived of target contact^{7,8}. Innervation of sympathetic neurons *in vitro* in the absence of target tissues reproduces only a subset of the changes that are observed with development *in vivo*^{9–11}. The current study demonstrates retrograde, target-specific regulation of the levels and functional profile of receptors expressed at antecedent interneuronal synapses.

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RESULTS

To test whether target contact alters the profile of nAChRs expressed by innervated autonomic neurons, we examined the nicotinic responses of embryonic chick sympathetic neurons innervated *in vitro* by explants of the pre-ganglionic, visceral motor nucleus (VMN) and cocultured with cardiac or renal target tissue. We first tested whether nicotinic transmission at VMN–sympathetic neuron synapses was altered by concurrent innervation of peripheral targets. Examination of spontaneous postsynaptic currents (sEPSCs) recorded in sympathetic neurons revealed differences in nAChR-mediated transmission depending on the presence or absence of target and on the particular target tissue contacted. In neurons contacting kidney, sEPSCs were somewhat larger than those recorded in innervated neurons lacking target and were considerably larger and faster than those detected at synapses formed in the presence of cardiac explants (Fig. 1).

We extended our analysis of the effects of target contact on nicotinic receptors and nAChR-mediated transmission at VMN–sympathetic neuron synapses. First, focusing on whether specific targets might differentially control expression of transmitter receptors by innervated sympathetic neurons, we assayed both ACh-evoked macroscopic currents and nAChR-mediated synaptic currents (Fig. 2). As shown previously, innervation of sympathetic neurons without target contact increased the number and altered the profile of expressed nAChR subtypes (refs. 2, 10 and D.S.M., P.D., L.W.R. & A.B. Brussard, unpublished data). Comparison of nAChR-mediated currents in innervated neurons contacting kidney, heart or no target revealed target-specific changes in nicotinic currents ($n = 29$, $n = 31$, $n = 33$, respectively; Fig. 2).

Macroscopic currents elicited by maximal concentrations of ACh give a gross measure of nAChR expression. The ACh-evoked currents of innervated sympathetic neurons contacting kidney were approximately threefold larger than those of innervated neurons contacting heart. In innervated neurons maintained without target, macroscopic currents were intermediate between those of innervated neurons contacting kidney and those contacting heart (Fig. 2a).

To refine our analysis, we examined both synaptic physiology and nAChR-subunit gene expression in individual innervated neurons contacting either heart or kidney (Figs. 2b and c and 3). By assaying nAChR physiology and subunit gene expression in the same neuron, these experiments tested for target-specific regulation of both synaptic nAChR expression and associated nicotinic currents. The properties of synaptic nAChRs were assayed by analysis of the sEPSCs, as direct recording of single-channel currents at synaptic sites is not possible.

Renal or cardiac tissue had opposite effects on amplitude distribution of interneuronal sEPSCs at synapses onto neurons contacting these target tissues (Fig. 2b). An sEPSC amplitude index was calculated for each synaptic pair from cumulative amplitude histograms of ~100 events (see Methods). The distribution of sEPSC amplitude indices of all innervated, kidney-contacting neurons were fit by summed Gaussian distributions with 52% of indices in the first mode, 34% in the second (centered at 15.9 ± 1.6 and 35.8 ± 1 , respectively) and 14% of indices ≥ 50 . In contrast, the amplitude indices for innervated neurons contacting heart were described by a single log-normal distribution centered at 11.63 ± 0.83 . Thus, sEPSCs in innervated neurons contacting cardiac tissue were significantly smaller than those recorded in innervated neurons contacting kidney. Amplitude indices of synaptic currents recorded in targetless, innervated neurons were intermediate in the overall distribution, with 87% falling within the first peak of a bimodal, Gaussian distribution. Thus, the amplitudes of spontaneous synaptic currents recorded at VMN–sympathetic neuron synapses differed depending on the specific target tissue contacted by the sympathetic neuron.

Synaptic current decay kinetics (τ_{syn} , a measure of synaptic nAChR opening duration) were also differentially regulated by contact with heart or with kidney tissue (Fig. 2c). In >80% of sympathetic neurons contacting kidney or lacking target contact, sEPSC analysis indicated fast open-time kinetics for nAChRs at these neuron–neuron synapses. In contrast, sEPSCs recorded in most neurons contacting heart were best fit by a combination of both fast and slow components, consistent with a mix of brief- and long-duration synaptic nAChR channels.

We tested whether differences in sEPSC amplitude and kinetics are due to differences in the profile of synaptic nAChR complexes and, as such, might be reflected in altered patterns of nAChR-subunit gene expression (Fig. 3). We measured levels of nAChR subunit mRNAs in each neuron by a quantitative PCR (qPCR) assay in which products were generated within the linear range of amplification. Confirming previous studies *in vivo*⁵ in cultured embryonic day 9 (E9) sympathetic neurons, $\alpha 3$, $\alpha 5$, $\alpha 7$ and $\beta 4$ subunits predominated.

Quantitation of nAChR subunit mRNAs in individual innervated neurons following electrophysiology revealed correlated changes in gene expression and biophysical properties of the synaptic nAChRs. Levels of $\alpha 3$ and $\alpha 7$ in neurons with uniformly fast synaptic currents (that is, innervated neurons without target or with kidney contact) were significantly higher than in neurons with both fast and slow currents (that is, innervated neurons contacting heart). Furthermore, innervated neurons contacting heart that had slow synaptic nAChRs expressed significantly higher levels of $\beta 4$ (Fig. 3a).

Previous studies demonstrated that autonomic neurons express a variety of nAChR complexes with distinct physiological profiles and subunit compositions^{6,7,9,12-19}. In view of the contribution of the $\alpha 3$ subunit to these heteromeric receptors, we examined whether levels of $\alpha 3$ relative to those of $\alpha 5$, $\alpha 7$ and/or $\beta 4$ might correlate with the differences in physiological profiles of synaptic nAChRs between innervated neurons contacting kidney and those with cardiac targets. Analysis of the ratio of $\alpha 3$ mRNA relative to $\alpha 5$, $\alpha 7$ and $\beta 4$ mRNAs revealed statistically significant, target-specific regulation of nAChR gene expression. Changes in nAChR subunit mRNAs were co-regulated with changes in the synaptic nAChR current kinetics induced by different target tissues (renal versus cardiac; Fig. 3a). The co-ordinate decline in the relative levels of $\alpha 3$ versus $\alpha 5$, $\alpha 7$ or $\beta 4$ subunits and the increase in the slow sEPSC contribution in sympathetic neurons innervating heart suggest target-specific induction of long-open-duration synaptic nAChRs that differ in subunit composition from nAChRs at interneuronal synapses on cells contacting kidney or without target.

Differences between innervated neurons contacting kidney and those contacting heart in the amplitude of nAChR-mediated synaptic current were also reflected in distinct expression profiles of nAChR subunit genes (Fig. 3b). Measurement of absolute levels of $\alpha 3$ and ratios of $\alpha 3$ to $\alpha 5$ and to $\beta 4$ revealed positive correlations with sEPSC amplitude in innervated neurons contacting kidney. In contrast, in innervated neurons contacting heart, relative expression level of $\alpha 3$ either to $\alpha 5$ or to $\alpha 5$ plus $\beta 4$ was constant, regardless of sEPSC amplitude. For innervated neurons without target, the curve of $\alpha 3/\alpha 5$ ratio versus sEPSC amplitude index was intermediate between that of innervated neurons contacting kidney and that of neurons contacting heart. This finding suggests that, in addition to the regulatory influence of innervation alone, each target tissue differentially regulates the profile of synaptic nAChRs^{2,7,9}.

The above analysis focuses on the distinct effects of peripheral targets on both the profile of synaptic nAChR channels and the levels of nAChR subunit gene expression by upstream, innervated neurons. The regulation of nAChR expression by each target is sufficiently distinct that comparison of subunit gene expression, independent of physiological profile, also reveals significant differences in nAChR expression (Fig. 3c). Thus, the ratio of $\alpha 3$ expression to that

of $\beta 4$ and $\alpha 5$ is approximately threefold higher in innervated neurons contacting renal tissue compared to those contacting heart. Higher levels of $\alpha 3$ than $\alpha 5$ may determine the nAChR profile expressed by sympathetic neurons following synaptogenesis (Fig. 3c).

To distinguish from effects of target on receptor expression, we examined target-induced changes in neuronal nAChRs independent of presynaptic input. In these experiments, sympathetic neurons were maintained *in vitro* in the presence of renal or cardiac tissue targets but without presynaptic input. This allowed direct assay of the biophysical properties of nAChR channels in neurons with or without target (Fig. 4a and b). Kidney contact increased the amplitude of ACh-evoked macroscopic currents and induced the expression of high-conductance, brief-open-duration nAChR channels that were not detected in targetless sympathetic neurons (Fig. 4a and b). Two conductance classes (~ 35 pS and ~ 70 pS) dominated the single-channel amplitude histograms of non-innervated neurons contacting kidney. Consistent with expression of a large number of low-affinity nAChRs, channel activity showed little decline during patch recordings. Likewise, increasing ACh concentration from $500 \mu\text{M}$ to 2.5 mM —normally a supramaximal concentration—elicited further increases in macroscopic current amplitude in neurons contacting kidney (Fig. 4a and b and data not shown).

The profile of nAChR single-channel currents recorded in neurons contacting cardiac tissue explants contrasted markedly with those obtained in targetless neurons or those with renal targets. The vast majority of nAChR opening events in patches from neurons innervating heart were of considerably longer duration than those detected in neurons contacting kidney and had low conductance (~ 15 pS; Fig. 4b).

Significant increases in the levels of expression of $\alpha 3$, $\alpha 5$ and $\beta 4$ were revealed by qPCR analysis of nAChR-subunit mRNAs in all neurons contacting renal explants. Contact with cardiac target upregulated the expression of $\alpha 5$, $\alpha 7$ and $\beta 4$ without affecting $\alpha 3$ expression (Fig. 4c).

Preliminary studies suggest that distinct mechanisms underlie differential effects of target on nicotine receptor expression. Application of a cardiac membrane fraction to input- and target-naïve sympathetic neurons mimicked the effects of heart coculture on nAChR expression, whereas soluble cardiac-derived factor(s) were without effect (for example, I_{ACh} + heart membrane, 475 ± 62 pA, $n = 18$ versus neurons alone, 744 ± 66 pA, $n = 29$). In contrast, neither soluble nor membrane-bound renal factors mimicked kidney contact (not shown). Analysis of potential mechanisms underlying the regulation of nAChR expression by kidney suggests that synaptic interaction between sympathetic neurons and renal target is required. Concurrent addition of α - and β -adrenergic receptor antagonists to sympathetic neuron–kidney cocultures eliminated inductive effects of kidney on nAChR subunit profile (for example, $\alpha 3$ mRNA in SNs + kidney, $240 \pm 30\%$ of control; in SNs + kidney + adrenergic blockers, $105 \pm 10\%$ of control, $n = 3$).

DISCUSSION

The principal conclusion of this study is that nAChR subunit expression is differentially regulated by target-derived signals. We further demonstrated that this target-specific regulation of nAChR channels occurs at both synaptic and non-synaptic sites. Changes in nAChR subunit expression in innervated and non-innervated neurons suggest that target-dependent regulation of neuronal nAChRs involves alterations in the subunit composition of both synaptic and non-synaptic nAChR complexes.

Neuronal nAChR channels expressed by sympathetic ganglion neurons can be distinguished on the basis of biophysics and/or pharmacology. Although post-translational mechanisms may

contribute to functional diversity of native nAChRs, various combinations of recombinant subunits yield nAChR channels with distinct conductance and kinetic properties^{12,13}. Thus alterations in levels of expression and/or assembly of specific nAChR subunits may underlie observed changes in synaptic and non-synaptic nAChR physiology.

The $\alpha 3$ subunit seems to be a key component of the various nAChR subtypes expressed by autonomic neurons¹⁴⁻¹⁷. Studies in heterologous systems indicate that homomeric and heteromeric complexes of $\alpha 7$ subunits can be formed^{18,19}. In addition, inclusion of $\alpha 5$ in hetero-oligomeric nAChRs comprising other α and β subunits yields receptors with lower agonist affinity and larger conductance²⁰⁻²⁴. In view of the diversity of potential nAChR subunit combinations, what might the current findings reveal about the composition of the physiologically distinct nAChRs induced by contact with distinct target tissues? Both targets induced significant changes relative to controls in the levels and profile of nAChRs expressed in synaptically naive sympathetic neurons. Induction of a novel class of high-conductance, brief-opening nAChRs, expression of receptors with low apparent affinity and significant upregulation of $\alpha 3$, $\alpha 5$ and $\beta 4$ are consistent with a substantial increase in the number of $\alpha 5$ -containing nAChR complexes in neurons contacting kidney. The expression of all but the smallest conductance class of nAChRs recorded in controls was suppressed in neurons contacting heart, despite increased levels of expression of $\alpha 5$, $\alpha 7$ and $\beta 4$.

The key feature of the profile of subunit gene expression in neurons contacting heart may be the lack of induction of $\alpha 3$, rather than the increased level of expression of $\alpha 5$, $\alpha 7$ and $\beta 4$. Thus, if $\alpha 3$ is a required component of nAChR complexes, and if its incorporation into more than one subtype of channel requires its expression in relative excess compared with other subunits, then only a single class of complexes might be formed. We propose that $\alpha 3$ co-assembles preferentially with $\beta 4$ relative to $\alpha 5$. Thus, in sympathetic neurons contacting cardiac tissue, the predominant complex formed is a small-conductance channel comprising $\alpha 3$ and $\beta 4$. Likewise, we propose that, by increasing relative $\alpha 3$ expression, contact with kidney expands the nAChR subtypes expressed to include $\alpha 5$ -containing complexes in addition to the more-favored $\alpha 3/\beta 4$ complexes. Thus, the novel ~70 pS channels detected in neurons contacting renal tissue may represent $\alpha 5$ -containing nAChRs. In sum, we propose that a surplus of $\alpha 3$ supports assembly of less-favored nAChR complexes, even when the levels of $\alpha 5$, $\alpha 7$ and/or $\beta 4$ are also increased by target contact.

Synaptic currents recorded from innervated neurons contacting kidney differed from those recorded in innervated neurons contacting heart. Likewise, the profile of subunit expression significantly differed between these two conditions. In particular, both absolute levels of $\alpha 3$ and the ratios of $\alpha 3$ to $\alpha 5$, $\alpha 7$ and $\beta 4$ were significantly higher in innervated neurons contacting kidney than in those contacting heart. Although specific subunit compositions of nAChRs expressed by innervated neurons are unknown, comparison of nAChR subunit expression with the physiological properties of the synaptic nAChRs clearly indicates co-ordinate and differential regulation of both parameters by target contact.

In the context of previous work, the current findings support models of target-induced changes in neuronal nicotinic receptors. First, previous single-channel and single-subunit deletion studies indicate that the 15 pS channel, which dominates the nAChR profile in non-innervated sympathetic neurons contacting heart, includes only $\alpha 3$ and $\beta 4$ subunits^{15,20,22,23}. Second, previous single-channel, subunit-deletion and heterologous expression studies indicate that the inclusion of $\alpha 5$ in nAChR complexes yields receptors with large conductance, brief open-duration and low agonist sensitivity^{18,21-23}. The increased number of large-amplitude sEPSCs recorded in sympathetic neurons contacting kidney is also consistent with enhanced inclusion of $\alpha 5$ in synaptic nAChRs. Furthermore, studies of recombinant nAChR channels in heterologous expression systems suggest that assembly of functional nAChRs from simple α /

β pairs is more efficient than formation of α/β complexes that include $\alpha 5$ ^{18,21-23}. Finally, the most striking evidence for target-dependent specification of nAChR channels at ganglionic synapses is the distinct kinetics of sEPSCs recorded in innervated neurons contacting heart versus those of sEPSCs in neurons contacting kidney. Slow components of synaptic currents recorded in innervated neurons contacting heart reflect the prolonged open-time kinetics of the underlying channels. Such channels with long open duration are detected neither in non-innervated neurons or in innervated neurons maintained in the absence of target (Fig. 2)^{6,11}. In contrast, nAChR channels with $\tau_o \geq 15$ ms are expressed by acutely dispersed neurons taken from animals at developmental stages at which both presynaptic input and target contact are established *in vivo*^{9,18,20}.

In conjunction with previous studies, these results demonstrate that both target and input control the expression of nicotinic receptors at interneuronal synapses—the former by retrograde, as yet unidentified signals^{5,7} and the latter via anterograde factors that likely include neuregulins^{2,7,10}. This study demonstrates that target tissues differentially control neurotransmitter receptor profiles at ‘upstream’ neuron–neuron synapses. Retrograde influence of peripheral targets on the strength of synaptic inputs is a striking example of how neuronal circuits and synaptic efficacy can be tailored to the specific functions they serve.

METHODS

Primary cultures

Sympathetic neurons from E9 chick embryos were cultured as described¹⁰. Presynaptic input was provided by addition of microexplants of the dorsal portion of chick E8 spinal cord¹¹.

Cardiac tissue was dissected from E11 chick embryos, teased into ~0.5-mm pieces and added to neuronal cultures after two days *in vitro*. Maintenance of a differentiated phenotype was assessed by immunohistochemistry using an anti-desmin antibody (Boehringer Mannheim). Synaptic interactions between individual sympathetic neurons and cardiocytes were confirmed by increased ‘beating’ rate with depolarization of the sympathetic input.

Renal tissue was dissected from E14 chick embryos, gently dispersed by two strokes in a homogenizer with a loose pestle and added to the dispersed neuronal cultures two days after plating. Integrity of the renal explants was confirmed by immunohistochemical staining with antibodies to keratin (keratin AE3; Boehringer Mannheim) and epithelial adhesion molecules prominently expressed in kidney (L-CAM, Sigma). Chick sympathetic neurons maintained *in vitro* retain their adrenergic phenotype and do not form functional synapses with one another whether alone or co-cultured with presynaptic input (ref. 10 and unpublished data) and/or with either cardiac or renal tissue explants (see below and data not shown).

PCR assays

Levels of nAChR subunit mRNAs were quantified relative to β -actin mRNA by PCR assay of single cell or multiple cell extracts. Assays were carried out in the linear range of amplification (Fig. 4). The cytoplasm of ~30 neurons (the target contact studies) or individual neurons was removed by applying negative pressure to a patch electrode. RNA was collected from the samples by breaking the pipet tip in an Eppendorf tube containing reaction buffer. mRNA was reverse-transcribed directly in 30 μ l final volume for collected neurons by 300 U SuperScript II (GibcoBRL) in the presence of 40 U RNasin (Promega), 20 μ M random hexamers (GibcoBRL) and 0.5 μ M of each dNTP for 1 h at 37°C. For assay of individual neurons, reverse transcription was scaled down to 5 μ l final volume. An aliquot of cDNA was amplified for 2 $\times$ 35 cycles in 50 μ l with 0.5 U *taq* Polymerase (Boehringer Mannheim), in the presence of 1 μ M of sense and antisense primers, 200 μ M of each dNTP, including [α -³²P] dATP and 1.5

mM MgCl₂ (94°C, 30 s; 45°C, 30 s; 72°C, 45 s) carrying over 10 µl of the amplification reaction. cDNA from single cells was amplified in a third round of 20 cycles. PCR products were separated from the unincorporated radioactivity by electrophoresis. DNA bands were excised, and incorporated radioactivity was quantified in a scintillation counter. Relative amplification efficiencies of each subunit cDNA were determined in control reactions, and mRNA measurements were corrected accordingly. Efficiency of amplification depends on the primer-template interaction, that is, $n = N_0 E^n$, where N is the amount of product, N_0 is the amount of template, E is the efficiency and n is the number of cycles. The amplification efficiencies for the nAChR subunit cDNAs relative to $\alpha 3$ were 2.63 for $\alpha 5$, 1.62 for $\alpha 7$ and 1 for $\beta 4$.

Primers, their sequence position and sequence references in GenBank were as follows: actin (L08165) sense (860)ATCTTTCTTGGGTATGGA, antisense (1135) ACATCTGCTGGAAGGTGG; $\alpha 3$ (ref. 25) sense (897) CAGAACACCCAAGACACA, antisense (1390)TGAAAATGAAAACAGAGG; $\alpha 5$ (J05642) sense (480) AACCTTCTTTCCCTTTGACC, antisense (1083) TTCCTTTTTCCTCCTTCTGA; $\alpha 7$ (X52295) sense (974)GGGGAAAAATGCCTAAAT, antisense (1478) GACAGCCTCTACAAAGTT; $\beta 4$ (J05643) sense (745)TATCCGTGCTGCTTGCTTTG, anti-sense (1256)CACTCTGGTCGTTGTCATCA.

Electrophysiology

Whole-cell recording and single-channel recording used the patch-clamp technique²⁶. For whole-cell recording, the internal solution included 150 mM KCl, 2 mM MgCl₂, 10 mM HEPES, and 1 mM EGTA at pH 7.2, supplemented with 5 mM Mg-ATP and 0.3 mM GTP. External solutions were composed of 140 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM HEPES at pH 7.2. ACh at 500 µM or 2.5 mM was dissolved in external solution and was applied by focal pressure ejection within 10 µm of the soma. The holding potential in whole-cell voltage-clamp recordings was set to -60 mV. Single-channel recordings used the cell-attached configuration with pipets containing external solution with 2.5 µM ACh. All recordings were carried out in the presence of 2 µM TTX. Synaptic currents were blocked by 50 µM mecamylamine, consistent with nAChR-mediated synaptic transmission (data not shown). Sampling of individual sEPSCs was carried out using analysis software written in Axobasic by A. Kyrozis and modified by R. Girod. Analysis was limited to events with rise times of < 3 ms. The decay time constants for sEPSCs were determined using the Simplex algorithm. Cumulative amplitude distributions for each cell were analyzed by plotting the amplitude of each event versus the fraction of total sEPSCs larger than that event. Area beneath the curve was integrated to provide an assessment of the sEPSC amplitude for each neuron (amplitude index) and to permit comparison among cells.

Statistical analysis

Amplitude index and synaptic current distributions were analyzed with Micrococal Origin. Normally distributed data of equal sample size were assessed for statistical significance by *t*-test. Some of the data are presented using non-parametric analysis, as the raw data do not conform to a normal distribution (Kolmogorov-Smirnov, Lilliefors test on standardized data, NPAR module of Systat and non-parametric algorithms in Micrococal Origin²⁷). In this format, each box includes all the data points of an experimental group. The bottom symbol shows the minimum value of the data group, the second symbol marks the first percentile, and the bottom of the vertical line marks the fifth percentile. The box denotes the range of data within the middle 50%; the bottom marks the twenty-fifth percentile, the middle line the fiftieth (nearest symbol indicates arithmetic mean) and the top, the seventy-fifth percentile. The top of the vertical line marks the ninety-fifth percentile, and symbols above it indicate the ninety-ninth and one-hundredth percentiles. Following the required log-linear transformation,

statistical significance was evaluated by ANOVA with a post-hoc test for multiple comparisons and group means with unequal sample size. Unless otherwise noted, the asterisk (*) indicates statistical significance at the $p < 0.02$ – 0.001 level in between-group comparisons.

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Fig. 1. Spontaneous synaptic currents from individual innervated sympathetic neurons: effect of target interactions. **(a)** Schematic diagram of cellular interactions and recording configurations (above); sample recordings from three innervated sympathetic neurons maintained *in vitro* in the presence or absence of the indicated targets (below). Synaptic currents were recorded from (left to right) an innervated sympathetic neuron in the absence of target tissue (input + SNs), an innervated sympathetic neuron contacting renal tissue (input + SNs + kidney) and an innervated sympathetic neuron contacting cardiac tissue (input + SNs + heart). Scale bar, 40 pA, 40 ms. **(b)** Histograms of the sEPSC amplitudes from the same three neurons (above). The sEPSC amplitude distributions are skewed, precluding the use of mean amplitude as a representative measure of sEPSC size. Cumulative amplitude distributions of the sEPSCs recorded from the same three neurons (below). The area of each distribution is calculated to yield an 'amplitude index' for each cell (see Methods). Amplitude indices for these three cells

are 24.3, 38.7 and 8.9 respectively. (c) Distributions of the decay time constants of the sEPSCs recorded from the same three neurons.

Fig. 2.

Target-specific regulation of nAChR-mediated macroscopic and synaptic currents. Effects of target contact on synaptic nAChRs were examined in innervated neurons without target ($n = 33$), contacting kidney ($n = 29$) and contacting heart ($n = 31$). **(a)** ACh-evoked macroscopic currents recorded in innervated sympathetic neurons without target or in those contacting either renal or cardiac target tissue. Responses of innervated neurons lacking target were similar to those of neurons contacting cardiac tissue; their distributions were unimodal with means of 949 ± 143 pA ($\chi^2 = 0.6$, $n = 17$) and 785 ± 53 ($\chi^2 = 0.3$, $n = 13$), respectively. In contrast, the distribution of ACh-elicited macroscopic currents of innervated neurons contacting renal tissue was bimodal; most responses were significantly larger than those recorded in innervated neurons with or without target contact (1760 ± 39 pA; $n = 7$ of 11, $\chi^2 = 0.02$). **(b)** sEPSC amplitude index values for innervated sympathetic neurons maintained without target (top) or with either renal (middle) or cardiac tissue (bottom) are shown. The amplitude index distribution for targetless, innervated neurons was well fit by the sum of two Gaussian distributions, with 87% of the values within the first peak centered at 20.1 ± 0.6 ($\chi^2 = 0.44$). The distribution of sEPSC amplitude indices for innervated neurons contacting kidney was multimodal, with 52% of the values in the first and 34% in the second Gaussian peak (at 15.9 ± 1.6 and at 35.8 ± 1.6 respectively; $\chi^2 = 1.04$) and 14% of the values ≥ 50 . In contrast, the distribution of the amplitude index values for innervated neurons contacting heart was described by a single log-normal distribution centered at 11.63 ± 0.83 ($\chi^2 = 0.61$). **(c)** Plots of the decay time constants (τ_{syn}) of sEPSCs recorded in innervated neurons contacting kidney or heart or in the absence of target. The time constants of synaptic events recorded in targetless neurons and in neurons contacting kidney reflect uniformly fast nAChR kinetics (no target, $\tau_{syn} = 2.08 \pm 0.05$; $n = 1546$, $\chi^2 = 2.8$; +kidney, $\tau_{syn} = 2.23 \pm 0.03$, $n = 2258$, $\chi^2 = 2.8$). In contrast, sEPSCs in most neurons contacting heart reflect roughly equal contributions of both

fast and slow events (48%, $\tau_f = 2.09 \pm 0.03$ ms; 42%, $\tau_{s1} = 16.9 \pm 0.8$ ms) with the slowest synaptic currents ($\tau_{s2} = 37.1 \pm 3$ ms) making up 9% of the currents recorded ($n = 699$; $\chi^2 = 0.9$).

Fig. 3.

Synaptic nAChR channel properties and subunit gene expression are regulated by target. After electrophysiology (Fig. 2), levels of nAChR subunit mRNA of each neuron were measured by quantitative PCR. **(a)** Effects of renal or cardiac target on nAChR expression. Innervated neurons with or without kidney target are characterized by uniformly fast kinetic sEPSCs and by significantly higher $\alpha 3$ and $\alpha 7$ and lower $\beta 4$ expression than innervated neurons with both fast and slow kinetic synaptic events (that is, those contacting heart). Relative expression levels of $\alpha 3$ to $\alpha 5 + \alpha 7 + \beta 4$ in individual innervated neurons with fast sEPSCs (+ kidney) are compared with those with both fast and slow τ_{syn} (+heart). Data were analyzed using non-parametric statistics and are presented as 'box plots' (see Methods). **(b)** Relationship between nAChR-subunit expression and sEPSC amplitude in innervated neurons with or without different targets. In innervated neurons either lacking target or contacting kidney, amplitude index values are positively correlated with ratio of $\alpha 3 / (\alpha 5 + \beta 4)$; $r = 0.93$ and 0.97 , respectively). In contrast, the ratio of $\alpha 3$ to $\alpha 5$ and $\beta 4$ is low in neurons contacting heart, regardless of sEPSC amplitude index. Likewise, the ratio of $\alpha 3 / \alpha 5$ is positively correlated with amplitude index in innervated neurons contacting kidney, whereas this ratio is constant over the full range of amplitudes in innervated neurons contacting heart. **(c)** Target regulation of nAChR subunit expression in innervated neurons. Innervated neurons contacting kidney have significantly higher ratios of $\alpha 3$ to $\alpha 5$ and/or to $\beta 4$ than innervated neurons contacting cardiac tissue. $*p \leq 0.01$; $**p \leq 0.002$.

Fig. 4.

Target contact differentially regulates the profile of nAChR channels and subunit gene expression in non-innervated sympathetic neurons. **(a)** Schematic diagram of cellular interactions and recording configurations (above) and sample macroscopic currents elicited by 2.5 mM ACh (below) from non-innervated sympathetic neurons maintained in the absence of target (SNs alone), neurons contacting kidney (SNs + kidney) and neurons contacting heart (SNs + heart). Amplitudes of currents evoked by 500 μ M ACh were significantly larger in neurons contacting renal tissue ($n = 27$) than those elicited in neurons maintained without target ($n = 49$, $p < 0.001$). In contrast, macroscopic currents recorded in neurons contacting cardiac tissue are significantly smaller than control ($n = 19$, $p < 0.004$). Increasing ACh concentration from 500 μ M to 2.5 mM significantly increased current in neurons with kidney, but had little effect under other culture conditions. Data were analyzed using non-parametric statistics and are presented as box plots (see Methods). Note the difference in scale on amplitude axes. **(b)** Uninnervated sympathetic neurons express three nAChR channel subtypes classified on the basis of distinct chord conductances, pharmacology and kinetics^{9,23,24}. Representative traces from single-channel recordings in the three experimental groups (top). The peaks in the amplitude distributions from control neurons correspond to ~15, 30 and 50 pS nAChR channels ($n = 11$; bottom). Neurons contacting kidney express two major conductance classes with relatively brief opening times: one 30–35 pS and the other ≥ 70 pS ($n = 25$)^{9,23,24}. In contrast,

neurons contacting heart primarily express longer-duration nAChRs of $\lesssim 15$ pS ($n = 9$). (c) The levels of nAChR subunit gene expression were measured in 30–40 neurons per experiment by qPCR of cytoplasmic RNA (see Methods and Fig. 5) and were plotted relative to actin mRNA levels. We assayed neurons contacting kidney ($n = 8$), contacting heart ($n = 9$) and without target ($n = 15$). Contact with kidney significantly increased $\alpha 3$, $\alpha 5$ and $\beta 4$ from control (targetless) levels. In contrast, contact with cardiac tissue had little effect on $\alpha 3$ mRNA levels but significantly upregulated $\alpha 5$, $\alpha 7$ and $\beta 4$ expression. $*p \leq 0.01$; $**p \leq 0.001$; $***p \leq 0.0001$.

Fig. 5.

Characterization of qPCR assay used to measure $\alpha 3$, $\alpha 5$, $\alpha 7$ and $\beta 4$ mRNA levels in individual neurons. **(a)** Above, an agarose gel showing the amplified actin, $\alpha 3$, $\alpha 5$, $\alpha 7$ and $\beta 4$ PCR products from a single innervated neuron. Below, agarose gel of a standard curve of PCR products amplified from decreasing amounts of subunit cDNAs. **(b)** Amplification of subunit mRNAs from a single neuron for $2 \times 35 + 1 \times 20$ cycles yields DNA product within the linear range of standard curves generated with 0.05 to 1 fg of template. **(c)** Standard curve over the entire range. Amplified product was quantified based on [^{32}P]dNTP incorporation.