Neuron, Vol. 43, 305–311, August 5, 2004, Copyright ©2004 by Cell Press

Tethering Naturally Occurring Peptide Toxins for Cell-Autonomous Modulation of Ion Channels and Receptors In Vivo

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Summary

The physiologies of cells depend on electrochemical signals carried by ion channels and receptors. Venomous animals produce an enormous variety of peptide toxins with high affinity for specific ion channels and receptors. The mammalian prototoxin lynx1 shares with α-bungarotoxin the ability to bind and modulate nicotinic receptors (nAChRs); however, lynx1 is tethered to the membrane via a GPI anchor. We show here that several classes of neurotoxins, including bungarotoxins and cobratoxins, retain their selective antagonistic properties when tethered to the membrane. Targeted elimination of nAChR function in zebrafish can be achieved with tethered α-bungarotoxin, silencing synaptic transmission without perturbing synapse formation. These studies harness the pharmacological properties of peptide toxins for use in genetic experiments. When combined with specific methods of cell and temporal expression, the extension of this approach to hundreds of naturally occurring peptide toxins opens a new landscape for cell-autonomous regulation of cellular physiology in vivo.

Introduction

Our current understanding of the development and function of the mammalian brain rests in large part on conclusions from experimental or accidental lesions that perturb CNS function. These studies have involved a wide variety of techniques, including the analysis of behavioral deficits in people suffering from disease or acute brain injury (Manns et al., 2003), sensory deprivation during development of experimental animals (Shatz and Stryker, 1978), pharmacologic manipulations of CNS receptors and ion channels (Bauer et al., 2002; Shatz and Stryker, 1988), and genetic ablations of specific CNS cell types in experimental animals (Champtiaux and Changeux, 2004; Kofuji et al., 2000; Nakazawa et al., 2004). This rich history and the advances in genetic techniques that enable in vivo manipulations of molecules, cells, and circuits (KOs, BACs, siRNA) provide a powerful incentive for the development of genetic approaches that extend our ability to alter the physiologic properties of specific neurons in vivo. For example, the ability to block presynaptic vesicle fusion using botulinum neurotoxins has been introduced as an effective approach to genetically interfere with neurotransmission in specific CNS cell types (Saint-Amant and Drapeau, 2001; Steinhardt et al., 1994). Likewise, targeted overexpression of K^+ channels has been shown to be highly effective at silencing neuronal activity both in mammalian and *Drosophila* excitable cells (Johns et al., 1999; Nadeau et al., 2000; Nitabach et al., 2002; Paradis et al., 2001), although in mammalian cells this approach can induce apoptotic cell death in neurons (Nadeau et al., 2000).

Based on the knowledge that the electrical properties of neurons are controlled by a remarkable variety of ionic currents carried by many specific ion channels and receptors (Catterall, 1999; Champtiaux and Changeux, 2004; Coetzee et al., 1999) and the fact that small specifically acting soluble peptide neurotoxins have evolved to block many of these currents (Adams and Olivera, 1994; McIntosh et al., 1999; Olivera et al., 1994; Terlau and Olivera, 2004; Tsetlin and Hucho, 2004), we sought to harness the potential of peptide toxins as a means to genetically manipulate the functional properties of CNS neurons. To do this, we generated fusion proteins based on the lynx1 prototoxin (Ibañez-Tallon et al., 2002; Miwa et al., 1999) but carrying functional domains from naturally occurring peptide neurotoxins. These chimeric toxins are tethered to the cell surface via a GPI anchor yet retain their ability to specifically block ligand and voltage-gated receptors and ion channels cell autonomously in *Xenopus* oocytes and in vivo in zebrafish muscle fibers.

Results

Generation of Chimeric Tethered Toxins

Tethered toxin constructs containing functional domains from several classes of neurotoxins were expressed on the cell surface using an N-terminal signal peptide, a flexible linker, and sequences controlling the addition of the lynx1 GPI anchor (Figure 1). To test the generality of this approach, tethered toxins specific for several different receptors and ion channels were prepared (Table 1). To target nAChRs, we have employed domains from the α- and κ-bungarotoxins and the α-conotoxins MII and PnIB (Servent et al., 1997; Terlau and Olivera, 2004; Tsetlin and Hucho, 2004). These were chosen because they have distinct specificities for nAChR subtypes, which would therefore allow manipulations of specific classes of these receptors in vivo. A tethered toxin construct carrying the broad spectrum Na^+ channel inhibitor μ-O-conotoxin MrVIA was also constructed, since this toxin has recently been shown to block rat skeletal muscle and neuronal voltage-gated Na^+ channels in *Xenopus* oocytes and in hippocampal pyramidal neurons (Terlau and Olivera, 2004; Terlau et al., 1996). The activity of this toxin, therefore, could be used to block Na^+ currents that are required for action potential generation and propagation. Finally, to target

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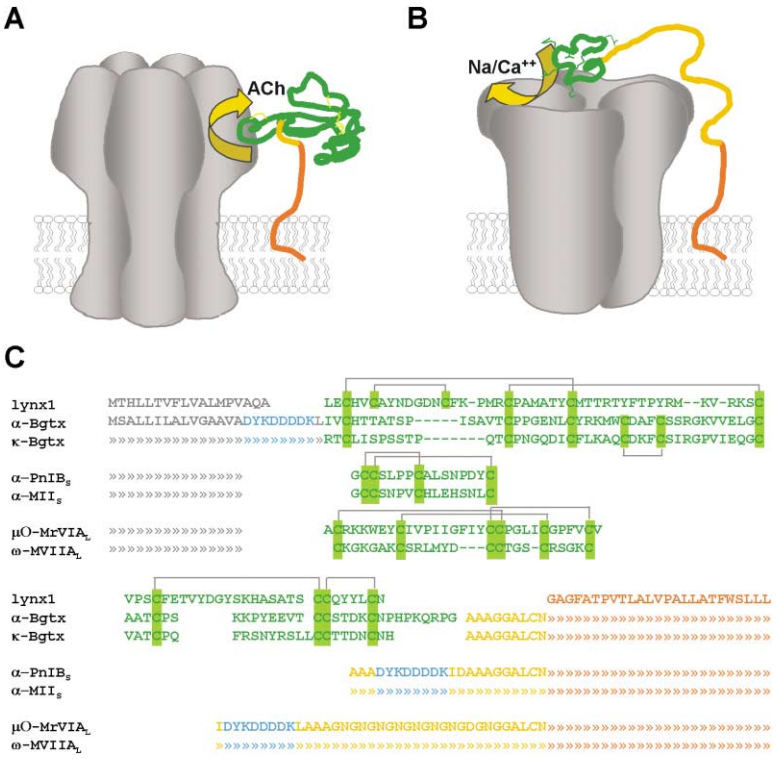

Figure 1. Construction of Tethered Toxins for Cell-Autonomous Inactivation of Ligand and Voltage-Gated Channels

(A) Model representing the binding of tethered α-bungarotoxin to the ACh binding site of the nAChR.

(B) Model representing the binding of tethered conotoxins to the channel pore of voltage-gated sodium and calcium channels.

(C) Amino acid alignments and disulfide bridges of lynx1 and toxins. Tethered toxin constructs include mature toxin sequences (green), synthetic polypeptide linkers of different lengths and composition (yellow), lynx1 hydrophobic C-terminal sequence for addition of the GPI anchor (orange), secretion signal sequences (gray), and epitope tag (blue).

voltage-gated Ca^{2+} channels, we have generated a tethered form of the ω-conotoxin MVIIA (Olivera et al., 1994). This toxin can differentiate N-type from P/Q-type, L-type, and T-type Ca^{2+} channels (Olivera et al., 1994). Specific blockades of Ca^{2+} currents can be used to examine a variety of crucial cellular functions, including mechanisms of presynaptic neurotransmitter release, signal transduction, and hormone secretion (Olivera et al., 1994; Tsien, 1983).

Specific Inactivation of nAChR Subtypes with Tethered Bungarotoxins and Conotoxins

Each of the tethered toxins was coinjected into *Xenopus* oocytes with its appropriate target(s) or controls and was assayed by two-electrode voltage clamp recordings. Given the strong structural homology of the bungarotoxins and lynx1 (Miwa et al., 1999), our previous demonstration that lynx1 modulates nAChRs when GPI anchored to the cell surface (Ibañez-Tallon et al., 2002), and the mode of binding of bungarotoxins to their target receptors (Figure 1A), we first tested whether these molecules could retain their activity and specificity when tethered to the cell surface. Recordings of nicotinic receptor activities in response to these tethered toxins confirmed this expectation (Figure 2). Thus, coexpression of tethered α-bungarotoxin (t-αBgtx) with muscle $\alpha1\beta1\gamma\delta$ or neuronal α7 nAChRs on the surface of oocytes completely blocks current flow in response to acetylcholine, but does not block $\alpha4\beta2$ nAChR function. In contrast, tethered κ-bungarotoxin (t-κBgtx) does not block $\alpha1\beta1\gamma\delta$ nAChR function when coexpressed in the oocyte system. Rather, it specifically blocks the neuronal nAChR composed of α7 subunits and partially blocks $\alpha4\beta2$ receptors, consistent with the relative affinities described in previous studies using soluble toxins (Table 1). The fact that the tethered bungarotoxins retained

Table 1. Toxins Used in This Study

Toxin	Receptor/Ion Channel	Affinity	Reference
α-bungarotoxin	$\alpha1\beta1\gamma\delta$ nAChR	0.4 nM	1
	α7 nAChR	0.95 nM	2
	$\alpha4\beta2$ nAChR	100 μM	3
κ-bungarotoxin	$\alpha1\beta1\gamma\delta$ nAChR	1 μM	4
	α7 nAChR	1–12 nM	2
	$\alpha4\beta2$ nAChR	>100 nM	5
α-conotoxin PnIB	α7 nAChR	61.3 nM	6
α-conotoxin MII	$\alpha3\beta2$ nAChR	0.5 nM	7
μO-conotoxin MrVIA	Na_v 1.2	200 nM	8
ω-conotoxin MVIIA	Ca_v 2.2	1 nM	9

References: 1, Lukas et al., 1981; 2, Servent et al., 1997; 3, Grutter et al., 2003; 4, Loring et al., 1986; 5, Grant et al., 1998; 6, Luo et al., 1999; 7, Cartier et al., 1996; 8, Terlau et al., 1996; 9, Sato et al., 2000.

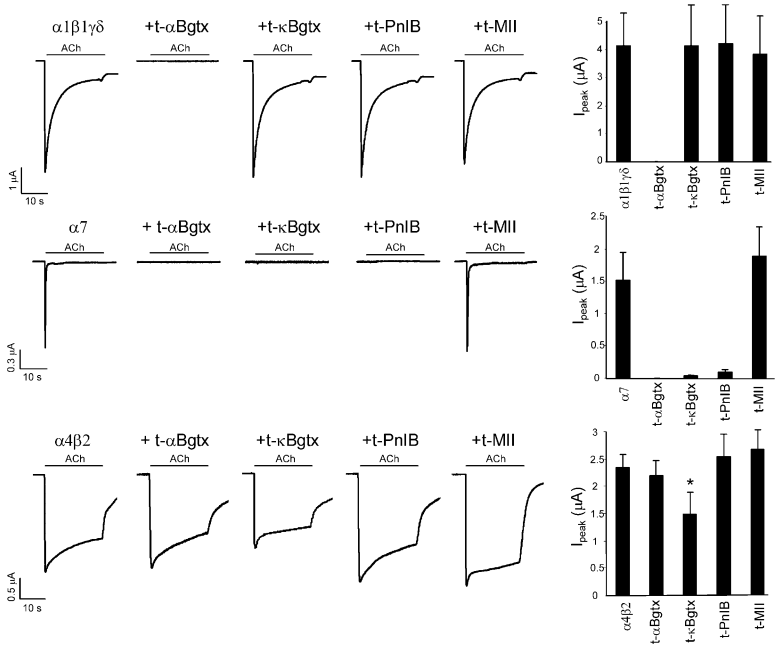

Figure 2. Tethered Toxins Specifically Inhibit nAChRs in *Xenopus* Oocytes

(Top) Coexpression of tethered toxins with muscle $\alpha1\beta1\gamma\delta$ nAChRs demonstrates that t-αBgtx completely blocks the muscle receptor. (Middle) Coexpression of tethered toxins with neuronal $\alpha7$ nAChRs shows that t-αBgtx, t-κBgtx, and t-PnIB inhibit the receptor, whereas t-MII has no effect. (Bottom) t-κBgtx partially inhibits $\alpha4\beta2$ nAChRs (Student's t test, $p = 0.0017$), while the other tethered toxins have no effect. Representative traces of ACh-evoked responses (20 s application) from individual oocytes are shown. Bar graphs represent the average ± SEM of the peak current obtained in ten oocytes for each case.

their efficacy and specificity for specific nAChRs prompted us to test whether a second structurally distinct class of toxins known to act on nAChRs could also retain their specificity when tethered to the cell surface (Figure 2). Thus, tethered conotoxins PnIB (t-PnIB) and MII (t-MII) were tested for their ability to inhibit nAChR currents in oocytes. As shown in Figure 2, t-PnIB retains its specificity for $\alpha7$ nAChRs, resulting in a block of these currents without affecting either the muscle or neuronal $\alpha4\beta2$ nAChR activities. As expected (Luo et al., 1999), t-MII does not inhibit the activity of these receptors.

Inactivation of Voltage-Gated Sodium and Calcium Channels with Tethered Conotoxins

The ability to use tethered conotoxins to manipulate their cognate target molecules presented the possibility of performing an astounding array of genetic experiments to manipulate the properties of cells in vivo. Thus, there are ~500 species of cone snails, each expressing a unique set of 50 to 200 peptide toxins, yielding a collection of nearly 50,000 conotoxins, of which less than 0.2% have been characterized (McIntosh et al., 1999). However, a significant proportion of these molecules (e.g., those that block voltage-gated ion channels) have been shown to block activity by binding in the vestibule of the ion channel to directly block ion flux (Olivera et al., 1994; Terlau and Olivera, 2004). Given the small size of the conotoxin functional domains and the increased distance and rotational flexibility required for a tethered toxin to bind properly within the vestibule of the channel (Figure 1B), it seemed probable that extension of this approach to this class of toxins might be problematic. To investigate this possibility, we next tested tethered toxins directed toward voltage-gated Na^+ and Ca^{2+} channels. In both cases, we have been able to construct tethered toxins that retained both their activity and specificity against their target channels. Thus, the tethered μ-O-conotoxin MrVIA (t-MrVIA) blocked >90% of the current flux through $Na_v1.2$ channels in oocytes, without affecting N-type Ca^{2+} channel or shaker K^+ channel function (Figure 3). In contrast, tethered ω-conotoxin MVIIA (t-MVIIA) coexpression completely blocked $Ca_v2.2$ (N-type) Ca^{2+} channels, without affecting the $Na_v1.2$ Na^+ or shaker K^+ channels. Taken together, our data indicate that toxins from several different classes can retain their specificity and activity when expressed as GPI-anchored molecules, effectively inhibiting currents that are fundamental to the physiologic activities of neurons and other cell types. It is important to note, however, that attempts to create tethered toxin constructs using toxin molecules that normally contain noncanonical amino acid residues have not generally been successful. For example, unsatisfactory results were obtained with several toxins in which *trans*-4-hydroxyproline was present in the venom, which was substituted by proline in our tethered toxin constructs. Thus, tethered toxin constructs for GID, PIIIA, GVIA, and RIIIK all exhibited reduced or no activity when tested in oocytes (data not shown). We believe that this is due to the requirement for hydroxyproline in these toxins, as has been shown in the case of conotoxin GIIIA (Wakamatsu et al., 1992), although further experimentation will be required to test this idea.

Tethered Toxins Function Cell Autonomously

Maximal utility of the tethered toxin approach for in vivo use requires that the toxins act in a cell-autonomous manner. To demonstrate that the tethered toxins are not released from the cell surface to affect nearby cells, oocytes coexpressing the tethered α-bungarotoxin construct and neuronal $\alpha7$ nAChRs were incubated overnight in the presence of oocytes expressing $\alpha7$ nAChRs only (Figure 4). Each oocyte was then separated from its neighbors, and recordings of nAChR activity in response to acetylcholine were measured. As shown in Figure 4, the receptors on oocytes coexpressing the

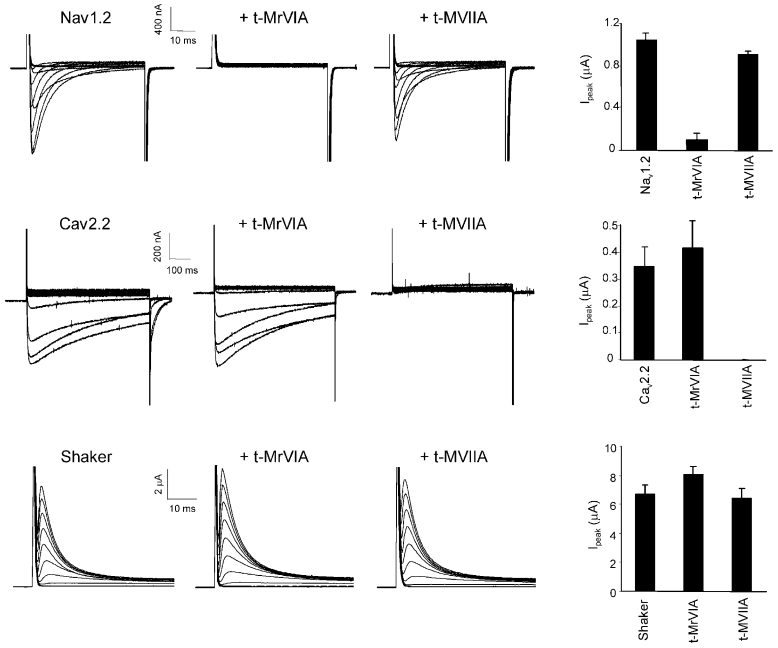

Figure 3. Tethered Toxins Specifically Inhibit Voltage-Gated Sodium and Calcium Channels in *Xenopus* Oocytes

(Top) Coexpression of tethered MrVIA potently blocks the sodium Na_v 1.2 voltage-gated channel, whereas t-MVIIA does not. (Middle) Coexpression of t-MVIIA completely blocks the calcium Ca_v 2.2 voltage-gated channel, whereas t-MrVIA has no effect. (Bottom) Potassium Shaker channels are not affected by coexpression of either t-MrVIA or t-MVIIA conotoxins. Representative traces of whole-cell currents recorded from individual oocytes are shown. Voltage steps ranged from −70 to 40 mV in steps of 10 mV from a holding potential of −90 mV. A prepulse to −110 mV was done for Shaker channel recordings. Bar graphs represent the average ± SEM of the peak current obtained in ten oocytes for each case.

tethered toxins had no activity in response to ligand, whereas those coincubated with oocytes expressing only the neuronal α7 nAChR exhibited normal responses to acetylcholine. These data demonstrate that α neurotoxins can retain their properties when expressed on the surface of cells via a GPI anchor and that they do not affect neighboring cells through release from the cell surface.

In Vivo Efficacy of Tethered Toxins

Zebrafish embryos were chosen to test the efficacy of the tethered toxin approach in vivo because of the ability to visualize single muscle fibers in whole fish and analyze their electrical activity using whole-cell recordings. In vivo block of muscle nAChRs by t-αBgtx represents a formidable challenge because of the large number and high density of receptors at the neuromuscular junction.

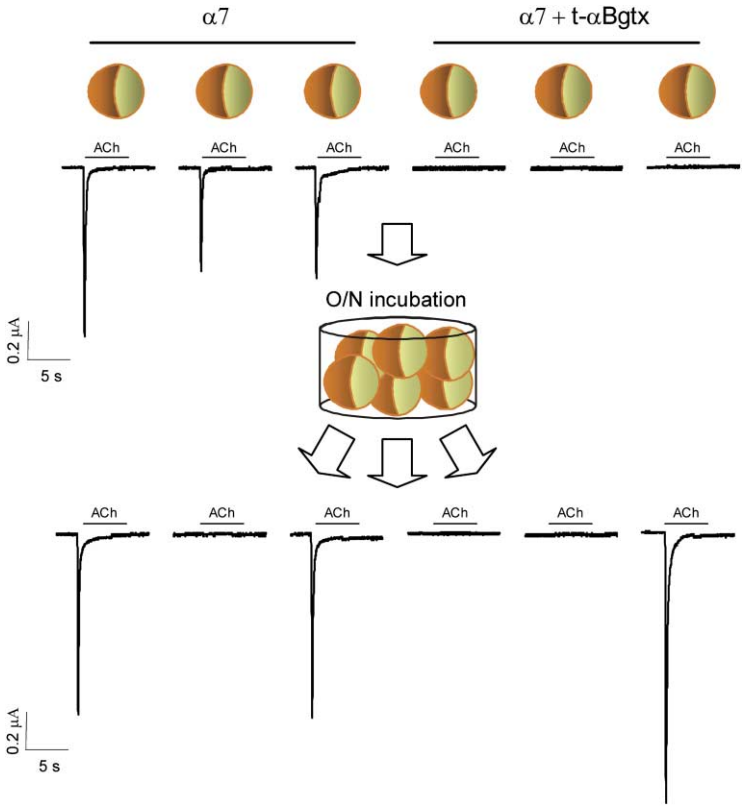

Figure 4. Tethered Toxins Are Not Cleaved from the Membrane

Oocytes injected with only α7 nAChRs or with α7 nAChRs plus tethered αBgtx were recorded to test the response to ACh. After overnight coincubation of the two types of oocytes, electrophysiology recordings were repeated. Again, three oocytes responded to ACh, and three oocytes did not respond, indicating that the tethered toxin acts only on coexpresssed receptors and it does not affect neighboring cells through release from the cell surface.

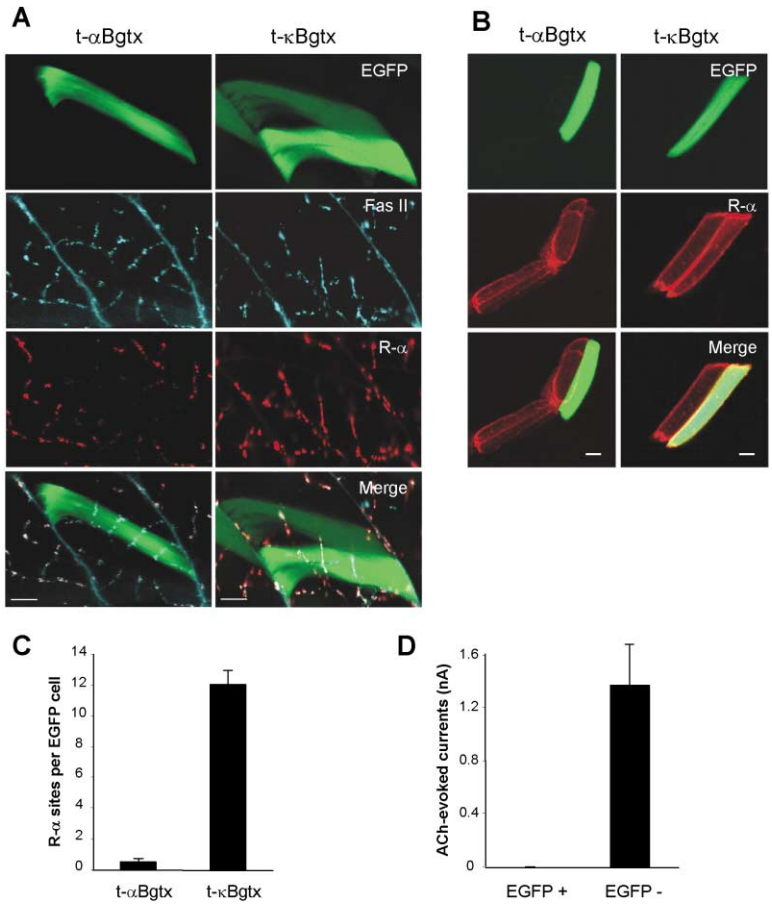

Figure 5. Functional Inactivation of Muscle nAChRs In Vivo

(A) Each field shows approximately two tail segments in intact embryos. Zebrafish embryos were injected with dual promoter constructs containing the CMV promoter upstream of EGFP and the α-actin promoter driving expression of either t-αBgtx (left panel) or t-κBgtx (right panel). Synaptic sites on fluorescent muscle cells expressing EGFP were identified by labeling of synaptic acetylcholinesterase using FasII (second row). Labeling of AChRs with rhodamine αBgtx (R-α; third row) reveals greatly reduced levels at identified synaptic sites in the muscle cell injected with tα-Bgtx. No colocalization of FasII and rhodamine αBgtx is detected in t-αBgtx injected muscle but it is prominent in t-κBgtx injected cells (bottom row).

(B) Labeling of nAChRs in partially dissociated muscle cells from the zebrafish mutant line *twitch once* that expresses receptors over the entire surface membrane. Rhodamine αBgtx labeling is effectively blocked in a single green fluorescent cell expressing t-αBgtx (left panel) and not in t-κBgtx injected cells (right panel).

(C) Bar graph indicating the average number ± SEM of rhodamine αBgtx labeled sites per green fluorescent muscle cell in fish injected with t-κBgtx (n = 41 cells) or t-αBgtx (n = 28 cells).

(D) Block of nAChRs in t-αBgtx injected fish assessed by electrophysiological recordings in dissociated muscle cells. The bar graph indicates the average ± SEM of ACh-evoked responses from ten EGFP-positive cells and from ten EGFP-negative cells.

Zebrafish embryos were injected with dual promoter constructs encoding either t-αBgtx or t-κBgtx driven by the muscle-specific α-actin promoter and cytoplasmic EGFP driven by the CMV promoter. As shown in Figure 5A, muscle fibers expressing EGFP were readily identified in fish injected with both tethered toxin constructs. Synaptic sites on EGFP fluorescent muscle cells and their immediate neighbors were identified by labeling with conjugated-fasciculin (FasII) that labels acetylcholinesterase at postsynaptic sites. The positions and morphology of synapses revealed by FasII binding were unaffected in the t-αBgtx and t-κBgtx expressing cells, indicating that the expression of tethered bungarotoxins did not interfere overtly with synapse development. However, muscle cells expressing t-αBgtx had greatly reduced to nonexistent levels of soluble rhod-Bgtx labeling, demonstrating that the muscle nicotinic receptors in the t-αBgtx expressing cells were occupied. t-κBgtx expressing cells retained normal levels of rhod-Bgtx labeling (Figures 5A and 5C). To provide further evidence that the block in rhod-Bgtx labeling was due to the occupation of the muscle nicotinic receptors by t-αBgtx, *twitch once* zebrafish mutants that expressed high levels of the receptor over the entire muscle surface were employed. As shown in Figure 5B, rhod-Bgtx binding is observed over the surface of control EGFP-negative muscle cells and of EGFP-positive cells expressing t-κBgtx, whereas no labeling is detected in EGFP-positive cells expressing t-αBgtx. Finally, nAChR function was directly tested in cells expressing t-αBgtx by electrophysiological responses to fast application of 10 μM ACh in acutely dissociated muscle cells from these fish. Peak current in nonfluorescent muscle cells revealed robust responses to ACh (mean current 1.4 nA, n = 10), whereas no response was recorded in fluorescent muscle cells coexpressing EGFP and t-αBgtx (mean current 0 nA, n = 10) (Figure 5D). Taken together, these data prove that the tethered bungarotoxins retain their specificity in vivo, that they act cell autonomously, and that t-αBgtx provides an effective block of its cognate receptor in vivo, even under conditions of extremely high receptor expression. These data also indicate that silencing of muscle nicotinic receptor activity in individual muscle cells during zebrafish development has no gross effect on the development or distribution of neuromuscular synapses.

Discussion

We have demonstrated here that peptide neurotoxins from several classes retain their activity and specificity for ligand-gated and voltage-gated ion channels when tethered to the cell membrane via a GPI anchor and that they act cell autonomously in *Xenopus* oocytes and zebrafish muscle fibers. The ability to tether these naturally occurring peptide neurotoxins to the cell surface in a manner that preserves their activity and specificity, combined with the use of BAC transgenic con-

structs to target expression to specific CNS cell types (Gong et al., 2003), allows tremendous flexibility in the genetic dissection of specific factors and pathways that influence the development and function of the mammalian CNS. For example, the expression of tethered bungarotoxins and α-conotoxins can allow simple genetic manipulations of specific nAChR classes to begin to unravel the complex contributions of this diverse group of receptors to the activities of specific cell types and circuits in vivo. Also, selective suppression of neuronal activity can be achieved using t-MrVIA to inhibit neuronal Na^+ channels that are required for the generation of the action potential or by using t-MVIIA to block Ca^{2+} channels that are required for neurotransmitter release (Terlau and Olivera, 2004). Extension of this approach to other toxins, such as those that block other ion channels, serotonin, and NMDA receptors, offers additional important experimental opportunities.

There are many advantages of the tethered toxin approach over other methods for the manipulation of cellular physiology. First, this approach harnesses the impressive functional diversity of the peptide neurotoxins, enabling simple manipulations of ion channels and receptors that mediate important physiologic processes within cells. For example, peptide toxins can differentially block heteromeric channels sharing one or more subunits; conversely, they can block several members of a given ion channel or receptor family. This enables manipulations of currents that would be extremely difficult (or impossible) to achieve using traditional genetic approaches. Second, peptide toxins can act either to block channel activity (as in the cases we have chosen for these studies) or to inhibit channel inactivation resulting in hyperexcitation of the targeted cells (McIntosh et al., 1999; Terlau and Olivera, 2004), offering the possibility of both loss- and gain-of-function studies in vivo. Third, novel toxin activities can be produced using chimeric toxins derived from the fusion of known peptide toxins (Sato et al., 2000). Given the small size of most peptide toxins, we believe that the creation of novel specificities by mutagenesis will extend the use of this approach to receptors and ion channels for which natural toxins have not been identified. Fourth, the small size of most tethered toxins and their simple incorporation into transgenic and viral constructs will allow their use in a wide variety of species for which gene targeting is not yet possible (Gong and Rong, 2003). For example, tethered toxins could be employed to examine the influence of receptor function or neuronal activity on behavior in transgenic rats, which are advantageous for certain studies of CNS function.

Two extensions of the present studies are of immediate interest. First, although reversible expression of the tethered toxins can be achieved using established methods (Mansuy and Bujard, 2000), development of strategies for the rapid regulation of these activities for use in short-term experiments remains an important goal. Second, the application of this strategy to other peptides, including hormones and neuropeptides, offers interesting opportunities for the analysis of the roles of these ligands in specific cell types. Thus, we anticipate that tethered toxins and other peptides will become critical instruments for the genetic dissection of CNS cells and circuits.

Experimental Procedures

Generation of Tethered Toxin Constructs

Construction of molecular chimeras between lynx1 and the snake bungarotoxins was done by replacing the sequence encoding lynx1 by the cDNAs of α- or κ-bungarotoxin in-frame between the secretion signal and lynx1 hydrophobic sequence for GPI attachment. A flag epitope sequence was introduced downstream of the secretion signal, and a short nine aa linker was inserted between the toxin and the sequence for GPI attachment. The constructs for tethered conotoxins were prepared as above, except that the flag epitope was inserted downstream of the toxin and that, for toxins against voltage-gated channels, a flexible linker of $(asn\text{-}gly)_n$ joining the mature toxin molecule to the GPI anchoring sequence was inserted between the toxin and the hydrophobic sequence for GPI attachment. The following double stranded oligonucleotides were used to generate the corresponding conotoxins: PnIB, GGATGTTGCAGTT TACCCCCTTGTGCACTAAGTAACCCGGACTATTGT; MII, GGATGTTG CAGTAATCCAGTATGTCACCTAGAGCATAGCAACCTTTGC; MrVIA, GCATGCCGGAAGAAGTGGGAGTACTGCATAGTGCCGATAATAGG ATTCATATACTGCTGTCCAGGACTTATATGCGGTCCATTCGTATG CGTC; MVIIA, TGCAAAGGCAAGGGCGCGAAGTGCTCCCGCCTCA TGTATGACTGTTGCACCGGATCGTGTAGGTCCGGTAAGTGC.

Injections and Whole-Cell Electrophysiology Recordings

The preparation of *Xenopus* oocytes, cRNA transcripts, and two-electrode voltage-clamp ACh recordings were done essentially as described (Ibañez-Tallon et al., 2002). cRNA injection mixes containing either receptor/channel subunit cRNAs or the same amount of receptor/channel and t-toxin (t-t) were prepared at the following ratios: for nAChRs [1α1: 0.5β1: 0.5γ: 0.5δ: 2t-t], [1α7: 3.5t-t], [0.5 α4: 0.5β2: 3.3 t-t], for sodium Nav1.2 [1α: 4t-t] calcium Cav2.2 N-type [1β3: $3.2\alpha1_B$: 1α2δ: 1t-t] for shaker [1sh: 4t-t]. Recordings of voltage-gated channels were performed as described (Lin et al., 1997; Nadal et al., 2001).

Zebrafish Experiments

Tethered bungarotoxins were transiently expressed in zebrafish embryos as described (Ono et al., 2002). The effectiveness of tethered αBgtx to bind and block AChRs in muscle was assessed by in vivo labeling with fluorescence-conjugated αBgtx and whole-cell recordings (Ono et al., 2001, 2002). Synaptic sites in intact fish were marked by treatment with 0.1 μM Alexa 647-conjugated Fasciculin 2 (Alomone Labs, Israel) for 45 min, which binds to acetylcholinesterase (Peng et al., 1999). Alternatively, muscle was dissociated by incubating skinned fish in 10 mg/ml collagenase (GIBCO) for 2–3 hr prior to gentle trituration. In some experiments, mutant *twitch once* fish that expressed large amounts of diffusely distributed acetylcholine receptors were used (Ono et al., 2002). In vivo imaging utilized an inverted Zeiss LSM 510 Meta microscope and a C-Apochromat 40× objective. Simultaneous measurements of EGFP (excitation 488/emission LP505), Alexa-647 (excitation 633/emission LP650), and rhodamine (excitation 543/emission BP560-590) fluorescence were provided by the Meta multitrack mode of acquisition.

Acknowledgments

We thank G.A. Grant for providing the κ-Bgtx expression plasmid; E. Hawrot for the α-Bgtx expression plasmid; A. Karlin for muscle nAChR subunits; L.W. Role for neuronal nAChR subunits; A.L. Goldin for Nav1.2 subunits; D. Lipscombe for Cav2.2 subunits; and B. Rudy for the shaker 29-4 channel. We are indebted to M. Nadal for invaluable help with voltage-clamp recordings. We would also like to thank B. Rudy and D. Besser for discussions; P. Vergani, Y. Amarillo, M. Mani, and C.S. Schwarz for technical help; and J. Walsh for administrative assistance. This work was supported by NIH for I.I.-T., J.M.M., P.B., and N.H. and by HHMI for I.I.-T., J.X., and N.H.

Received: May 28, 2004
Revised: July 6, 2004
Accepted: July 7, 2004
Published: August 4, 2004

References

Adams, M.E., and Olivera, B.M. (1994). Neurotoxins: overview of an emerging research technology. Trends Neurosci. *17*, 151–155.

Bauer, E.P., Schafe, G.E., and LeDoux, J.E. (2002). NMDA receptors and L-type voltage-gated calcium channels contribute to long-term potentiation and different components of fear memory formation in the lateral amygdala. J. Neurosci. *22*, 5239–5249.

Cartier, G.E., Yoshikami, D., Gray, W.R., Luo, S., Olivera, B.M., and McIntosh, J.M. (1996). A new alpha-conotoxin which targets alpha3-beta2 nicotinic acetylcholine receptors. J. Biol. Chem. *271*, 7522–7528.

Catterall, W.A. (1999). Molecular properties of brain sodium channels: an important target for anticonvulsant drugs. Adv. Neurol. *79*, 441–456.

Champtiaux, N., and Changeux, J.P. (2004). Knockout and knockin mice to investigate the role of nicotinic receptors in the central nervous system. Prog. Brain Res. *145*, 235–251.

Coetzee, W.A., Amarillo, Y., Chiu, J., Chow, A., Lau, D., McCormack, T., Moreno, H., Nadal, M.S., Ozaita, A., Pountney, D., et al. (1999). Molecular diversity of K+ channels. Ann. N Y Acad. Sci. *868*, 233–285.

Gong, M., and Rong, Y.S. (2003). Targeting multi-cellular organisms. Curr. Opin. Genet. Dev. *13*, 215–220.

Gong, S., Zheng, C., Doughty, M.L., Losos, K., Didkovsky, N., Schambra, U.B., Nowak, N.J., Joyner, A., Leblanc, G., Hatten, M.E., and Heintz, N. (2003). A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. Nature *425*, 917–925.

Grant, G.A., Luetje, C.W., Summers, R., and Xu, X.L. (1998). Differential roles for disulfide bonds in the structural integrity and biological activity of kappa-Bungarotoxin, a neuronal nicotinic acetylcholine receptor antagonist. Biochemistry *37*, 12166–12171.

Grutter, T., Prado de Carvalho, L., Le Novere, N., Corringer, P.J., Edelstein, S., and Changeux, J.P. (2003). An H-bond between two residues from different loops of the acetylcholine binding site contributes to the activation mechanism of nicotinic receptors. EMBO J. *22*, 1990–2003.

Ibañez-Tallon, I., Miwa, J.M., Wang, H.L., Adams, N.C., Crabtree, G.W., Sine, S.M., and Heintz, N. (2002). Novel modulation of neuronal nicotinic acetylcholine receptors by association with the endogenous prototoxin lynx1. Neuron *33*, 893–903.

Johns, D.C., Marx, R., Mains, R.E., O'Rourke, B., and Marban, E. (1999). Inducible genetic suppression of neuronal excitability. J. Neurosci. *19*, 1691–1697.

Kofuji, P., Ceelen, P., Zahs, K.R., Surbeck, L.W., Lester, H.A., and Newman, E.A. (2000). Genetic inactivation of an inwardly rectifying potassium channel (Kir4.1 subunit) in mice: phenotypic impact in retina. J. Neurosci. *20*, 5733–5740.

Lin, Z., Haus, S., Edgerton, J., and Lipscombe, D. (1997). Identification of functionally distinct isoforms of the N-type Ca^{2+} channel in rat sympathetic ganglia and brain. Neuron *18*, 153–166.

Loring, R.H., Andrews, D., Lane, W., and Zigmond, R.E. (1986). Amino acid sequence of toxin F, a snake venom toxin that blocks neuronal nicotinic receptors. Brain Res. *385*, 30–37.

Lukas, R.J., Morimoto, H., Hanley, M.R., and Bennett, E.L. (1981). Radiolabeled alpha-bungarotoxin derivatives: kinetic interaction with nicotinic acetylcholine receptors. Biochemistry *20*, 7373–7378.

Luo, S., Nguyen, T.A., Cartier, G.E., Olivera, B.M., Yoshikami, D., and McIntosh, J.M. (1999). Single-residue alteration in alpha-conotoxin PnIA switches its nAChR subtype selectivity. Biochemistry *38*, 14542–14548.

Manns, J.R., Hopkins, R.O., Reed, J.M., Kitchener, E.G., and Squire, L.R. (2003). Recognition memory and the human hippocampus. Neuron *37*, 171–180.

Mansuy, I.M., and Bujard, H. (2000). Tetracycline-regulated gene expression in the brain. Curr. Opin. Neurobiol. *10*, 593–596.

McIntosh, J.M., Santos, A.D., and Olivera, B.M. (1999). Conus peptides targeted to specific nicotinic acetylcholine receptor subtypes. Annu. Rev. Biochem. *68*, 59–88.

Miwa, J.M., Ibañez-Tallon, I., Crabtree, G.W., Sanchez, R., Sali, A., Role, L.W., and Heintz, N. (1999). lynx1, an endogenous toxin-like modulator of nicotinic acetylcholine receptors in the mammalian CNS. Neuron *23*, 105–114.

Nadal, M.S., Amarillo, Y., Vega-Saenz de Miera, E., and Rudy, B. (2001). Evidence for the presence of a novel Kv4-mediated A-type K(+) channel-modifying factor. J. Physiol. *537*, 801–809.

Nadeau, H., McKinney, S., Anderson, D.J., and Lester, H.A. (2000). ROMK1 (Kir1.1) causes apoptosis and chronic silencing of hippocampal neurons. J. Neurophysiol. *84*, 1062–1075.

Nakazawa, K., McHugh, T.J., Wilson, M.A., and Tonegawa, S. (2004). NMDA receptors, place cells and hippocampal spatial memory. Nat. Rev. Neurosci. *5*, 361–372.

Nitabach, M.N., Blau, J., and Holmes, T.C. (2002). Electrical silencing of *Drosophila* pacemaker neurons stops the free-running circadian clock. Cell *109*, 485–495.

Olivera, B.M., Miljanich, G.P., Ramachandran, J., and Adams, M.E. (1994). Calcium channel diversity and neurotransmitter release: the omega-conotoxins and omega-agatoxins. Annu. Rev. Biochem. *63*, 823–867.

Ono, F., Higashijima, S., Shcherbatko, A., Fetcho, J.R., and Brehm, P. (2001). Paralytic zebrafish lacking acetylcholine receptors fail to localize rapsyn clusters to the synapse. J. Neurosci. *21*, 5439–5448.

Ono, F., Shcherbatko, A., Higashijima, S., Mandel, G., and Brehm, P. (2002). The Zebrafish motility mutant twitch once reveals new roles for rapsyn in synaptic function. J. Neurosci. *22*, 6491–6498.

Paradis, S., Sweeney, S.T., and Davis, G.W. (2001). Homeostatic control of presynaptic release is triggered by postsynaptic membrane depolarization. Neuron *30*, 737–749.

Peng, H.B., Xie, H., Rossi, S.G., and Rotundo, R.L. (1999). Acetylcholinesterase clustering at the neuromuscular junction involves perlecan and dystroglycan. J. Cell Biol. *145*, 911–921.

Saint-Amant, L., and Drapeau, P. (2001). Synchronization of an embryonic network of identified spinal interneurons solely by electrical coupling. Neuron *31*, 1035–1046.

Sato, K., Raymond, C., Martin-Moutot, N., Sasaki, T., Ohtake, A., Minami, K., Van Renterghem, C., Takahashi, M., and Seagar, M.J. (2000). Binding of six chimeric analogs of omega-conotoxin MVIIA and MVIIC to N- and P/Q-type calcium channels. Biochem. Biophys. Res. Commun. *269*, 254–256.

Servent, D., Winckler-Dietrich, V., Hu, H.Y., Kessler, P., Drevet, P., Bertrand, D., and Menez, A. (1997). Only snake curarimimetic toxins with a fifth disulfide bond have high affinity for the neuronal alpha7 nicotinic receptor. J. Biol. Chem. *272*, 24279–24286.

Shatz, C.J., and Stryker, M.P. (1978). Ocular dominance in layer IV of the cat's visual cortex and the effects of monocular deprivation. J. Physiol. *281*, 267–283.

Shatz, C.J., and Stryker, M.P. (1988). Prenatal tetrodotoxin infusion blocks segregation of retinogeniculate afferents. Science *242*, 87–89.

Steinhardt, R.A., Bi, G., and Alderton, J.M. (1994). Cell membrane resealing by a vesicular mechanism similar to neurotransmitter release. Science *263*, 390–393.

Terlau, H., and Olivera, B.M. (2004). Conus venoms: a rich source of novel ion channel-targeted peptides. Physiol. Rev. *84*, 41–68.

Terlau, H., Stocker, M., Shon, K.J., McIntosh, J.M., and Olivera, B.M. (1996). MicroO-conotoxin MrVIA inhibits mammalian sodium channels, but not through site I. J. Neurophysiol. *76*, 1423–1429.

Tsetlin, V.I., and Hucho, F. (2004). Snake and snail toxins acting on nicotinic acetylcholine receptors: fundamental aspects and medical applications. FEBS Lett. *557*, 9–13.

Tsien, R.W. (1983). Calcium channels in excitable cell membranes. Annu. Rev. Physiol. *45*, 341–358.

Wakamatsu, K., Kohda, D., Hatanaka, H., Lancelin, J.M., Ishida, Y., Oya, M., Nakamura, H., Inagaki, F., and Sato, K. (1992). Structure-activity relationships of mu-conotoxin GIIIA: structure determination of active and inactive sodium channel blocker peptides by NMR and simulated annealing calculations. Biochemistry *31*, 12577–12584.