Interactions between presynaptic Ca\(^{2+}\) channels, cytoplasmic messengers and proteins of the synaptic vesicle release complex

Scott E. Jarvis and Gerald W. Zamponi

Influenza of Ca\(^{2+}\) through presynaptic voltage-gated Ca\(^{2+}\) channels is a key step in rapid neurotransmitter release. The amount of Ca\(^{2+}\) entering through these channels is modulated by a plethora of intracellular messenger molecules, including \(\beta\gamma\)-subunits of G proteins, and protein kinases. In addition, Ca\(^{2+}\) channels bind physically to proteins of the vesicle-release machinery in a Ca\(^{2+}\)-dependent manner, which can, in turn, regulate the activity of Ca\(^{2+}\) channels. Recent evidence suggests that second messengers and presynaptic vesicle-release proteins do not regulate Ca\(^{2+}\) channel activity as independent entities, but that there is extensive crosstalk between these two mechanisms. The complex interactions between second messengers, vesicle-release proteins and voltage-gated Ca\(^{2+}\) channels might provide multiple avenues for fine-tuning Ca\(^{2+}\) entry into the presynaptic terminal and, consequently, neurotransmission.

Ca\(^{2+}\) influx through neuronal voltage-dependent Ca\(^{2+}\) channels mediates a range of cytoplasmic responses, including the release of neurotransmitters, activation of Ca\(^{2+}\)-dependent enzymes and regulation of neuronal excitability\(^1,2\). Several disorders have been linked to excessive Ca\(^{2+}\) entry into the cytosol, and mutations and deletions in genes encoding Ca\(^{2+}\) channels are associated with etiologies such as migraine, cerebellar ataxia, night blindness and epilepsy\(^3\). As a consequence, Ca\(^{2+}\) channels are key pharmacological targets and a detailed understanding of the mechanisms that control Ca\(^{2+}\) channel activity is of fundamental importance.

There are multiple types of Ca\(^{2+}\) channels with unique physiological roles in the CNS. They have been classified by their distinct electrophysiological and pharmacological profiles into T-, N-, L-, Q-, P- and R-types\(^4\). The T-type (or low-voltage-activated) channels activate at potentials more negative than \(-40\) mV, have a small unitary conductance and inactivate rapidly upon opening. By contrast, N-, L-, R-, Q- and P-type channels activate at more positive potentials and are therefore termed high-voltage activated. Although these channels have overlapping biophysical profiles, they are distinguished by their pharmacologies\(^4\): N-type channels are specifically blocked by \(\omega\)-conotoxin GVIA; P/Q-type channels are potently, but differentially, blocked by \(\omega\)-agatoxin IVA; L-type channels can be either inhibited or activated by dihydropyridines; and R-type channels are defined by their resistance to these blockers.

A more precise classification has become possible with the advent of molecular cloning techniques that identify the genetic makeup of voltage-gated Ca\(^{2+}\) channels\(^5,6\) (Box 1). As a result, significant advances in our understanding of the structural determinants of Ca\(^{2+}\) channel function and their roles in the functions of excitable tissues have been gained through mutagenesis studies and Ca\(^{2+}\) channel gene knockouts\(^8,9\). It is now widely accepted that N- and P/Q-type Ca\(^{2+}\) channels are the predominant species in presynaptic nerve termini\(^10,11\) and that these channels couple physically to proteins that form the release machinery for synaptic vesicles\(^12\). Ca\(^{2+}\) entry through these channels and the ensuing disruption of the Ca\(^{2+}\)-channel–vesicle protein complex are key steps in fast neurotransmitter release\(^12\). Hence, the cellular mechanisms that regulate the activities of Ca\(^{2+}\) channels and their association with vesicle-release proteins serve to fine tune neurotransmission.

In this article, recent advances in our understanding of the complex interactions between presynaptic Ca\(^{2+}\) channels, cytoplasmic messengers and the neurotransmitter release machinery will be reviewed.

Regulation of presynaptic Ca\(^{2+}\) channels by intracellular messengers

It is well established that the activity of presynaptic Ca\(^{2+}\) channels is modulated extensively by activation of cytoplasmic messenger molecules. Perhaps the most widely documented means of regulating Ca\(^{2+}\) channel activity is the direct inhibition of N- and P/Q-type Ca\(^{2+}\) channels by G proteins\(^14\). At the whole-cell level, activation of G-protein-coupled receptors (GPCRs) results in a decreased current amplitude and slowed activation and inactivation kinetics of these channels (Box 2). Moreover, direct inhibition of G proteins is strongly dependent on membrane potential\(^14\) and can be relieved temporarily by strong membrane depolarizations or rapid trains of action potentials\(^15\). Although this type of modulation was first reported two decades ago\(^16\), many of the underlying mechanisms have been resolved only recently (Box 2). The direct inhibition of presynaptic Ca\(^{2+}\) channels by \(\beta\gamma\)-subunits of G proteins (G\(\beta\gamma\)) has been classified by their distinct electrophysiological and pharmacological profiles into T-, N-, L-, Q-, P- and R-types\(^4\). The T-type (or low-voltage-activated) channels activate at potentials more negative than \(-40\) mV, have a small unitary conductance and inactivate rapidly upon opening. By contrast, N-, L-, R-, Q- and P-type channels activate at more positive potentials and are therefore termed high-voltage activated. Although these channels have overlapping biophysical profiles, they are distinguished by their pharmacologies\(^4\): N-type channels are specifically blocked by \(\omega\)-conotoxin GVIA; P/Q-type channels are potently, but differentially, blocked by \(\omega\)-agatoxin IVA; L-type channels can be either inhibited or activated by dihydropyridines; and R-type channels are defined by their resistance to these blockers.

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Ca²⁺ channels can contain only the five genes encoding putative neuronal α₁-subunit. The α₁-subunit contains four homologous domains (I–IV) that are connected via large cytoplasmic linker regions. Each of the four domains comprises six putative membrane-spanning helices (termed S1–S6) plus a pore-forming P-loop region. The α₁-subunit determines the major functional properties of the channel and defines the Ca²⁺ channel subtype, whereas the other subunits modulate the properties of α₁ (Ref. 1). Several different neuronal Ca²⁺ channel α₁-subunits have been cloned and expressed functionally, revealing that alternative splicing of the gene encoding the α₁-subunit generates both P- and Q-type Ca²⁺ channels; the gene encoding the α₁-subunit forms N-type channels, the genes encoding α₁C, α₁D, and α₁E-subunits generate L-type channels, the genes encoding α₁O, α₁P, and α₁L-subunits form T-type channels; and the gene encoding the α₁-subunit is thought to generate an R-type channel. Four distinct types of ancillary β-subunits (termed β₁–β₄), three genes encoding α₂-δ-subunits and five genes encoding putative neuronal γ-subunits have also been cloned from rat brain. Although the precise roles of the α₂-δ- and γ-subunits and their sites of interaction on the Ca²⁺ channel α₁-subunit remain to be determined, coexpression of the β-subunit modulates the function of the α₁-subunit, which is apparent as an increase in current density (through masking of an endoplasmatic-reticulum-retention signal on the α₁-subunit), modulation of activation and inactivation kinetics, effects on pharmacological properties and modulation of second messenger regulation. The main site of interaction of the β-subunit on the Ca²⁺ channel α₁-subunit is localized to the cytoplasmic loop connecting domains I and II, with additional interactions occurring in the C-terminal region.

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Box 1. Structure and subunit composition of voltage-gated Ca²⁺ channels

High-voltage-activated Ca²⁺ channels are heteromultimeric proteins formed by association of multiple subunits (α₁, α₂-δ, β and γ) (Fig. I), whereas low-voltage-activated Ca²⁺ channels can contain only the α₁-subunit. The α₁-subunit contains four homologous domains (I–IV) that are connected via large cytoplasmic linker regions. Each of the four domains comprises six putative membrane-spanning helices (termed S1–S6) plus a pore-forming P-loop region. The α₁-subunit determines the major functional properties of the channel and defines the Ca²⁺ channel subtype, whereas the other subunits modulate the properties of α₁ (Ref. 1). Several different neuronal Ca²⁺ channel α₁-subunits have been cloned and expressed functionally, revealing that alternative splicing of the gene encoding the α₁-subunit generates both P- and Q-type Ca²⁺ channels; the gene encoding the α₁-subunit forms N-type channels, the genes encoding α₁C, α₁D, and α₁E-subunits generate L-type channels, the genes encoding α₁O, α₁P, and α₁L-subunits form T-type channels; and the gene encoding the α₁-subunit is thought to generate an R-type channel. Four distinct types of ancillary β-subunits (termed β₁–β₄), three genes encoding α₂-δ-subunits and five genes encoding putative neuronal γ-subunits have also been cloned from rat brain. Although the precise roles of the α₂-δ- and γ-subunits and their sites of interaction on the Ca²⁺ channel α₁-subunit remain to be determined, coexpression of the β-subunit modulates the function of the α₁-subunit, which is apparent as an increase in current density (through masking of an endoplasmatic-reticulum-retention signal on the α₁-subunit), modulation of activation and inactivation kinetics, effects on pharmacological properties and modulation of second messenger regulation. The main site of interaction of the β-subunit on the Ca²⁺ channel α₁-subunit is localized to the cytoplasmic loop connecting domains I and II, with additional interactions occurring in the C-terminal region.

Important consequences for neurotransmission. For example, opioid receptor agonists such as morphine depress transmission of pain signals via inhibition of N-type Ca²⁺ channels through the G-protein pathway. Further, the relief of tonic G-protein inhibition following repetitive membrane depolarization might contribute to the magnitude of paired pulse facilitation at the synaptic terminal, indicating a role in short-term plasticity.

By contrast, the activation of protein kinase C (PK C) upregulates the activities of N-type Ca²⁺ channels. This effect can occur via two mechanisms. First, in some isoforms of the N-type Ca²⁺ channel, there appears to be an intrinsic increase in channel activity following PKC-dependent phosphorylation. Second, the activation of PKC antagonizes G-protein-mediated inhibition of the channel, resulting in an increased level of current activity by removal of tonic G-protein inhibition. The direct upregulation might be due to phosphorylation of either one of two residues (Thr422 or Ser425) located within one of the G-protein-binding sites in the N-type Ca²⁺ channel domain I–I1 linker (Box 2), whereas the effect on G-protein inhibition is mediated exclusively by Thr422 (Ref. 22).

Interestingly, this phenomenon appears to be a unique feature of the Gβ1-subunit. Thus, the extent of crosstalk between G protein and PKC pathways could vary with receptor type depending on how effectively a GPCR is coupled to this Gβ1-subunit isoform. Together with the notion that the different types of Gβ1-subunits inhibit N- and P/Q-type channels to varying degrees (Box 2), this might offer the potential of fine-tuning the amount of Ca²⁺ entering the presynaptic terminal and, hence, neurotransmission.

Recently, a GPCR-mediated inhibition of N-type Ca²⁺ channels has been reported that is independent of voltage. This phenomenon can also be triggered by cytoplasmic application of Gβγ and appears to occur concomitantly with the direct G-protein inhibition described above but is not reversed by membrane depolarization. It has been suggested that this occurs via a Gβγ-mediated activation of tyrosine kinase phosphorylation of the α₁-subunit of the N-type channel, which results in depression of current activity through interaction with a regulator of protein kinase (RG51)27. However, the exact molecular mechanisms that underlie this inhibition are still under investigation, and it is not
clear if P/Q-type channels, or other types of Ca\(^{2+}\) channels, are subject to this type of regulation. Furthermore, it is not known if this pathway is dependent on the G\(\beta\)-subunit isoform. Nonetheless, tyrosine phosphorylation has the propensity to emerge as another important regulatory mechanism of Ca\(^{2+}\) homeostasis in the presynaptic nerve terminal.

**Interactions between Ca\(^{2+}\) channels and synaptic proteins**

The release of neurotransmitter from presynaptic nerve terminals depends crucially on the concentration of free intracellular Ca\(^{2+}\) at the sites of release. To permit a localized increase of Ca\(^{2+}\) at the release sites without overloading the cell with cytotoxic levels of Ca\(^{2+}\), vesicles must be physically docked near the sources of Ca\(^{2+}\) influx, that is, presynaptic Ca\(^{2+}\) channels12. Indeed, two proteins of the presynaptic vesicle-release complex, syntaxin 1A and SNAP-25 (25 kDa synaptosomal-associated protein) (also termed t-SNARE proteins), interact physically with the domain II–III linker region of both \(\alpha_{1}\)A and \(\alpha_{1}\)A channels in a Ca\(^{2+}\)-dependent manner28,29 (Table 1). These proteins concomitantly interact with the proteins synaptotagmin and synaptobrevin (termed v-SNARE proteins) on the vesicle membrane, thereby bringing the vesicles close to the channels and, thus, the source of Ca\(^{2+}\) (Refs 12,30). Entry of Ca\(^{2+}\) through these channels is thought to result in a disruption of the synaptic protein core complex, leading to vesicle fusion with the plasma membrane13,31,32. Consistent with this, neurotransmission is inhibited by intracellular application of synthetic peptides directed against the region of the N-type Ca\(^{2+}\) channel that binds syntaxin33,34. Thus, the association of presynaptic Ca\(^{2+}\) channels with SNARE proteins is fundamentally important for neurotransmitter release.

Syntaxin 1A and SNAP-25 not only tether vesicles to the channels but also appear to modulate channel activity. Coexpression of P/Q-type or N-type Ca\(^{2+}\) channels with syntaxin 1A results in a ~15 mV hyperpolarizing shift in the midpoint of the steady-state inactivation curve of the channels, thereby reducing the probability of channel opening35–38. Similar effects were reported for the syntaxin 1B isoform and for SNAP-25; however, when both syntaxin 1A and SNAP-25 were coexpressed, the hyperpolarizing shift is ablated, restoring the...
Box 2. Molecular determinants of inhibition of presynaptic Ca\(^{2+}\) channels by G proteins

Expression studies indicate that inhibition of presynaptic Ca\(^{2+}\) channels by G proteins is mediated by direct interactions between the dimer formed by G-protein \(\beta\gamma\)-subunits\(^{\text{a,b}}\) and the linker region between domains I and II of the Ca\(^{2+}\) channel \(\alpha\)-subunit\(^{\text{c,d}}\), with G\(\beta\) being the active G-protein species (Fig. I). Additional regions on the Ca\(^{2+}\) channel \(\alpha\)-subunit that participate in modulation of G\(\beta\gamma\) could include the N-terminus\(^{\text{e}}\), the C-terminal region\(^{\text{i}}\) and portions of domain I (Refs h,i,j). Kinetic analyses of the G-protein effects at the single-channel level suggest that 1:1 binding of G\(\beta\gamma\) to the Ca\(^{2+}\) channel \(\alpha\)-subunit stabilizes the closed conformation of the channel and that virtually all of the effects observed at the whole cell level might be due to an increased first latency to opening of the channel. Moreover, G proteins must physically dissociate from the channel complex before channel opening (but see Ref. k). For the most part, direct G-protein inhibition of Ca\(^{2+}\) channels is seen most extensively with N- and P/Q-type channels\(^{,}\), although \(\alpha\)\(\text{I}\) (R-type) Ca\(^{2+}\) channels are also susceptible under certain circumstances\(^{\text{m,n}}\). It was shown recently that the degree of inhibition is dependent on the type of G-protein \(\beta\)-subunit\(^{\text{p,q}}\), with a much less pronounced dependence on the nature of the G\(\gamma\)-subunit\(^{\text{b}}\). Furthermore, different types of G-protein \(\beta\)-subunits differentially affect N-type and P/Q-type channels, with N-type channels typically undergoing a larger degree of inhibition\(^{\text{r}}\). Recent evidence indicates that a family of regulators of G-protein signaling (RGS) proteins might modulate G-protein inhibition of Ca\(^{2+}\) channels. For receptor-mediated inhibition, some RGS proteins increase the rate of GTP hydrolysis of the G\(\alpha\)-subunit, thus favoring its reassociation with the G\(\beta\)-subunit and antagonizing their effect on the channel\(^{\text{s,t}}\). In addition, several RGS proteins contain a G-protein \(\gamma\)-subunit-like domain that competes with G\(\gamma\) for the G\(\beta\)-subunit, thereby possibly preventing the effects of G\(\beta\gamma\) on Ca\(^{2+}\) channels\(^{\text{u,v}}\). Despite considerable efforts\(^{,}\), the exact structural determinants of the G\(\beta\gamma\)-subunit that governs interactions with N-type Ca\(^{2+}\) channels have remained largely elusive.

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be cautious in extrapolating these effects to intact neurons. Moreover, intact presynaptic terminals contain additional proteins that could modulate the effects of SNARE proteins on voltage-gated Ca\(^{2+}\) channels. For example, it was recently shown that two modular adaptor proteins, Mint1 and CASK (Ca\(^{2+}\)-calmodulin-dependent serine protein kinase), interact with the C-termini of presynaptic Ca\(^{2+}\) channels (Table 1) but not other Ca\(^{2+}\) channel isoforms\(^{38}\). Although direct effects of these interactions on Ca\(^{2+}\) channel function are not yet described, they could profoundly affect either Ca\(^{2+}\) channel activity or their functional interactions with SNARE proteins. The interaction between presynaptic Ca\(^{2+}\) channels and SNARE proteins is not necessarily unidirectional. It has been shown that the expression
of some isoforms of α1A, but not other types of Ca2+ channels, triggers the expression of syntaxin 1A in tsA-201 cells in a Ca2+-dependent manner. In addition, selective blockade of endogenous P/Q-type channels in cerebellar granule cells with ω-agatoxin IVA decreases endogenous levels of syntaxin 1A. These effects are dependent on activation of cAMP-dependent protein kinase (PKA), calmodulin-independent protein kinase II (CaMKII) and mitogen-activated protein kinase, and are consistent with the involvement of the cAMP response element-binding protein (CREB) pathway. The notion that syntaxin 1A expression depresses activity of P/Q-type Ca2+ channels suggests that an activity-dependent feedback mechanism could contribute to synaptic activity and the regulation of presynaptic Ca2+ levels.

**Coupling between second messenger regulation and SNARE protein interactions**

The synaptic protein interaction (synprint) site in the domain II–III linker of N-type Ca2+ channels that binds both syntaxin 1A and SNAP-25 contains several putative phosphorylation sites for threonine and serine kinases. Under certain conditions, phosphorylation of fusion proteins of this region by PKC and CaMKII results in a complete loss of syntaxin 1A and SNAP-25 binding in vitro, whereas phosphorylation by either PKA or protein kinase G is ineffective. Under physiological conditions, activation of PKC does not appear to completely prevent binding of syntaxin 1A to the channel but, nonetheless, it prevents the hyperpolarizing shift in steady-state inactivation.
Table 1. Protein interaction sites on the α1A-subunit of the N-type Ca\(^{2+}\) channel

<table>
<thead>
<tr>
<th>Protein</th>
<th>Region</th>
<th>Amino acid position in the rat α1A-subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+}) channel β-subunit</td>
<td>I–II linker</td>
<td>378–396</td>
</tr>
<tr>
<td>Ca(^{2+}) channel β-subunit</td>
<td>C-terminal</td>
<td>2013–2069</td>
</tr>
<tr>
<td>G-protein βγ-subunits</td>
<td>I–II linker</td>
<td>372–389, 410–428</td>
</tr>
<tr>
<td>G-protein βγ-subunits</td>
<td>C-terminal</td>
<td>2013–2069</td>
</tr>
<tr>
<td>Syntaxin 1A</td>
<td>II–III linker</td>
<td>718–963</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>II–III linker</td>
<td>718–963</td>
</tr>
<tr>
<td>Cysteine string protein</td>
<td>II–III linker</td>
<td>718–963</td>
</tr>
<tr>
<td>Mint1</td>
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<td>2331–2336</td>
</tr>
<tr>
<td>CASK</td>
<td>C-terminal</td>
<td>2020–2233</td>
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</tbody>
</table>

*Abbreviations: CASK, Ca\(^{2+}\)-calmodulin-dependent serine protein kinase; SNAP-25, 25 kDa synaptosomal-associated protein.

mediated by syntaxin 1A (Ref. 38). This is consistent with at least a partial disruption of syntaxin-1A–N-type-channel interactions in vivo and, together with the PKC-mediated enhancement of channel activity described earlier, provides an additional avenue for increasing N-type current amplitude. However, the PKC-mediated disruption of syntaxin 1A and SNAP-25 binding suggests that the increased Ca\(^{2+}\) influx is unlikely to participate in neurotransmission, but could modulate other Ca\(^{2+}\)-dependent processes.

Whereas PKC antagonizes syntaxin binding, the interactions between syntaxin 1A and N-type Ca\(^{2+}\) channels appear to promote inhibition of these channels by G proteins. The first indication of an interaction between syntaxin and G proteins came from experiments in chick ciliary ganglion cells where inhibition of N-type Ca\(^{2+}\) channels by Gβγ is abolished following activation of PKC or in the presence of botulinum toxin C1, which prevents interaction of N-type channels with Gβγ. This indicated that syntaxin 1A might be an important factor in direct G-protein inhibition of N-type Ca\(^{2+}\) channels. Indeed, coexpression of N-type Ca\(^{2+}\) channels with syntaxin 1A promotes a tonic inhibition of the channels by Gβγ that depends on the association of syntaxin 1A with the synprint region of the channel (Ref. 42). However, the presence of syntaxin 1A is not a prerequisite of G-protein inhibition. Rather, biochemical studies showing that syntaxin 1A can physically interact with Gβγ (Ref. 43) support a role as a chaperone between Gβγ and N-type Ca\(^{2+}\) channels. In neurons of the chick dorsal root ganglion, intracellular application of purified Gβγ only promotes voltage-independent inhibition of N-type channels unless syntaxin 1A is exogenously expressed (Ref. 26). Interestingly, these neurons only contain the syntaxin 1B isoform, which does not promote a G-protein-dependent inhibition of N-type channels in transient expression systems. Together with the effects of botulinum toxin in ciliary ganglia, this indicates that the effects of syntaxin 1A on G-protein-mediated inhibition are a physiological feature of N-type Ca\(^{2+}\) channels.

More recently, cysteine string protein (CSP), another synaptic vesicle protein, was shown to promote G-protein-dependent inhibition of N-type channels (Ref. 38). Like syntaxin 1A, CSP interacts with the synprint regions of N-type and P/Q-type Ca\(^{2+}\) channels (Ref. 42, 44, 45) (Table 1) and physically interacts with Gβγ in vitro. This suggests that these two proteins could act via a similar mechanism. Although knock-out of the gene encoding CSP in Drosophila causes impaired neurotransmission (Ref. 46), the precise role of CSP is poorly understood and its effects on G-protein signaling in vivo remain to be established.

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Concluding remarks

Regulation of the activity of Ca\(^{2+}\) channels in the presynaptic nerve terminal and the interactions between these channels and the vesicle-release machinery are complex (Fig. 1). Many research groups are working on the molecular interactions between SNARE proteins; however, a detailed understanding of neurotransmission requires the incorporation of these interactions with those of second messengers and voltage-gated Ca\(^{2+}\) channels.

Fig. 1. Functional interactions between N-type Ca\(^{2+}\) channels, second messengers and SNARE proteins. Gβγ-subunits inhibit N-type Ca\(^{2+}\) channels, whereas protein kinase C (PKC) upregulates channel activity both directly and by antagonizing the inhibition of the channel by Gβγ. Syntaxin 1A inhibits N-type channels by promoting inactivation and by enhancing their inhibition by G proteins. The effect of syntaxin 1A on Ca\(^{2+}\) channel inactivation is abolished following activation of PKC or in the presence of SNAP-25 or nSec-1. Abbreviations: CSP, cysteine string protein; SNAP-25, 25 kDa synaptosomal-associated protein.
Although we are only beginning to unravel the functional significance of these complexities, they could provide a tremendous potential for the precise regulation of Ca\(^{2+}\) influx into synapses and thus the fine-tuning of neurotransmitter release. Much of our current knowledge is based on in vitro work and on studies in functional expression systems. The extent to which these interactions occur in vivo and how they affect neurotransmission will be greatly facilitated by the increased availability of knockout models and viral-expression techniques and it is likely that our understanding of the molecular mechanisms that modulate neurotransmission will advance significantly in the near future.

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