

## How to Lose Your Hippocampus by Working on Chloride Channels

Chloride channels are the problem children of ion channels. By comparison, the well-behaved  $K^+$ ,  $Ca^{2+}$ , and  $Na^+$  channels are understood to high levels of precision, with most of the remaining questions surrounding the detailed movements of their amino acids during gating. Even the holy grail of ion channels, the structural mechanism of cation selectivity, has largely been laid to rest by crystallography, while the chloride channels continue to provide confounding results at the level of site-directed mutagenesis. In this issue of *Neuron*, a knock-out (KO) mouse of one chloride channel subtype reminds us of the importance of this recalcitrant group of channels (Stobrawa et al., 2001). Stobrawa, Jentsch, and colleagues, in attempting to straighten out a controversy surrounding the role of swelling-activated chloride channels, knocked out the CIC-3 gene and stumbled across the way to completely eliminate the hippocampus. But the paper is a double-edged sword that will also open the veins of controversy surrounding swelling-activated chloride channels.

Ion channels are electrical switches that rapidly change transmembrane voltages. In doing so, they are the fastest signal transduction mechanism known to biology, permitting roughly ten million ions to flow through each proteinaceous hole per second. While most ion channel research has been on those spanning the plasma membranes, many ion channels function in intracellular membranes such as the nucleus, mitochondria, and endoplasmic reticulum. Due to the difficulties in the direct measurement of these channels, much less is known about their detailed physiological roles. Intracellular channels, like their plasma membrane counterparts, change transmembrane potentials and ultimately alter the distribution of important ions such as  $Ca^{2+}$  and  $H^+$ .

The chloride-selective channels are a particularly diverse group of proteins that have been constructed by multisubunit ligand-gated proteins ( $\gamma$ -aminobutyric acid, glycine channels), the cystic fibrosis transmembrane regulator (CFTR) protein, and the  $Cl^-$  channel (CLC) class of chloride channels. The mammalian CLC genes are a family of nine, divided by sequence similarity into three branches (Jentsch et al., 1999). Unlike cation channel genes, they encode 12 transmembrane-spanning channel subunits and their selectivity and gating are poorly understood. Alterations in CIC-1 give rise to myotonia congenita, while mutant CIC-5 results in Dent's disease, a defect in kidney salt transport. One of the most troublesome subfamilies of the nine mammalian genes is comprised of CIC-3, -4, and -5, with CIC-3 being alternatively spliced at the amino terminus to produce long and short forms. CIC-5 disruption in mice produces a defect in renal proximal tubular endocytosis, including calciotropic hormones, probably as a

result of defective endosomal acidification (Piwon et al., 2000).

Prior to the elucidation of the CLC genes, electrophysiologists had characterized a number of chloride currents roughly divided into voltage activated (CIC-1 being the firmly established and responsible member), calcium activated, and swelling activated. With the discovery of Cl channel genes, experimentalists have been busy trying to match the known currents with their molecular counterparts. But a number of problems unique to Cl channels have led to a murky functional subclassification. First, intracellular Cl concentrations are not as firmly set as many cations, yielding a range of Nernst potentials from  $-15$  to  $-65$  mV. Selectivity between halide ions (F, Br, Cl, I) is low and, although used to try to distinguish channel types, is prone to error. Chloride channels are ubiquitous—no mammalian cell or cell line is known to lack them. There are no highly specific toxins or small molecule blockers. Whole-cell  $Cl^-$  channel kinetics are confusingly similar and, since many are regulated by nucleotides and kinases, slowly change with time. Finally,  $Cl^-$  channels may be heteromultimers of  $Cl^-$  channel protein subunits. The presence of numerous and complex  $Cl^-$  currents in the common expression system, *Xenopus laevis* oocytes, has misled many investigators into confusing their newly minted clones with intrinsic currents. One of the most sought-after Cl channels is the swelling-activated or volume-sensitive outwardly rectifying anion conductance (for review, see Hume et al., 2000). These problems have contributed to the prior misidentification, in my opinion, of this swelling-activated chloride current as being several different proteins, including both the human multidrug resistance P-glycoprotein (Valverde et al., 1992) an pICln, a protein which was expression cloned based on the ability to activate chloride sensitive outward currents in *Xenopus* oocytes (Paulmichl et al., 1992). CIC-2 appears to be volume activated, but is not the typical swelling-activated channel commonly observed in many cells (Grunder et al., 1992).

After the CLC gene family was discovered by Jentsch and colleagues, Kawasaki et al. (1994) reported the expression of CIC-3 in oocytes and CHO cells. The authors reported a large weakly outward rectifying chloride current that did not inactivate. The current was completely blocked by activation of protein kinase C. Subsequently, Duan et al. (1997) reported that expression of CIC-3 in NIH/3T3 fibroblasts gave rise to huge (30 nA or 500 pA/pF) outwardly rectifying, steeply inactivating currents when exposed to hypotonic solutions and concluded that CIC-3 was the swelling-induced channel. Subsequently, the same group used an anti-CIC-3 antibody to block the swelling-activated chloride current in oocytes, and smooth and cardiac muscle (Duan et al., 2001). Wang et al. (2000) used CIC-3 antisense in ciliary epithelial cells to decrease CIC-3 expression (observed as a decrease mainly in nuclear immunofluorescence) and noted a concomitant decrease in swelling-activated current. However, these authors noted major differences between the swelling-activated Cl current of the epithe-

lial cells (in kinetics and sensitivity to inhibition by ATP and PKC) and those reported for CIC-3 by Duan et al. (1997) and concluded that CIC-3 was not the only, or even the major, swelling-activated current in the epithelial cells. Weinman and colleagues (Shimada et al., 2000) expressed both the short and long forms of CIC-3 and recorded constitutively active currents. The short form, virtually identical to all previously studied CIC-3, was strongly outwardly rectifying but did not inactivate, unlike the current recorded by Duan et al. (1997). Reassuringly, the currents expressed by Shimada et al. (2000) resembled the currents of its cousins, CIC-4 and CIC-5 (Friedrich et al., 1999). Interestingly, several groups have failed to find any functional plasma membrane current (see, e.g., Friedrich et al., 1999) when CIC-3 is expressed, but as usual, negative results are not published in detail.

Thus, leading up to the current paper, the field could be divided into proponents of CIC-3 being *the* swelling-activated current, those who believe it is *only* an intracellular channel, and those who believe it is a *component* of the swelling-activated current. It does not seem likely that all the groups' data can be completely reconciled by proposing missing ancillary subunits or heteromultimerization between CLC subtypes. Undoubtedly, the field has suffered from the problems encountered with separating chloride currents as mentioned above, but it is also likely that the field would have benefited from reporting all recordings, including negatives, of cells in which the protein was known to be expressed by GFP tagging. Alternatively, double blind recordings of the swelling-activated current in expressing and control cells would probably have simplified the current picture.

Now Stobrawa, Jentsch, and colleagues in this edition of *Neuron* report the disruption of the CIC-3 gene in mice. RNase protection assays and immunoblotting membrane proteins verified a complete absence of normal CIC-3 in *Clcn3*<sup>-/-</sup> (KO) mice. The authors show that CIC-3 gene deletion resulted in practically complete hippocampal and retinal degeneration. The authors carefully and completely document these effects at the level of gross morphology, function, and behavior.

This paper will come as a challenge to the proponents of CIC-3 as the swelling-activated current since Stobrawa et al. (2001) measure normal swelling-activated currents in hepatocytes and pancreatic acinar cells of the CIC-3 KO mice (see their Figure 6). Perhaps CIC-4 or CIC-5 was upregulated to compensate for CIC-3 and comprise the swelling-activated current still present? The authors mitigate against this conclusion since CIC-4 and -5 do not closely resemble the swelling-activated current and they found no evidence of compensatory increased expression of CIC-4 or -5 in brains of KO mice. Stobrawa et al. (2001) clearly and forcefully argue for the role of CIC-3 as a purely intracellular channel. By immunostaining, they find that CIC-3 is present in intracellular membranes as identified the endosomal markers rab-4 and lamp-1, as well as in synaptic vesicles identified by synaptophysin. Furthermore, the CIC-3 protein was not detected in the plasma membrane.

The authors go to substantial length to try to understand the defect that results in neuronal cell death, in the hippocampus between 2 weeks and 2 months and in the retina between 2 weeks and 1 month after birth.

By 2 months, the hippocampus is gone, leaving a large cavity next to the brain ventricles. By 1 month, the retinal photoreceptors have degenerated. Stobrawa et al. (2001) demonstrate that synaptic vesicle acidification is impaired and suggest that CIC-3 is required for vesicular acidification in order to balance charge differences induced by proton pumping. They show that known synaptic GABA transporter and H-ATPase expression is not altered, but that the glutamate transporter protein level is decreased by half. The primary hypothesis that the synaptic vesicles in the hippocampus are overloaded with glutamate. This is an indirect consequence of the fact that V-type H-ATPase acidifies the synaptic vesicle but needs CIC-3 to exchange the anion chloride in order to preserve charge balance. The degeneration of retinal cells may be more complex, but again hinges on the idea that acidification of vesicles is necessary for their normal targeting and function. Regardless of the detailed mechanisms for neuronal cell death in CIC-3 KO mice, it may provide a useful model for studying the function of the hippocampus. Standard spatial memory tests such as the Morris water maze are not feasible since the mice are blind, but it may be possible to make a targeted KO that spares the retina but still eliminates the hippocampus.

For the field of chloride channels, this paper will stimulate research on many levels. In the short term, arguments will be refined about the role of CIC-3. In my view, the short form of CIC-3 is probably both a plasma membrane and intracellular channel in overexpression systems. It almost certainly plays a physiological role in wild-type cells as an intracellular channel. In some cells, it may be part of the complement of plasma membrane currents, but whether as a monomer or heteromultimeric subunit will have to be sorted out in more detail. Given the preponderance of evidence, it seems unlikely that it is the long-sought swelling-activated chloride conductance. But the antibody data of Duan et al. (2001) is still a major piece of support for its role in swelling and begs for replication by other laboratories. An important control will be to test CIC-3 KO tissues for specificity of the antibody. Are there any more candidates for this channel besides pGp, pICln, CIC-2, or CIC-3? None seem obvious, and I suspect its elucidation will be both contentious and difficult. Regardless of the outcome, Stobrawa et al. (2001) have shown once again that chloride channels are full of surprises.

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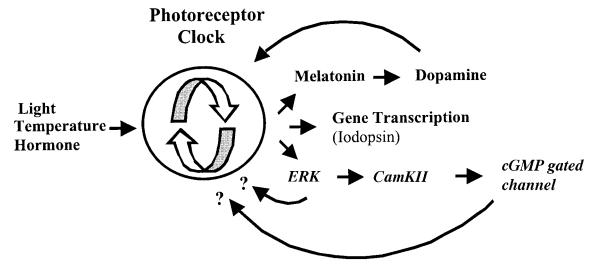
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## Coupling an Activated MAP Kinase to Circadian Clock Output

Circadian rhythms, generally detected as ~24 hr oscillations in behavioral, physiological, or biochemical processes, are widespread in organisms ranging in complexity from the blue-green algae to flowering plants and man. Endogenous circadian clocks drive such rhythms. Recently, the analysis of model organisms has resulted in two major generalizations (reviewed in Dunlap, 1999). First, circadian clocks appear to be cell autonomous oscillators. Even in multicellular systems where overt physiological rhythms require intercellular signaling, rhythm generation is fundamentally a cellular process. Second, at their core circadian clocks involve self-sustained oscillations in gene expression. For example, in flies and mice, the bHLH transcriptional activators CLOCK and BMAL (also called CYCLE) drive transcription of the clock genes *Period*, *Timeless*, or *Cryptochrome*. The resulting proteins dimerize (in different combinations depending on the species) and inhibit CLOCK/BMAL activated transcription. This alternation of positive and negative regulation results in persistent rhythms of mRNA and protein, which represent the core oscillation.

To be physiological, relevant oscillations in clock gene expression must be entrained (synchronized) to environmental cycles such as light and must be coupled to important output pathways (see figure). The molecular details of signaling pathways coupling circadian clocks to both inputs and outputs represents fertile ground and data appear to be accumulating rapidly. A major advance for photoreceptor clocks is reported by Ko et al. (2001) in this issue of *Neuron*.

The retina has long been recognized as the site of a



Hypothetical Relationship of a Photoreceptor Circadian Clock to Input and Output Pathways

Dopamine can be regulated by melatonin and is known to directly effect the clock. Feedback of other output pathways is hypothetical.

circadian clock that controls a wide range of local rhythms (Besharse and Iuvone, 1983). The synthesis of melatonin, rod-cone dominance, retinomotor movements, components of the electroretinogram, visual sensitivity, gene transcription, and photoreceptor membrane turnover all exhibit persistent rhythms in constant conditions (reviewed in Cahill and Besharse, 1995). Furthermore, circadian clock properties have been localized to retinal photoreceptors in both the African clawed frog (Cahill and Besharse, 1993) and chicken (Pierce et al., 1993). In the chicken, a rhythm of transcription of the cone photopigment gene (*iodopsin*) persists in dissociated culture under constant conditions implicating cones as clock cells. Now Ko et al., using a similar culture system, show that the Erk form of mitogen activated protein kinase and  $Ca^{2+}$ /calmodulin-dependent protein kinase II (*CamKII*) are part of a signaling pathway regulating a rhythm in the gating properties of the cGMP-gated channel.

Using excised patches from the cell bodies of embryonic photoreceptors, Ko et al. show an ~2-fold higher affinity of the cGMP-gated channel for its ligand at night. The rhythm persists for at least 2 days in constant darkness, can be entrained to light–dark cycles, and results from posttranslational modification of the channel. This is the first report of a circadian rhythm at the primary level of visual transduction, but its functional consequences for vision are not entirely clear. The photoreceptors in this analysis lack fully formed outer segments, but appear to be cones. The properties of the channel match those of mature cones, and the cultures are enriched in cones. Assuming that a similar rhythm occurs in fully differentiated cones, the measured affinity difference could result in a notable difference in the photoreceptor dark current and responsiveness to light at different times of day. It is also likely that events occurring at the level of photoreceptor transduction in cones could contribute to more complex circadian changes seen in the avian electroretinogram.

Ko et al. also demonstrate antiphasic rhythms in the phosphorylated (active) forms of Erk and CamKII with peak activities during the night and day respectively. This along with the finding that selective inhibition of the phosphorylation and activation of Erk or CamKII blocks changes in channel affinity in a manner dependent on time of day, strongly suggests that Erk and CamKII are components of a pathway that increases

channel affinity at night and decreases it during the day. Interestingly, inhibition of Erk phosphorylation also inhibits rhythmic changes in CamKII, while inhibition of CamKII phosphorylation does not affect changes in p-Erk. Based on this finding, the authors suggest that Erk is upstream of CamKII in a signaling pathway from the clock (see figure).

The simplest and most plausible interpretation is that the cultured photoreceptors are cell autonomous oscillators driving changes in Erk and CamKII to control physiology at the level of the cGMP-gated channel. A potential caveat is that the measured changes were from mixed cell cultures. Although the cultures were enriched in photoreceptors, the factors driving circadian changes in Erk and CamKII are not known and could involve intercellular signals. Within the intact retina, the diffusible modulators melatonin and dopamine, driven at least in part by circadian oscillators, play such a role, and dopamine receptors that modulate cAMP levels are found on photoreceptors (Cahill and Besharse, 1993, 1995). Molecular definition of the mechanisms that couple the "clock" to changes in Erk and CamKII phosphorylation would clarify this issue.

Like most significant contributions, that of Ko, Ko, and Dryer may prove most important in raising new questions. For example, how does Erk and CamKII activity control channel gating? Previous work has shown that the cGMP-gated channel of rods can be modified by phosphorylation (Molokanova et al., 1997) and by Ca<sup>2+</sup>/calmodulin binding (Hsu and Molday, 1993). However, in the absence of direct analysis of channel phosphorylation by Erk and CamKII, it remains possible that the effects are indirect.

Likewise, the molecular mechanism coupling the clock to rhythmic changes in Erk and CamKII activity is not directly addressed. Circadian clocks often control rhythmicity through transcriptional regulation of downstream genes, and this can occur through a mechanism similar to that of CLOCK/BMAL regulated transcription of oscillating clock genes. However, Erk protein abundance does not vary during the day; it is p-Erk that is rhythmically controlled. Thus, one must look upstream of MEK1, the enzyme responsible for activating Erk, or to the phosphatases necessary for Erk dephosphorylation. It is also likely that cross-talk between Erk and other signaling pathways is involved. For example, dopamine-induced decreases in cAMP at night are known to mediate phase shifts in the core photoreceptor oscillator (Hasegawa and Cahill, 1999), and the cAMP pathway can lead to increased activation of Erk in some system.

Recent reports relating Erk activation to circadian phase shifting suggests a more general role for p-Erk in clock cells. For example, a rhythm in p-Erk similar to that reported by Ko et al. has also been seen in chick pinealocytes. Here light acutely reduces p-Erk at night, and inhibition of Erk phosphorylation appears to phase shift the clock (Sanada et al., 2000). p-Erk has also been implicated in circadian phase shifting in the mammalian suprachiasmatic nucleus (Obrietan et al., 1998). Interestingly, cross-talk with a cAMP signaling system involved in phase shifting (Ginty et al., 1993) may be of significance in the mammalian systems.

The studies on phase shifting differ from that of Ko et al. in that p-Erk is proposed to be in an "input" path-

way to the clock. Although based on different systems that could use p-Erk signaling in different ways, these data raise the interesting question of whether p-Erk plays a role both as an "output" and an "input" of the clock. Recent modeling of circadian clocks suggests that rhythmic clock outputs can feedback onto the core oscillator to increase its overall stability (Roenneberg and Mellow, 2000). What is needed now is to determine whether p-Erk plays a role in both phase shifting and in output pathways in the same cellular clock system.

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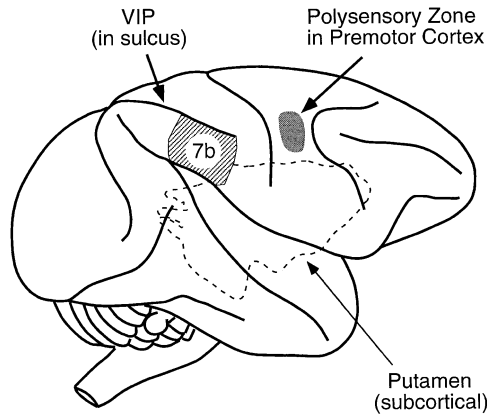
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## A System of Multimodal Areas in the Primate Brain

The primate cerebral cortex has traditionally been divided into separate territories for vision, touch, audition and movement. These functions are known to overlap in many parts of cortex, but until recently the regions of overlap were not well studied. In this issue of *Neuron*, Bremner et al. (2000) report a major advance in understanding at least one set of areas in the human brain in which the senses are integrated. This finding joins a growing set of work in monkeys and humans on the integration of the senses with each other and with the control of movement.

Vision, touch, and audition converge in many areas of the monkey brain, including the deep layers of the

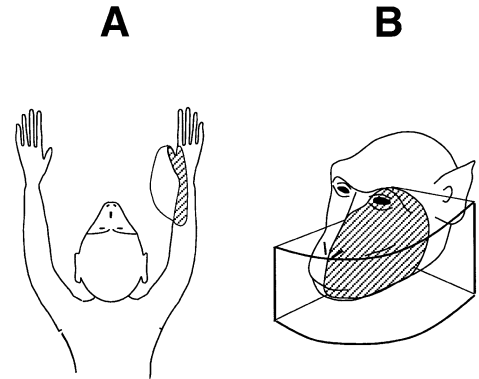


Side View of a Macaque Monkey Brain Showing the Location of Four Interconnected Multimodal Areas  
VIP: ventral intraparietal area.

superior colliculus, the superior temporal polysensory area, the putamen, the intraparietal sulcus, and parts of premotor cortex (Graziano and Gross, 1996). Multimodal responses can also be induced in a variety of brain areas with appropriate training. Tell a person to imagine a picture of a cat, and visual cortex becomes active; in this case, an entrained auditory signal activates a part of cortex that is usually only visual (Goldenberg et al., 1989). Train a monkey to associate a felt object or a sound with a picture, and visual cortical areas respond to these nonvisual stimuli (Heanny et al., 1988; Colombo and Gross, 1994). In other words, perhaps as a function of mental imagery, the neurons can respond to a range of sensory stimuli.

Among this catalog of multimodal brain areas, one interconnected set of areas stands out for its distinctive response properties. These areas are shown in the first figure in a side view of the monkey brain. Neurons in these areas respond to the sight, sound, and feel of objects moving in the space near the body, usually within reaching distance (Graziano and Gross, 1998). These responses do not depend on training the monkey on a task and are robust even in anesthetized monkeys. Most of these multimodal neurons are directionally selective. They may respond, for example, to a tactile stimulus swept across the skin in one direction, but not in the opposite direction. The same neurons will usually have a matching directional preference for visual stimuli moving in the space near the tactile receptive field. Some neurons prefer visual stimuli moving toward or away from the body.

These multimodal response have been studied most thoroughly in monkey premotor cortex (Rizzolatti et al., 1981; Graziano et al., 1997). Though sensory responses can be found throughout all of premotor cortex, especially in trained monkeys, this particular type of multimodal response to nearby objects is found only in a few clusters usually just posterior to the bend in the arcuate sulcus. The second figure shows the response properties of two bimodal, visual-tactile neurons studied in premotor cortex. In each case, the neuron responded when the monkey saw or felt an object near a particular part of the body. Other neurons in premotor cortex are



Two Examples of Bimodal, Visual-Tactile Neurons from Premotor Cortex

In both cases, the tactile receptive field (stippled) matched the location of the visual receptive field (outlined).

trimodal, responding to tactile, visual, and auditory stimuli. Remarkably, these trimodal neurons are able to distinguish the distance to the sound source; they respond best to nearby sounds regardless of the intensity of the stimulus (Graziano et al., 1999).

Almost identical neuronal responses encoding the space near the body have been described in the putamen and also in areas 7b and VIP in the parietal lobe (Colby et al., 1993; Graziano and Gross, 1996). These brain areas are all monosynaptically interconnected and therefore appear to form a single integrated system. However, even though the sensory properties of these neurons have been extensively characterized, their function is still unknown. One hypothesis is that they detect and localize threatening objects near the body and help to organize a defensive reaction.

Does the human brain also contain a set of areas that processes the sight, sound, and feel of objects moving in the space near the body? Bremmer et al. (2000) presented visual, tactile, and auditory stimuli to human subjects in an MRI scanner. These three types of stimulation were presented on separate trials. The tactile stimulus was a stream of air blowing across the face. The visual stimulus was a display of moving dots on a screen. The auditory stimulus, presented through headphones, gave the illusion of a sound source travelling in front of the subject's face from one side to the other. As expected, the moving visual stimulus, when compared to a control stimulus that was stationary, activated areas in the occipital, temporal, and parietal lobes that are known to be visually responsive. The tactile stimulus, when compared to a resting control condition, activated primary and secondary somatosensory areas. The auditory stimulus, when compared to a resting control condition, activated primary and secondary auditory areas. Thus, the traditional, separate territories for vision, touch, and audition were engaged.

In addition to these expected activations in the primary and secondary sensory areas, other presumably higher-order brain areas were also activated. Several brain areas in particular appeared to be multimodal. They became active whether visual, tactile, or auditory stimuli were presented. One of these multimodal areas

was located in the intraparietal sulcus, closely matching the location of the multimodal area VIP in the monkey brain as shown in the first figure. A second area was in a restricted part of premotor cortex, closely matching the multimodal region of monkey premotor cortex. A third area was in the upper bank of the lateral fissure. The correspondence to the monkey brain is less clear in this case. Part of monkey area 7b extends into the upper bank of the lateral fissure and therefore perhaps corresponds to this region of activation in the human brain. There are, however, other areas in the monkey lateral fissure that may also be multimodal but that are almost totally unstudied (Robinson and Burton, 1980). Clearly more monkey single-neuron experiments are now needed to explore this relatively unknown territory.

In summary, Bremmer et al. (2000) show that the human brain contains a system of multimodal areas similar to those in the monkey brain. The function of these areas is not yet clear. As in the case of the monkey, perhaps they play a role in detecting nearby threatening objects and coordinating a defensive reaction.

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