place, unless by a few percent" (*22*, p. 133).

In light of these observations, the postulate that CBF is regulated by and for the sake of metabolic rate must also be reconsidered. The disproportionate increase in CBF that accompanies physiological neural activation causes Po_2 and pH to rise and Pco_2 to fall, rather than the reverse (*2*), arguing strongly against glucose oxidation as a regulator of CBF under physiological conditions. Paulson and Newman, however, have proposed a mechanism independent of metabolic rate by which physiological changes in neural activity may regulate regional changes in CBF (*23*): K^+ released by neural firing is taken into astroglial processes surrounding the neuron, siphoned through soma, and released from processes (end-feet) abutting the capillary, which is highly sensitive to K^+ concentration.

In conclusion, traditional concepts of the dynamic regulation of cerebral metabolism and blood flow must be reconsidered. Although resting energy needs are supported by glucose oxidation, transient increases in neural activity preferentially induce glycolysis and glycogen formation. This result implies that the acute energy expenditures of neural activity are far less than has been inferred from the large increases in glucose uptake and the high (4.1:1) resting-state O_2:glucose molar ratio. Finally, blood flow increases during neural activity are regulated by a mechanism, and serve a need, other than oxidative metabolism.

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Fertilization Events Induced by Neurotransmitters After Injection of mRNA in *Xenopus* Eggs

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Fertilization initiates in the egg a dramatic increase in intracellular calcium that opens ion channels and causes exocytosis. To explore the possibility that these events might involve a receptor-mediated pathway, receptors for serotonin or acetylcholine (M1 muscarinic) were expressed in the *Xenopus* egg; serotonin or acetylcholine then could initiate a series of responses similar to those normally initiated by sperm. Thus, there may be an endogenous receptor in the egg membrane that is activated by sperm, and the serotonin or M1 muscarinic receptor may replace the sperm receptor in this pathway.

THE ACTIVATION OF THE EGG BY THE sperm is similar to interactions of neurotransmitters and hormones with membrane receptors; in particular, fertilization appears to activate a guanine nucleotide binding (G) protein leading to inositol 1,4,5-trisphosphate (IP_3) production and Ca^{2+} release (*1, 2*). Release of Ca^{2+} then causes diverse responses in the egg, including ion channel opening and cortical granule exocytosis (*3*). Although a receptor that mediates the binding of sperm to the egg's extracellular coat has been identified (*4*), it is not known whether there is a receptor in the plasma membrane of the egg that mediates the activation process.

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Receptors for neurotransmitters, hormones, and light that act by means of a G protein show striking structural similarities (*5*). We therefore examined the possibility that an analogous receptor mediates sperm interaction with the egg plasma membrane, leading to activation of the egg. Certain G protein–related receptors stimulate IP_3 production and Ca^{2+} release (*5*). These include the type 1C serotonin (5HT) receptor and the type M1 muscarinic acetylcholine (ACh) receptor (*6–10*); we therefore chose these for expression in the egg membrane.

Because a full-length cDNA coding for the 5HT receptor was not available, we

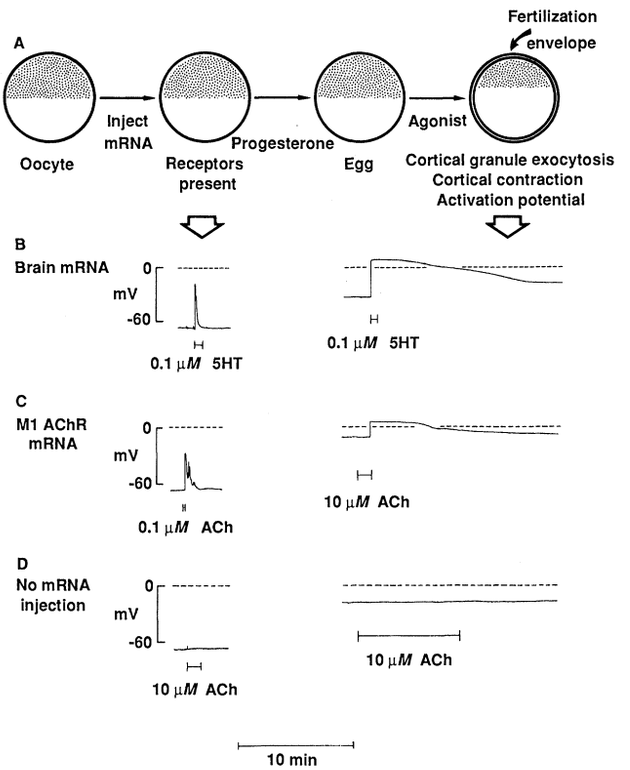

Fig. 1. Activation of *Xenopus* eggs by 5HT or ACh after injection of mRNA. (**A**) The experimental design. (**B**) Responses to 5HT in oocytes and eggs that were injected with rat brain mRNA. (**C**) Responses to ACh in oocytes and eggs that were injected with M1 ACh receptor mRNA. (**D**) A control oocyte and egg that were not injected with mRNA; no response to ACh was seen (*21*). Fully grown oocytes (≥1.3 mm in diameter) were dissected from the ovaries of frogs primed 4 days before by injection of pregnant mare serum gonadotropin (100 IU). The ovaries were treated with collagenase (20 mg/ml for 0 to 60 min), and then the follicular layers around the oocytes were manually removed. The oocytes were injected with ~20 ng of polyadenylated mRNA from rat brain (*22*) or with ~1 ng of mRNA specific for the M1 muscarinic ACh receptor (*11*). After 2 to 3 days of incubation at 18° to 20°C, we tested the oocytes for the presence of 5HT or ACh receptors by perfusing 0.1 μ*M* 5HT (creatinine sulfate complex) or 0.1 to 10 μ*M* ACh through the bath for 10 to 60 s while recording membrane potential. Oocytes were maintained in oocyte Ringer solution (OR2) containing 115 m*M* NaCl, 2.5 m*M* KCl, 1.8 m*M* $CaCl_2$, 10 m*M* Hepes (*p*H 7.4), and gentamycin (50 μg/ml). The oocytes were then matured by treatment with progesterone (5 μg/ml) for 15 min, followed by overnight incubation at 20°C. Mature eggs were identified by the presence of a white spot at the animal pole. Just before recording, the mature eggs were transferred to a low ionic strength fertilization medium (F1) containing 31 m*M* NaCl, 1.8 m*M* KCl, 0.5 m*M* NaH_2PO_4, 1.9 m*M* NaOH, 1.0 m*M* $CaCl_2$, 0.06 m*M* $MgCl_2$, and 10 m*M* tricine (*p*H 7.8). Then 0.1 μ*M* 5HT or 10 μ*M* ACh was perfused through the bath while we recorded membrane potential and observed the egg with a stereomicroscope to detect the elevation of the fertilization envelope and the cortical contraction.

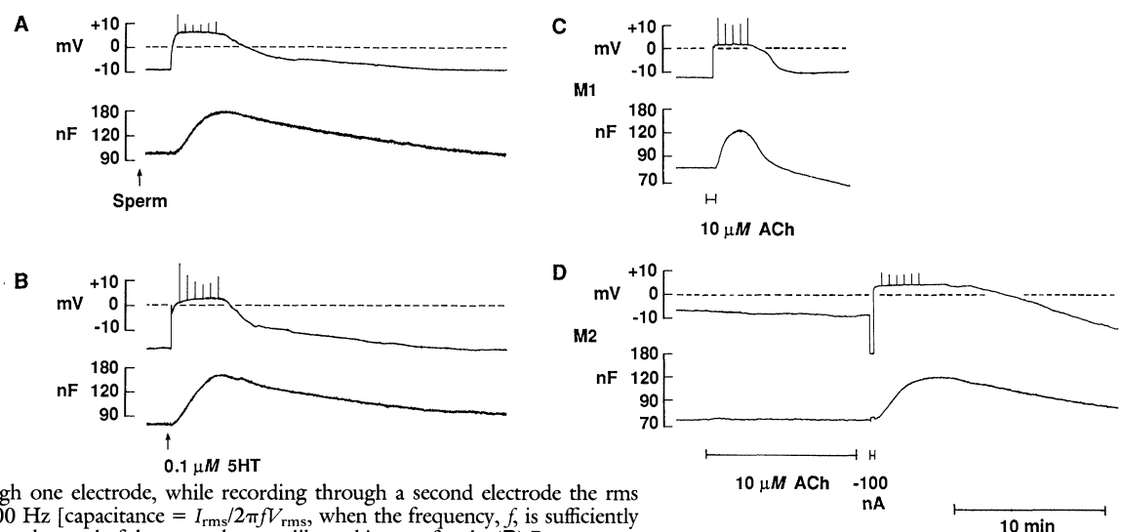

Fig. 2. Membrane potential, conductance, and capacitance during activation of *Xenopus* eggs with sperm, 5HT, or ACh. (**A**) Response to fertilization. Mature ovulated eggs were inseminated in F1 medium with sperm obtained from a dissected testis. (Upper trace) Membrane potential. The superimposed pulses are the voltage responses to applied pulses of 100-nA amplitude and 200-ms duration, which were used to measure conductance. (Lower trace) Capacitance, measured by applying a 400-Hz, 36-nA [root mean square (rms)] ac signal through one electrode, while recording through a second electrode the rms voltage bandpass filtered at 400 Hz [capacitance = $I_{rms}/2\pi f V_{rms}$, when the frequency, *f*, is sufficiently high (*14*)]. The trace is an inverted record of the rms voltage, calibrated in nanofarads. (**B**) Response to 5HT. Oocytes were injected with brain mRNA, and then matured as described in the legend to Fig. 1. (Upper trace) Membrane potential and conductance test pulses. (Lower trace) Capacitance in response to 0.1 μ*M* 5HT. The results in (A) and (B) are representative of 11 experiments with sperm and 13 experiments with 5HT. Initial capacitance values for the fertilization series averaged 89 ± 12 nF (SD); capacitance increased to a peak 1.8 ± 0.1 times the original and returned to the initial value at 17 ± 5 min. For the 5HT series, the initial capacitance was 92 ± 10 nF, the peak was 1.7 ± 0.2 times the initial value, and the time to return to the initial value was 13 ± 9 min. The peak voltages and conductances were +6 ± 3 mV (54 ± 13 μS) and +7 ± 5 mV (23 ± 6 μS) for fertilization and 5HT activation, respectively (*23*). (**C**) Activation in response to ACh in an egg previously injected with ~1 ng of M1 ACh receptor mRNA. For 12 cells from which such records were made during activation of M1 mRNA–injected cells by ACh, the initial capacitance was 76 ± 5 nF; the peak was 1.5 ± 0.1 times the initial value; and the time to return to the initial value was 8 ± 2 min (*24*). The peak voltage was +1 ± 3 mV, and the peak conductance was 17 ± 4 μS. (**D**) No activation in response to ACh in an egg previously injected with ~1 ng of M2 ACh receptor mRNA. The egg was subsequently activated by applying a pulse of −100-nA amplitude and 15-s duration across the plasma membrane (*25*).

injected total polyadenylated mRNA from rat brain into *Xenopus* oocytes to express this receptor (*6, 7*). To express ACh receptors, we injected a specific mRNA synthesized from cloned DNA for the human M1 muscarinic receptor (*10, 11*). Two days after they had been injected with mRNA, we tested the immature oocytes for the presence of 5HT or ACh receptors by measuring the membrane depolarization in response to 0.1 μM 5HT or 0.1 to 10 μM ACh (Fig. 1). Such oocytes depolarized from a resting potential of about −75 mV and reached a peak of about −25 mV. This depolarization was due to stimulation of a G protein, resulting in an IP_3-mediated increase in intracellular Ca^{2+}, causing the opening of Cl^- channels (*6–9*).

The mRNA-injected oocytes were induced to mature to eggs by addition of progesterone. Measurement of the membrane potential of the matured eggs in response to 0.1 μM 5HT or 10 μM ACh showed characteristics similar to a fertilization potential (Figs. 1 and 2). As is typical of mature eggs (*12, 13*), the resting potential was about −20 mV; upon addition of sperm or agonists, the membrane depolarized to about +5 mV and returned to 0 mV after about 5 to 10 min. Accompanying the agonist-induced change in potential was an increase in membrane conductance of about 50- to 200-fold. This conductance increase was indistinguishable from that occurring during fertilization (Fig. 2), which has been shown to be a Cl^- conductance (*12, 13*).

After the rise of the activation potential, the mRNA-injected, agonist-treated eggs underwent cortical granule exocytosis. This could be seen morphologically in the living egg by the resulting elevation of the fertilization envelope (Figs. 1A and 3A) and by the disappearance of cortical granules in histological sections (Fig. 3A). To compare the extent and timing of exocytosis in response to 5HT or ACh with that occurring at fertilization, we measured membrane capacitance. Membrane capacitance is proportional to plasma membrane surface area, which increases due to addition of cortical granule membrane (*14, 15*). We measured capacitance by applying a 400-Hz ac and measuring the amplitude of the resulting ac voltage (Fig. 2). Either 5HT (0.1 μM) or ACh (10 μM) caused membrane capacitance to increase to about 1.6 times the original value; the peak capacitance was reached about 3 min after the rise of the activation potential (Fig. 2, B and C). These responses were similar to those obtained after fertilization (Fig. 2A).

Cortical granule exocytosis at fertilization is followed by a period of endocytosis. Endocytosis results in a decrease in membrane surface area, which has been detected as a decrease in membrane capacitance (*15, 16*). After 5HT or ACh application, capacitance decreased with a time course similar to that after fertilization (Fig. 2, A to C). Thus, the neurotransmitters caused the same endocytotic process as that initiated by fertilization. Like fertilization, 5HT or ACh also caused a contraction of the egg cortex, producing a transient retraction of the pigmented zone in the animal half of the egg several minutes after fertilization (*17*) (Figs. 1 and 3B).

Control eggs not injected with mRNA did not activate in response to 5HT or ACh (Fig. 1D and Table 1). As an additional control, we injected oocytes with synthetic mRNA coding for the M2 muscarinic ACh receptor (*10, 11*). The M2 ACh receptor stimulates phosphatidylinositol metabolism only weakly (*9, 18*). Eggs injected with M2 mRNA were not activated when ACh was applied (Fig. 2D and Table 1). To demonstrate their viability, control eggs that failed to respond to 5HT or ACh were subsequently activated with the calcium ionophore A23187 or with electric shock (Fig. 2D).

In summary, application of 5HT or ACh to eggs into which we had introduced corresponding receptors by injection of mRNA caused at least four of the characteristic responses to fertilization: an activation potential, cortical granule exocytosis, endocytosis, and cortical contraction. We propose that the exogenously introduced 5HT or ACh receptor interacts with an egg G protein, thus initiating the activation response. We suggest that there is an endogenous receptor in the egg membrane, which is activated by sperm, and that the 5HT or ACh receptor substitutes for the initial component of the endogenous pathway.

These results contribute to our understanding of a central problem of fertilization, that is, how the sperm activates the egg. Two hypotheses have been proposed. One is that an activating component from the sperm cytoplasm might be introduced into the egg cytoplasm by means of sperm-egg fusion (*19*). An alternative idea is that activation of a receptor protein in the egg membrane, independent of the fusion event, initiates egg activation. Observations that an externally applied sperm protein fraction can activate eggs of the marine worm *Urechis* support this latter mechanism (*20*). Although our results do not definitively re-

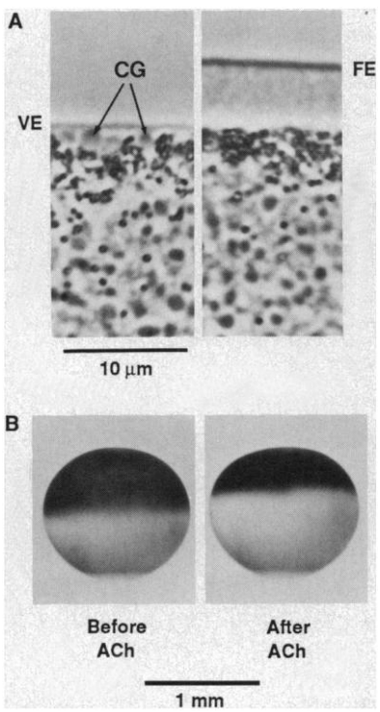

Fig. 3. (**A**) Cortical granule exocytosis and fertilization envelope elevation in response to ACh in eggs injected with M1 ACh receptor mRNA. The left panel shows an egg injected with mRNA and fixed without addition of ACh. Cortical granules (CG) underlie the egg vitelline envelope (VE). The right panel shows an egg injected with mRNA and fixed 20 min after addition of 10 μM ACh. Cortical granules are no longer present, and the vitelline envelope has elevated, forming the fertilization envelope (FE). Similar results were seen in two other M1 ACh receptor mRNA–injected eggs fixed after addition of 10 μM ACh. A noninjected control egg exposed to ACh before fixation showed intact cortical granules and no elevated envelope. Eggs were fixed in Smith's fixative, embedded in JB-4 resin, sectioned at 2 μm, and stained with periodic acid Schiff's reagent. (**B**) Cortical contraction in response to ACh in an egg injected with M1 ACh receptor mRNA. The egg was photographed before and 22 min after addition of 10 μM ACh. In response to ACh, the pigmented half of the egg contracted toward the animal pole, as occurs at fertilization.

Table 1. Responses of *Xenopus* eggs to agonists after injection of mRNA for corresponding receptors; ACh receptor, AchR.

Injection	Agonist	Fraction of eggs activated
Rat brain polyadenylated mRNA	5HT (0.1 μM)	28/29
No injection	5HT (10 μM)	0/6
M1 AChR mRNA	ACh (10 μM)	20/38*
M2 AChR mRNA	ACh (10 μM)	0/8†
No injection	ACh (10 μM)	0/8

*Expression of the M1 ACh receptor mRNA was variable in the six batches of oocytes injected (six frogs); this was seen from the concentration of ACh that was required to depolarize the immature oocyte membrane. Correspondingly, some batches of mature eggs showed a higher fraction of eggs responding. In one experiment, a batch of eggs that failed to respond to ACh at 3 days after injection responded on the fourth day. †For the M2 ACh receptor mRNA, results were obtained from three frogs. No binding studies were done to quantitate the expression of the M2 ACh receptor mRNA relative to that of M1, so the M2 series should be interpreted primarily as a sham message injection control.

solve this question, they also support the receptor hypothesis.

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21. In noninjected immature oocytes, small ACh responses have been reported, even after apparent defolliculation. These responses were rarely observed in our experiments and, when present, were much smaller than those seen after mRNA injection. Responses to ACh were never seen in noninjected mature eggs. Responses to 5HT in noninjected immature oocytes have been reported to be absent or very small; they were not seen in our experiments. [K. Kusano, R. Miledi, J. Stinnakre, *J. Physiol. (London)* **328**, 143 (1982); N. Dascal and S. Cohen, *Pfluegers Arch.* **409**, 512 (1987); (*6–9*)].
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23. The somewhat smaller conductance seen with 5HT could be related to these eggs having been matured in vitro, compared to in vivo maturation for the fertilization series. Biological variation is also probably an important factor because a previous study (*13*) reported a peak conductance of 13 μS during fertilization of *Xenopus* eggs in F1 medium.
24. The series of experiments with M1 (Fig. 2C) was done at a different season than the series of experiments with sperm and 5HT (Fig. 2, A and B); this may account for the approximately 20% smaller initial capacitance values in the M1 series. Another study of the capacitance of the unfertilized *Xenopus* egg gave an even smaller value (55 nF) (*15*), supporting the idea that there may be considerable biological variation among frogs. The shorter duration of the capacitance response may be due to biological variation or may be a real difference between sperm and ACh activation.
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RH 5849, a Nonsteroidal Ecdysone Agonist: Effects on a *Drosophila* Cell Line

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The steroid molting hormone 20-hydroxyecdysone is the physiological inducer of molting and metamorphosis in insects. In ecdysone-sensitive *Drosophila* K_c cells, the insecticide RH 5849 (1,2-dibenzoyl-1-*tert*-butylhydrazine) mimics the action of 20-hydroxyecdysone by causing the formation of processes, an inhibition of cell proliferation, and induction of acetylcholinesterase. RH 5849 also competes with [^{3}H]ponasterone A for high-affinity ecdysone receptor sites from K_c cell extracts. Resistant cell populations selected by growth in the continued presence of either RH 5849 or 20-hydroxyecdysone are insensitive to both compounds and exhibit a decreased titer of measurable ecdysone receptors. Although it is less potent than 20-hydroxyecdysone in both whole-cell and cell-free receptor assays, RH 5849 is the first nonsteroidal ecdysone agonist.

THE TWO NONPEPTIDE HORMONES known to regulate insect metamorphosis and development are the sesquiterpenoid juvenile hormone and the steroid molting hormone 20-hydroxyecdysone (Fig. 1) (*1*). Juvenile hormone, which is responsible for maintenance of a larval or nymphal state in immature molting insects, has been the subject of intensive chemical research and has served as a model for an entire class of structurally diverse molecules that mimic juvenile hormone (*2*). By contrast, although the use of ecdysones as insecticides has been considered (*3*), progress has been hampered by the structural complexity and synthetic inaccessibility of the active steroids for commercial scale field application. In addition, insects have developed powerful mechanisms for catabolizing and clearing ecdysones between molts (*4*). Thus the molting hormones have received little attention from the pesticide industry.

The discovery made over 50 years ago that keto-phenanthrenes can have estrogenic effects spawned both an active area of basic biomedical research and an approach to novel drug development (*5*). However, the absence of such nonsteroidal ecdysone agonists and antagonists has prevented similar advances in the field of insect endocrinol-

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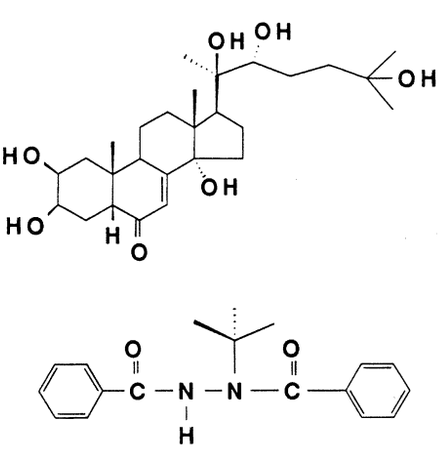

Fig. 1. Structures of (**top**) the invertebrate molting hormone 20-hydroxyecdysone and (**bottom**) RH 5849.