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Serotonin stimulates mitochondrial transport in hippocampal neurons

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Axonal transport of mitochondria is critical for proper neuronal function. However, little is known about the extracellular signals that regulate this process. In the present study, we show that the neuromodulator serotonin (5-HT) greatly enhances mitochondrial movement in the axons of rat hippocampal neurons *in vitro*. Administration of a 5-HT1A receptor antagonist inhibited mitochondrial movement, whereas addition of fluoxetine, a selective serotonin reuptake inhibitor, promoted mitochondrial movement. 5-HT receptors are known to activate the Akt/Protein kinase B pathway. Consistent with this, directional mitochondrial movement was almost completely blocked by a specific Akt inhibitor. Moreover, an inhibitor of glycogen synthase kinase-3β (GSK3β), a kinase whose activity is blocked by Akt-mediated phosphorylation, promoted mitochondrial movement. These findings show that 5-HT1A receptor activation stimulates mitochondrial movement in hippocampal neurons by inhibiting GSK3β activity via Akt. Our findings suggest that 5-HT may mediate the redistribution of energy sources within responsive neurons, a possibility that has significant implications for understanding the global biological effects of this important neuromodulator.

Keywords: Serotonin; 5-HT; Akt; GSK3β; Mitochondria; Axonal transport

Introduction

Cellular function critically depends on the provision of adenosine triphosphate (ATP) by mitochondria. In neurons, where the requirement for energy is distributed over an extensive network of axons and dendrites, the transport of mitochondria might be expected to play a crucial role. In fact, it has been proposed that aberrant mitochondrial trafficking in neurons may underlie many neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and Huntington's disease (Bendiske et al., 2002; Reynolds et al., 2004; Trimmer and Borland, 2005). Signals that regulate mitochondrial trafficking in neurons, however, remain poorly characterized.

Serotonin (5-hydroxytryptamine or 5-HT) is an important neuromodulator that is involved in many aspects of neural function, including the regulation of anxiety, depression, appetite, sleep, and circadian rhythm (Osborne and Hamon, 1988). Indeed, a well-known class of antidepressants, the so-called selective serotonin reuptake inhibitors (SSRIs), acts by inhibiting the reuptake of 5-HT. It is known that the distinct cellular effects of 5-HT are mediated by various 5-HT receptors, each of which can trigger multiple intracellular signaling pathways (Barnes and Sharp, 1999). Among the diverse 5-HT receptor subtypes, the 5-HT1A receptor, which is expressed on neurons in the limbic region of the brain – particularly in the hippocampus – is of special interest because of its involvement in a variety of biological processes, including sleep (Roman et al., 2005; Roman et al., 2006), learning, and memory (Meneses and Perez-Garcia, 2007). Dysfunction of 5-HT1A has been implicated in a number of pathologies, including Alzheimer's disease (Kepe et al., 2006) and transient ischemia (Schaper et al., 2000). Although the cellular effects of 5-HT and its receptors have been extensively investigated (Barnes and Sharp, 1999), few studies have explored the relationship between this neuromodulator and mitochondrial trafficking in neurons.

An early study reported that 5-HT is involved in the regulation of actin-dependent mitochondrial movement in Malpighian tubule cells of the insect *Rhodnius prolixus* (Bradley and Satir, 1981). In rat cortical neurons *in vitro*, 5-HT was shown to mobilize NMDA receptor transport in a microtubule-dependent manner, indicating that it can regulate cytoskeleton reorganization and protein trafficking (Yuen et al., 2005). Based on these observations, as well as the established importance of 5-HT and mitochondrial movement in modulating neuronal function, we investigated whether 5-HT can act as an extracellular signal that modulates mitochondrial trafficking in neurons.

We examined the effect of 5-HT signals on mitochondrial transport by dynamically imaging mitochondria in mature hippocampal neurons *in vitro*. To accomplish this, mitochondria were stably labeled by transducing neurons with a lentivirus encoding a mitochondrially targeted enhanced yellow fluorescent protein (mitoEYFP); transduced cells were then imaged using fluorescence microscopy. We found that 5-HT promoted axonal transport of mitochondria in neurons by activating the Akt-GSK3β pathway, primarily via the 5-HT1A receptor. Since administration of a selective 5-HT reuptake inhibitor in the absence of added 5-HT

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promoted mitochondrial movement, it appears that 5-HT is already present in this particular experimental system, and may act as an intrinsic regulator of mitochondrial trafficking. We speculate that some of the known effects of 5-HT in the nervous system may involve regulating the distribution of ATP within neurons by controlling axonal trafficking of mitochondria.

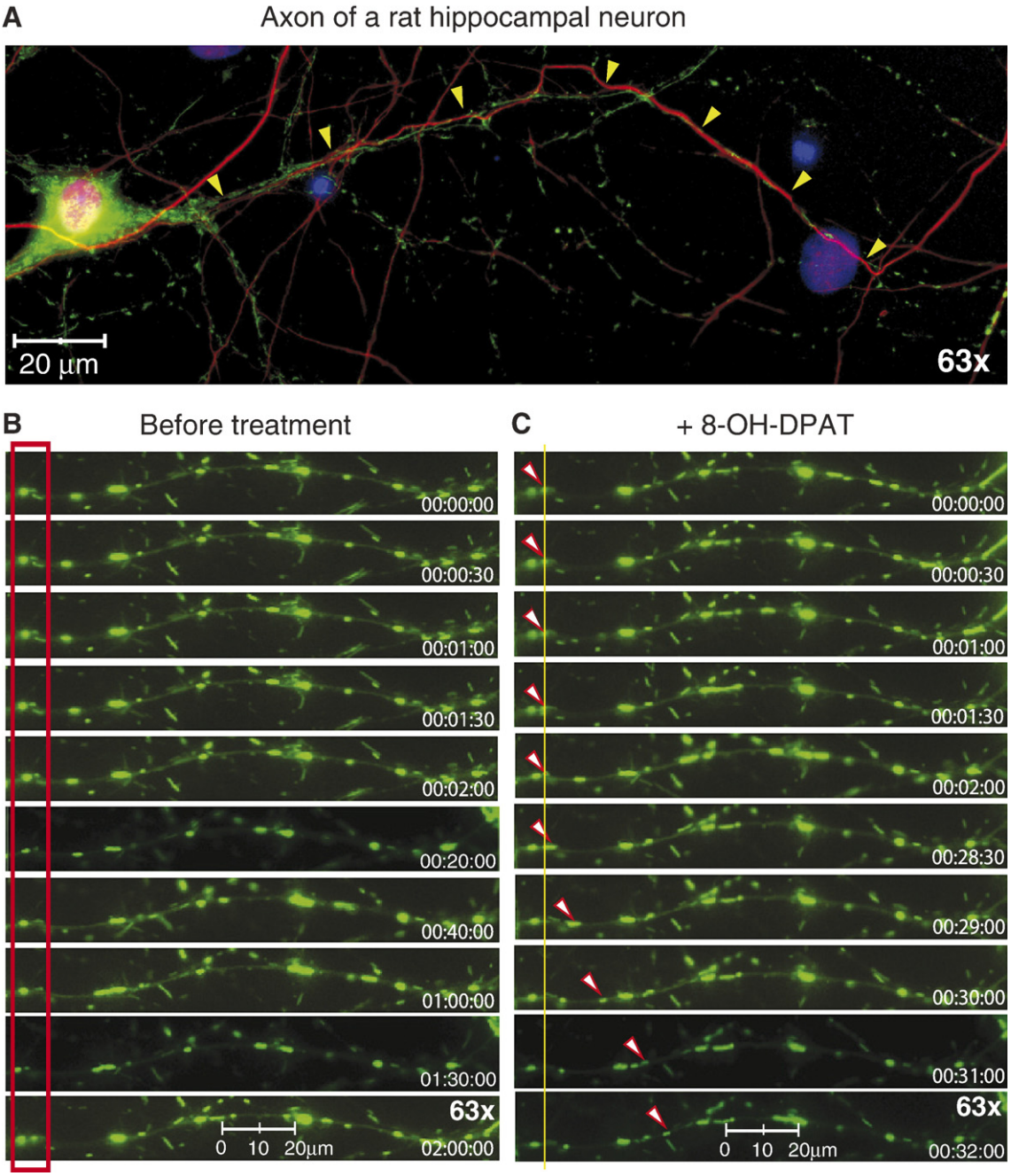

Fig. 1. (A) Identification of the axon of a typical rat hippocampal neuron in culture. Mitochondria labeled with EYFP protein (infected with EYFP-encoding vector lentivirus at day 17 *in vitro*) are shown in green; axons immunolabeled with phospho-neurofilament antibody are shown in red. Extent of typical axon is indicated by yellow arrowheads. Image is a composite of four overlapping micrographs. (B and C) Time-lapse imaging of axonal mitochondria. Frames from a typical time-lapse image series showing mitochondria in the axon of a cultured hippocampal neuron before (B) and after (C) treatment with the 5-HT1A receptor agonist 8-OH-DPAT (10 nM). (B) Red rectangle highlights a stationary mitochondrion (left) and an oscillatory mitochondrion (right) over multiple time points. (C) The mitochondrion observed to be oscillatory in B is moving anterogradely (i.e., toward the axon terminal) after treatment with 8-OH-DPAT (red-bordered white arrowheads). The vertical yellow line on the left side of the image series indicates the starting position of the moving mitochondrion. The neuron shown here was infected with a lentivirus containing mitochondrially targeted EYFP protein (mitochondria are labeled green). Time intervals are shown in the lower right-hand corner of each frame. Magnification (63×) is indicated at the lower right corner of panel. Although images were acquired every 30 s, larger intervals are shown after the fifth frame in B and C to indicate that, in an untreated neuron, the marked mitochondrion is oscillatory over the entire duration of the pre-treatment imaging session (B), and that, approximately 28 min after treatment, the oscillatory mitochondrion starts moving (C).

Results

Long-term observation of mitochondrial movement in hippocampal neurons

A number of model systems have been established to study mitochondrial movement in neurons (Morris and Hollenbeck, 1995; Ligon and Steward, 2000; Rintoul et al., 2003; Miller and Sheetz, 2006). Frequently, in such systems, vital dyes, such as MitoTracker® or JC-1, have been used to track mitochondria in relatively immature neurons. However, long-term observation of mitochondrial movement over a period of hours is difficult because of a loss of signal due to photobleaching and the eventual diffusion of dye out of the mitochondria. Furthermore, in dye-based experiments, all of the mitochondria in a culture are stained, which can make it difficult to track movement in individual axons and dendrites, particularly in older neurons (>14 days *in vitro*), which form a dense network of processes. To overcome the foregoing limitations of dye-based

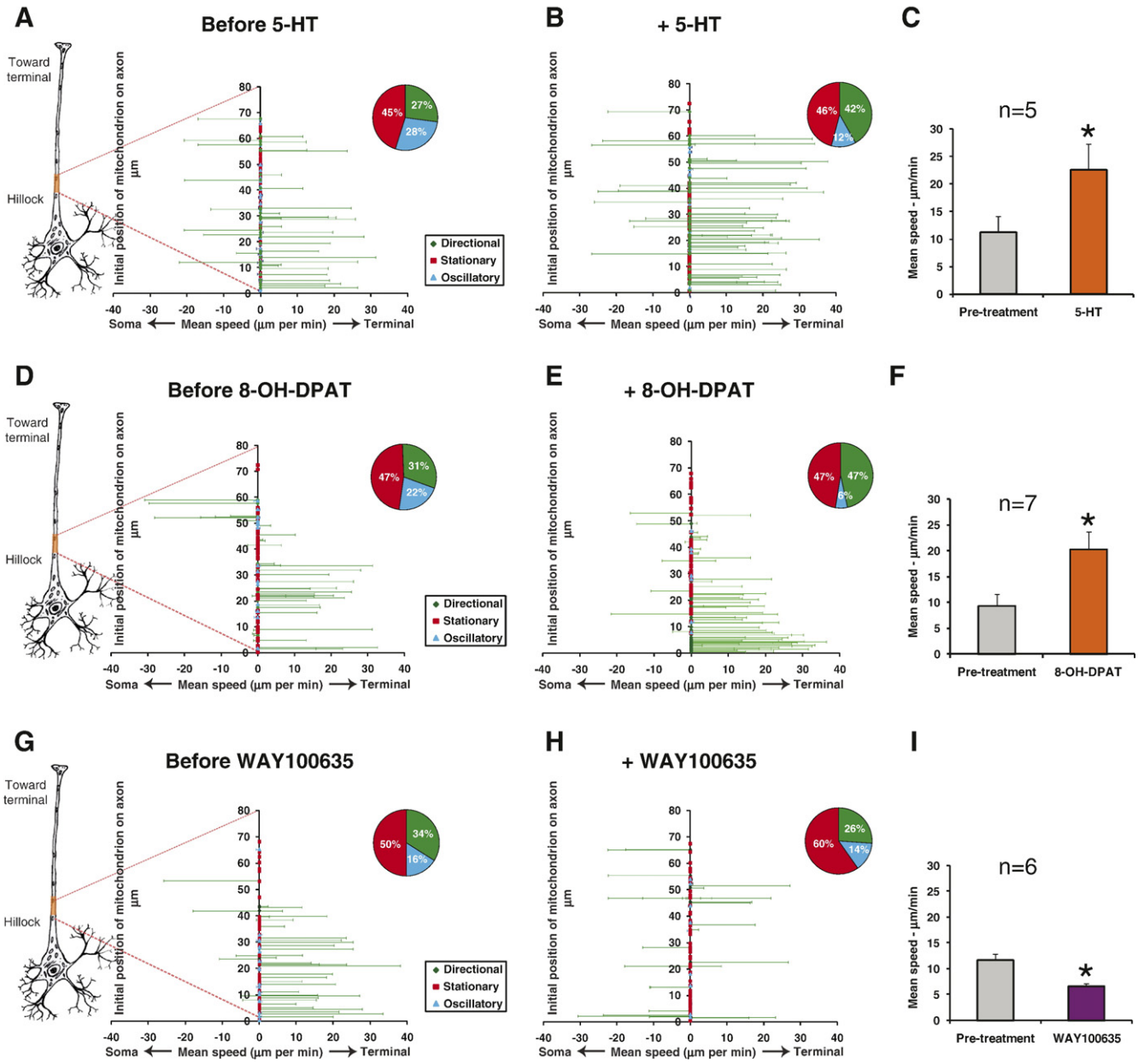

Fig. 2. (A to I) 5-HT (29 nM) and the 5-HT1A receptor agonist 8-OH-DPAT (10 nM) promote anterograde axonal transport of mitochondria in hippocampal neurons, whereas the 5-HT1A receptor antagonist WAY100635 inhibits mitochondrial transport. (A to F) Mean speeds (µm/min) of individual mitochondria before and after treatment with 5-HT (A, B), 8-OH-DPAT (D, E), and WAY100635 (10 nM) (G, H). Pie chart insets show the percentage of stationary (red), oscillatory (blue), and directionally moving (green) mitochondria in all pooled experiments. (C, F, I) Mean speed (µm/min) of all directionally moving mitochondria from pooled experiments before and after treatment with 5-HT ($n=5$, paired t-test; $p<0.02$) (C), 8-OH-DPAT ($n=7$, paired t-test; $p<0.02$) (F), and WAY100635 ($n=6$, paired t-test; $p<0.02$) (I). The red dotted lines projecting from the highlighted region of the schematic axon to the Y-axis of each graph indicate the approximate location (~100–150 µm from the soma) and extent (~50–80 µm) of the axon segment that was imaged.

methodologies, we employed a lentiviral vector encoding EYFP linked to a mitochondria-targeting peptide (MitoEYFP). Recombinant lentiviruses have several useful advantages over other gene transfer systems (Baum et al., 2006). They efficiently transduce post-mitotic cells, and by stably integrating into the genome of infected cells, generally assure long-term transgene expression. By adjusting the relative number of viral particles, it was possible to label all of the mitochondria in a limited number of cells in cultures of mature hippocampal neurons. These particular neurons are characterized by well-differentiated axons and dendrites (Fig. 1A), and, at the time of infection (>day 14 *in vitro*), are spontaneously active (Li et al., 2004b), making them a suitable experimental system for investigating mitochondrial dynamics in functional neurons. The stability of EYFP fluorescence all but precludes the degree of photobleaching associated with the use of vital dyes, and thereby allows continuous observation of mitochondrial movement over a period of several hours. In our experiments, even with a capture rate of one image every 30 s, no phototoxicity was observed after more than 6 h of continuous observation (results not shown).

The 5-HT1A receptor agonist promotes mitochondrial transport, whereas its antagonist inhibits transport

In each experiment, a segment of an axon (typically 50–80 μm in length) was continuously monitored for 2 h before and 2 h after administration of 5-HT (29 nM), a 5-HT1A receptor agonist (8-OH-DPAT; 10 nM), or a 5-HT1A receptor antagonist (WAY100635; 10 nM). Axons were identified by their length and morphology. Specifically, processes of uniform thickness that extended for distances of over 250 μm without significant branching were considered to be axons. This was confirmed by immunocytochemistry with a specific axonal marker (e.g., phospho-neurofilament) (Fig. 1A). Images were captured every 30 s throughout each 2 h session and the resultant image series were converted into Quicktime movie files (Figs. 1B, C; representative time-lapse movies are available in the Supplementary data). As detailed in Experimental methods, all of the fluorescently labeled mitochondria in the axonal segment under observation were individually masked in each time-lapse image and tracked using the particle tracking program module in the Slidebook™ image acquisition and analysis program. Assignments of particle identity made by this program were checked manually, and all ambiguous assignments were discarded. The mobility of each mitochondrion was then evaluated over the entire time course of a given experiment. The mean speed and direction of movement of each mitochondrion, number of mobile mitochondria, and average rate of movement of the total population of directionally moving mitochondrial were all extracted from the collected imaging data.

Mitochondria were categorized into three groups: a stationary population, an oscillatory population, and a population that exhibited directional movement. A mitochondrion was classified as stationary if it moved less than 0.2 μm (or 2 pixels; 1 pixel = 0.10235 μm under

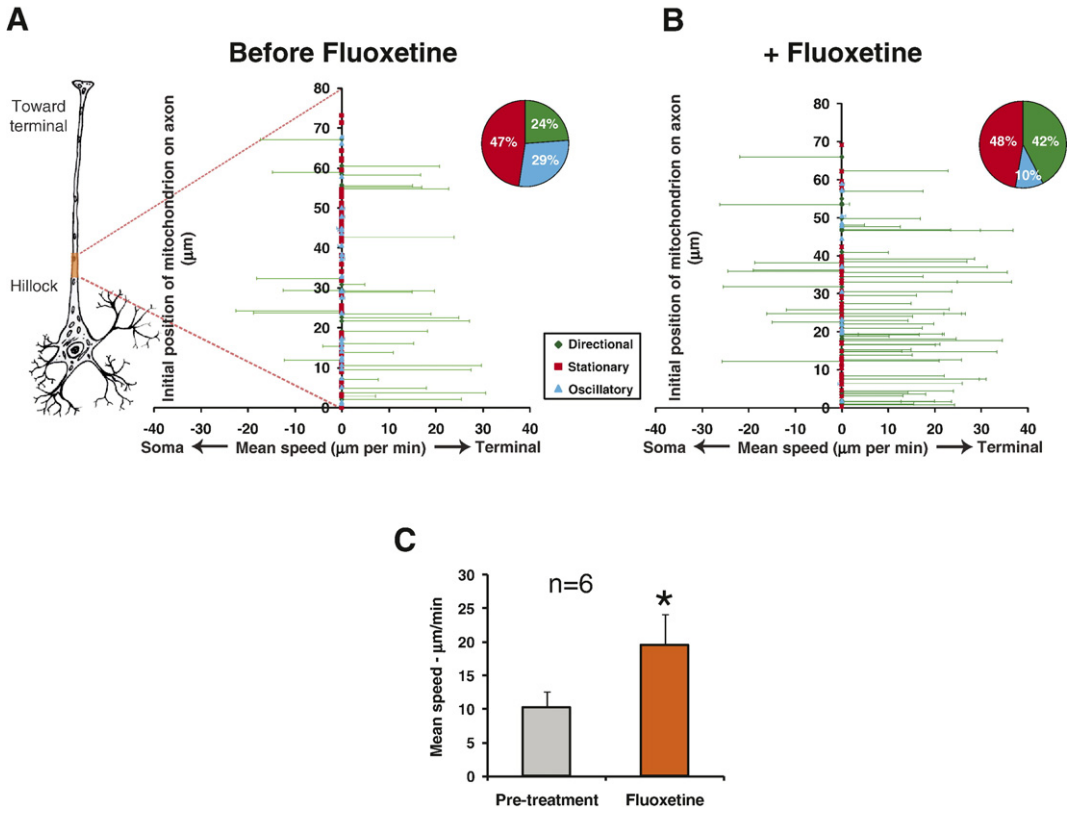

Fig. 3. (A to C) Fluoxetine, a selective serotonin reuptake inhibitor, promotes anterograde axonal transport of mitochondria in hippocampal neurons. (A, B) Mean speed (μm/min) of individual mitochondria before (A) and after (B) treatment with fluoxetine (3 μM). (C) Mean speed (μm/min) of all directionally moving mitochondria from pooled experiments before and after fluoxetine treatments ($n=6$, paired t-test; $p<0.02$). The red dotted lines projecting from the highlighted region of the schematic axon to the Y-axis of each graph indicate the approximate location (~100–150 μm from the soma) and extent (~50–80 μm) of the axon segment that was imaged.

63× magnification). A mitochondrion was considered to be oscillatory if it moved 0.2 μm to a maximum of 2.5 μm bidirectionally around a fixed point during the period of observation. A mitochondrion was considered to move directionally if it was observed to travel a distance greater than 2.5 μm within the axon. Prior to drug treatment, most mitochondria observed (~70%) were either stationary or moved in low-amplitude oscillations (Figs. 2A, D, G, insets). Following the administration of 5-HT (29 nM) (compare Fig. 2A and B, insets), there was a 15% increase in the size of the directionally moving population and 16% decrease in the size of the oscillatory population. There was no obvious change in the percentage of stationary mitochondria. These data were obtained from five paired time-lapse imaging experiments (five separately prepared cultures, each imaged for 2 h before and 2 h after 5-HT treatment; 279 mitochondria tracked). As shown in Fig. 2E, after treatment with the 5-HT1A receptor agonist, 8-OH-DPAT (10 nM), there was a 16% decrease in the oscillatory population and a 16% increase in the directionally moving population, with no obvious change in the percentage of stationary mitochondria. These data were obtained from the analysis of time-lapse images from seven paired time-lapse imaging experiments (seven separately prepared cultures, each imaged for 2 h before and 2 h after treatment with 8-OH-DPAT; 224 mitochondria tracked). The mean speed of each mitochondrion over a 2-h period was plotted against its initial position in the axon segment. The population exhibiting directional movement fell into three categories: net anterograde, net retrograde, and high amplitude bi-directional. Very few mitochondria exhibited high amplitude bi-directional movement; their contribution to the overall percentage change in the number of moving mitochondria was therefore negligible. As shown in Fig. 2, it is evident that most mitochondria moved in an anterograde direction before and after drug treatment. For each treatment, the proportion of anterograde and retrograde movement within the directionally moving population is summarized in Supplementary Table 1.

Administration of the 5-HT1A receptor-specific antagonist WAY100635 (10 nM) in the absence of exogenous 5-HT markedly inhibited directional mitochondrial movement (compare Fig. 2G and H). Based on an analysis of time-lapse images from five separate paired experiments (five separately prepared cultures, each imaged for 2 h before and 2 h after treatment with WAY100635; 252 mitochondria tracked), it appeared that blocking the 5-HT1A receptor caused previously mobile mitochondria to stop moving without affecting the population of oscillating mitochondria.

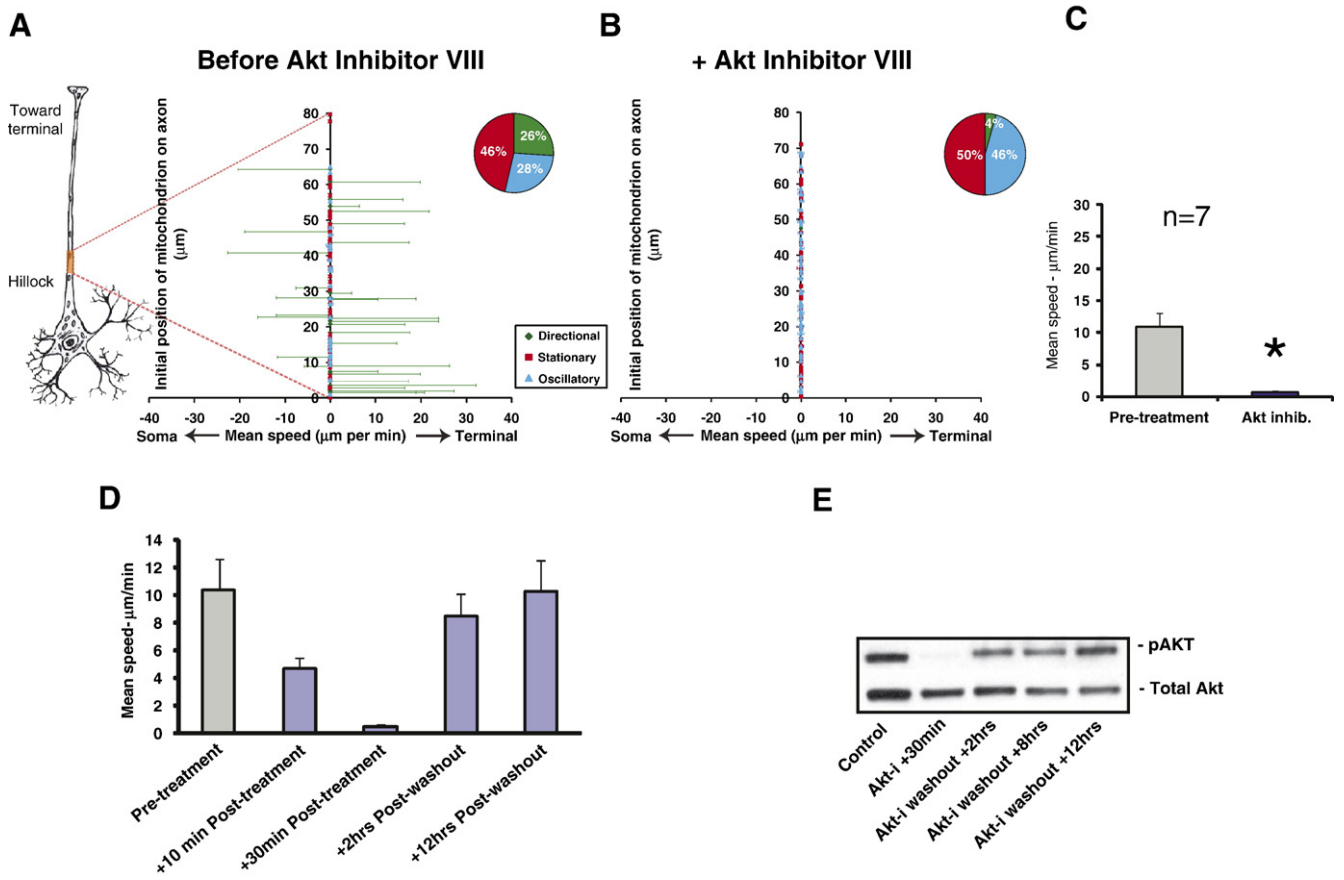

Fig. 4. (A to D) Akt inhibition blocks axonal transport of mitochondria in hippocampal neurons and does not induce apoptosis. (A, B) Mean speed (μm/min) of individual mitochondria before and after treatment with Akt Inhibitor VIII (5 μM). (C) Mean speed (μm/min) of all directionally moving mitochondria from pooled experiments before and after treatment with Akt inhibitor VIII ($n=7$, paired t-test; $p<0.02$). (D) Hippocampal neurons exhibit recovery 2 h after the washout of the Akt inhibitor; mitochondrial movement returns to normal levels within 12 h of washout. The red dotted lines projecting from the highlighted region of the schematic axon to the Y-axis of each graph indicate the approximate location (~100–150 μm from the soma) and extent (~50–80 μm) of the axon that was imaged. (E) Western blot analysis shows inhibition of Akt activation (i.e., decreased serine-473 phosphorylation) 30 min after adding Akt Inhibitor VIII (5 μM) and recovery of Akt activity after washout of inhibitor.

After treatment with 5-HT, the overall average speed of all directionally moving axonal mitochondria increased by 100% (Fig. 2C; $n=5$, paired t-test; $p<0.02$); after 8-OH-DPAT treatment, the average speed of this population increased by 115% (Fig. 2F; $n=7$, paired t-test; $p<0.02$). In contrast, administration of WAY100635 resulted in a 43% decrease in the average speed of all directionally moving mitochondria (Fig. 2I; $n=5$, paired t-test; $p<0.02$). Although 8-OH-DPAT has been shown to activate both the 5-HT7 and 5-HT1A receptor subtypes, the effect of WAY100635 we observed strongly suggests that 5-HT stimulates mitochondrial movement primarily through the 5-HT1A receptor subtype and not the 5-HT7 receptor subtype. This was confirmed by the fact that, in the presence of the 5-HT1A receptor antagonist, administration of 8-OH-DPAT had no observable effect on mitochondrial movement (see Supplementary Fig. 1A). Furthermore, in our investigations of other 5-HT receptor subtypes, agonists or antagonists to 5-HT1B and 5-HT2 did not have any observable effect on mitochondrial transport (Supplementary Figs. 1B–D).

A striking observation concerned the intracellular distribution of the most strongly affected population of mitochondria. As depicted in Fig. 2E, following the administration of 8-OH-DPAT, it seems that oscillating mitochondria closer to the cell body were induced to move anterogradely at a greater rate than those more distal to the cell body. This may relate to the observation that, in the axons of hippocampal neurons, 5-HT1A receptors are clustered close to the soma (Azmitia et al., 1996).

Although 5-HT appeared to have a significant effect on mitochondrial transport in the axons of hippocampal neurons, the same phenomenon was not observed in dendrites (data not shown). Taken together, these findings suggest that, in hippocampal neurons, 5-HT modulates axonal mitochondrial transport primarily through the activation of the 5-HT1A receptor.

Fluoxetine, a selective serotonin reuptake inhibitor, also promotes mitochondrial movement

The observed effect of WAY100635 on mitochondrial transport strongly suggests that mitochondrial movement prior to adding 5-HT is influenced by 5-HT already present in the culture system. To further investigate this possibility, we treated cells with fluoxetine (3 μM), which would be expected to increase extracellular levels of 5-HT. We found that, like 5-HT and the 5-HT1A receptor agonist, fluoxetine also greatly enhanced mitochondrial movement in neu-

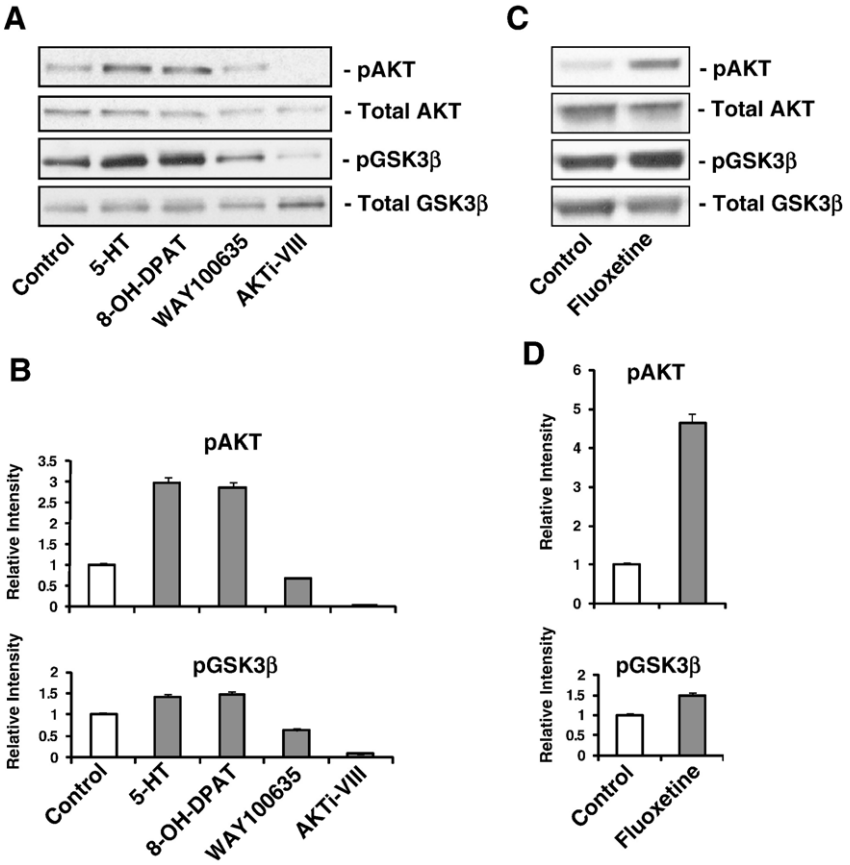

Fig. 5. Western blot analysis shows that 5-HT signals are coupled to the activation of Akt and inactivation of GSK3β in hippocampal neurons. (A) Treatment with 5-HT (29 nM) or the 5-HT1A receptor agonist (8-OH-DPAT, 10^{-8} M) for 30 min leads to the activation (i.e., increased serine-473 phosphorylation) of Akt and the inactivation (i.e., increased serine-9 phosphorylation) of GSK3β, whereas treatment with the 5-HT1A antagonist (WAY100635, 10^{-8} M) or Akt Inhibitor VIII (5 μM) for 30 min leads to the inactivation of Akt and activation of GSK3β. (B) Quantification of Western blots ($n>3$) showing the effect of 5-HT signals on the phosphorylation of Akt (top) and GSK3β (bottom). (C) Treatment with fluoxetine (3 μM) for 30 min leads to the activation of Akt and the inactivation of GSK3β. Whole cell extracts from hippocampal neuronal cultures (DIV 17) were used in Western blots. (D) Quantification of Western blots ($n>3$) showing the effect of the 5-HT reuptake inhibitor fluoxetine on phosphorylation of Akt (top) and GSK3β (bottom). In C and D, intensity values were normalized to total Akt or GSK3β values.

rons (Fig. 3). An analysis of images from six paired time-lapse imaging experiments (six separately prepared cultures, each imaged for 2 h before and 2 h after the fluoxetine treatment; 270 mitochondria tracked) showed an 18% increase in the size of the directionally moving population and a 19% decrease in the size of the oscillatory population (Figs. 3A, B). After administration of fluoxetine, there was a 90% increase in the average speed of all directionally moving mitochondria (Fig. 3C; $n=6$, paired t-test; $p<0.02$). This effect was similar to that observed after the administration of the 5-HT1A receptor agonist.

Akt inhibition blocks directional mitochondrial movement

In general, 5-HT has been associated with short-term signaling pathways through second messengers, such as cAMP and Ca^{2+}. There is increasing evidence that some 5-HT receptor subtypes can couple to signaling pathways normally associated with neuronal growth factors, such as the extracellular-regulated kinase (ERK) and Akt pathways (Cowen et al., 2005). In hippocampal neuronal cultures, it has been shown that 5-HT1A receptor activation by 5-HT or 8-OH-DPAT involves the activation of Akt by phosphatidylinositol 3-kinase (PI3K) (Cowen et al., 2005). In studies of mitochondrial movement, several lines of evidence point to the involvement of PI3K in mitochondrial motility and docking (Chada and Hollenbeck, 2004; Malaiyandi et al., 2005). Given these observations, we investigated whether Akt plays a role in regulating mitochondrial movement. Incubation of cultured hippocampal neurons with the selective Akt inhibitor, 1,3-Dihydro-1-(1-(4-(6-phenyl-1*H*-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2*H*-benzimidazol-2-one (5 μM) (Barnett et al., 2005), for 30 min almost completely inhibited directional mitochondrial movement; the remaining observable directional movement was sporadic, and travel distances were only slightly greater than the higher end of the observed oscillatory movement range (Figs. 4A, B). Analysis of seven paired time-lapse imaging experiments (seven separately prepared cultures, each imaged for 2 h before and 2 h after drug treatment; 263 mitochondria tracked) showed that there was an 18% increase in the size of the population of oscillating mitochondria after administration of the Akt inhibitor. Moreover, treatment with the Akt inhibitor blocked mitochondrial movement in axons, even in the presence of the 5-HT1A receptor agonist 8-OH-DPAT.

To exclude the possibility that changes in mitochondrial motility were merely an artifact of neuronal apoptosis induced by Akt inhibition, a recovery experiment was performed. As shown in Fig. 4D, mitochondrial movement approached normal levels 2 h after the washout of the Akt inhibitor; fully normal levels were achieved within 12 h. Western blot analysis confirmed that the inhibitor completely abrogated the phosphorylation of serine-473 on Akt (Fig. 4E, lane 2). These findings strongly suggest that Akt plays an important role in maintaining directional mitochondrial movement in the axons of hippocampal neurons.

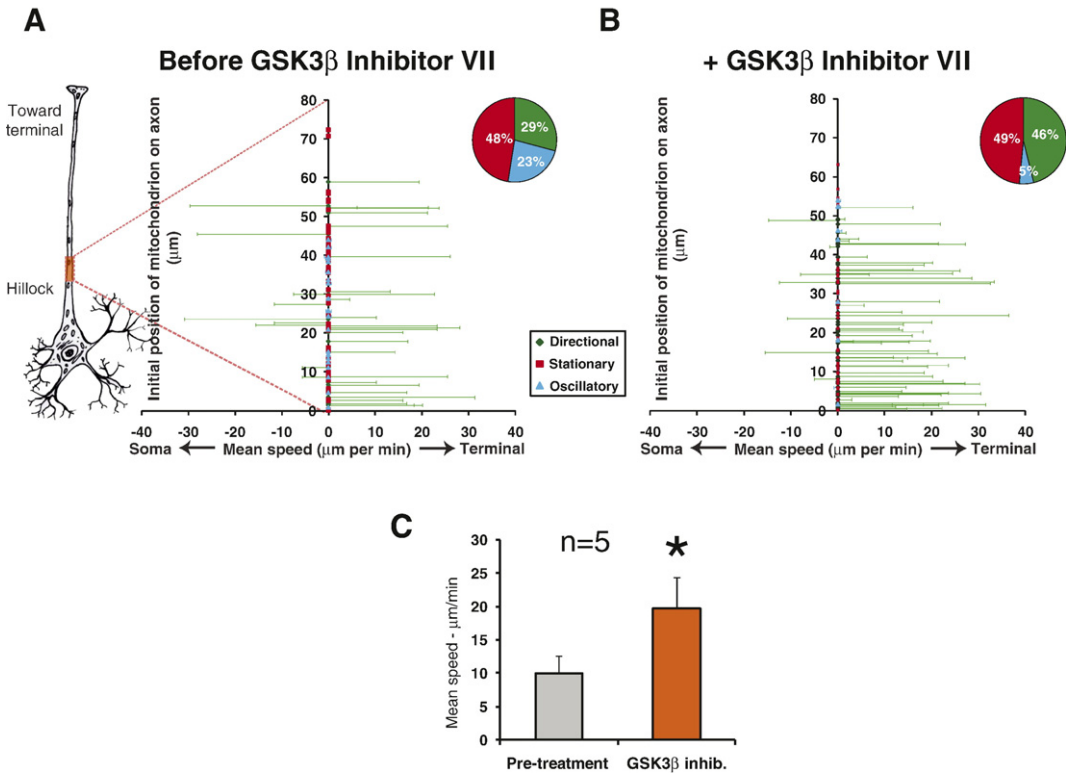

Fig. 6. (A to C) Inhibition of GSK3β promotes anterograde axonal transport of mitochondria in hippocampal neurons. (A, B) Mean speed (μm/min) of individual mitochondria before (A) and after (B) treatment with GSK3β Inhibitor VII (0.05 μM). (C) Mean speed (μm/min) of all directionally moving mitochondria from pooled experiments before and after treatment with GSK3β Inhibitor VII ($n=5$, paired t-test; $p<0.02$). The red dotted lines projecting from the highlighted region of the schematic axon to the Y-axis of each axis indicate the approximate location (~100–150 μm from the soma) and extent (~50–80 μm) of the axon that was imaged.

GSK3β is a target of Akt in hippocampal neurons

It is well established that mitochondrial movement is regulated by interactions between molecular motor proteins and elements of the cytoskeleton, such as microtubules and actin filaments (Hollenbeck and Saxton, 2005). In light of this, it has been suggested that the phosphorylation status of motor proteins plays an important role in regulating mitochondrial movement. However, to date, few kinases or phosphatases have been confirmed as key players in this interaction. The results of our Akt inhibition experiments indicate that Akt could play an important role in regulating mitochondrial movement, although no direct connection between Akt and motor protein phosphorylation has been previously reported. GSK3β, a known Akt substrate, is one possible candidate as a mediator of the effects of Akt – and therefore 5-HT – on motor protein phosphorylation. Phosphorylation of serine-9 on GSK3β by Akt has been shown to inhibit GSK3β activity (Cross et al., 1995). It has also been reported that the phosphorylation of the kinesin light chain (KLC) by GSK3β inhibits attachment of kinesin to cargoes (Morfini et al., 2002). Activation of Akt could therefore lead to a decrease in KLC phosphorylation and to a corresponding increase in cargo mobility.

Western blot analysis showed that the inhibition of Akt (as evidenced by a loss of serine-473 phosphorylation) clearly reduced serine-9 phosphorylation on GSK3β (Fig. 5A, lanes 1 and 5), indicating that GSK3β is a direct substrate of Akt in hippocampal neurons. Moreover, we found that 5-HT (29 nM), 8-OH-DPAT (10 nM), or fluoxetine (3 μM) increased both Akt serine-473 phosphorylation and, concurrently, GSK3β serine-9 phosphorylation (Fig. 5A, compare lanes 2 and 3; Fig. 5B, lanes 1 and 2). Conversely, the administration of the 5-HT1A receptor antagonist, WAY100635, caused a decrease in serine-473 phosphorylation of Akt and a corresponding decrease in serine-9 phosphorylation of GSK3β (Fig. 5A, lane 4). These data are consistent with the hypothesis that 5-HT stimulates movement of mitochondria by modulating the activity of GSK3β via Akt activity.

GSK3β inhibition enhances mitochondrial movement

To directly assess the influence of GSK3β on axonal mitochondrial transport, we treated primary hippocampal cultures with GSK3β inhibitor VII (α-4-Dibromoacetophenone; 0.05 μM) (Conde et al., 2003) and observed axonal mitochondria using live cell fluorescence microscopy and time-lapse imaging. As shown in Fig. 6, an analysis of five paired time-lapse imaging experiments (five separately prepared cultures, each imaged for 2 h before and 2 h after treatment; 250 mitochondria tracked) indicates that the number of moving mitochondria, as well as the rate and extent of mitochondrial transport, increased markedly after treatment with the GSK3β inhibitor. Whereas the percentage of stationary mitochondria remained the same after treatment, there was a 17% decrease in the size of the oscillatory population and a commensurate 17% increase in the size of the population showing directional movement (Figs. 6A, B). In addition, the mean speed of all moving mitochondria increased approximately two-fold after treatment (Fig. 6C; $n=5$, paired t-test; $p<0.02$). In contrast to the effects of both the 5-HT1A receptor agonist and antagonist, which were most pronounced in mitochondria closest to the axon hillock (Figs. 2D, E, G, H), changes in the mean speed of individual mitochondria after treatment with the GSK3β inhibitor were more uniformly distributed across the entire lengths of the axon segments imaged (Figs. 6A, B).

Discussion

Long-term observation of mitochondrial movement

In the present study, we employed a lentivirus-encoded mitoEYFP transgene to label mitochondria in order to measure their movement in hippocampal neurons that were sufficiently mature to possess differentiated axons and dendrites and be spontaneously active. This approach overcomes some of the drawbacks of using a vital dye-labeling method and makes long-term observation by fluorescence microscopy feasible. After monitoring a population of mitochondria in the same axonal segment over 4 h, three dynamically distinct populations of mitochondria became evident. We categorized these as stationary, oscillatory, and directionally moving. Administration of 5-HT, 8-OH-DPAT, fluoxetine, and a GSK3β inhibitor increased the number of mitochondria that moved directionally. This effect appears to involve the recruitment of mitochondria from the oscillating population. Conversely, blockade of Akt activity appears to decrease motility, driving highly mobile mitochondria into the oscillatory population. We speculate that the oscillatory population of mitochondria may serve as a buffering system which allows neurons to adapt to changing local energy requirements (i.e., intense pre-synaptic activity) by enabling easy recruitment to a highly mobile state for transport to distant regions such as axon terminals. In such instances, it would be of interest to know whether mitochondria have an increased number of bound motor proteins, and if so, to identify such motor proteins. In the majority of our experiments, the stationary population, as a proportion of the total mitochondrial population, showed almost no change (~50%). This suggests that the stationary population may be tightly anchored to the cytoskeleton, possibly through tethering to actin filaments (Chada and Hollenbeck, 2004), which would be expected to constrain movement. These mitochondria may be associated with synapses, as has been previously reported (Chang et al., 2006). Co-expression of a pre-synaptic marker that could be used in live neurons would allow us to investigate this possibility.

Specificity of 5-HT receptor subtype in the regulation of mitochondrial movement

We found that, in hippocampal neurons, 5-HT or a 5-HT1A receptor agonist stimulated mitochondrial transport. No significant change in mitochondrial trafficking was observed after administration of agonists or antagonists to the 5-HT1B or 5-HT2 receptor subtypes, suggesting that 5-HT enhances mitochondrial movement primarily through activation of the 5-HT1A receptor. The fact that 8-OH-DPAT, the receptor agonist to 5-HT1A used in our experiments, is also an agonist to the 5-HT7 receptor subtype would seem to leave open the possibility that stimulation of 5-HT7 could have contributed to the effect of enhanced mitochondrial movement we observed. However, the inhibitory effect of the 5-HT1A receptor antagonist – which is specific to the 5-HT1A receptor subtype – all but precludes this possibility. Interestingly, the findings that the 5-HT1A receptor antagonist dampened mitochondrial movement down to levels below those observed in untreated cells and that administration of the selective serotonin reuptake inhibitor fluoxetine enhanced mitochondrial movement suggest the presence of endogenous 5-HT in our culture system. It is conceivable that this endogenous 5-HT was contained in the glia-conditioned media we used. Indeed, it has been shown that

hippocampal astrocytes are capable of 5-HT uptake and that this uptake is fluoxetine-sensitive (Amundson et al., 1992; Anderson et al., 1992), suggesting that astrocytes in our cultures could have been a possible source of endogenous 5-HT.

The function of 5-HT-stimulated mitochondrial movement

In previous studies, a number of parameters have been used to characterize mitochondrial movement, including velocity, duration of movement, and directionality (De Vos et al., 2003; Li et al., 2004b). In the present study, we found that all three of these parameters were affected by the presence of 5-HT or the 5-HT1A receptor agonist 8-OH-DPAT (Figs. 2A–F). What would be the physiological function of such a modulation of mitochondrial transport? It is believed that mitochondria tend to accumulate in the active regions of a neuron; well characterized examples of this include the growth cones of developing neurons (Morris and Hollenbeck, 1993) and the protrusion of active neuronal spines (Li et al., 2004b). It would be interesting to know if other neurotransmitters or neuromodulators have similar effects on mitochondrial movement.

5-HT and other known extracellular signals that regulate mitochondrial movement

It is notable that all of the factors known to regulate mitochondrial movement are linked to the PI3K pathway. In the case of nerve growth factor (NGF), it seems that PI3K is involved in a mitochondrial "docking" effect through its two possible downstream targets, GSK3β and mitogen-activated protein kinase (MAPK), which can activate and inactivate motor proteins, respectively (Chada and Hollenbeck, 2004). Although PI3K is also involved in the activation of Akt in 5-HT signaling (Cowen et al., 2005), the fact that the MAPK pathway is not activated in hippocampal neurons might account for the absence of this docking effect on mitochondria after 5-HT stimulation. The inhibition of mitochondrial movement by excess levels of glutamate or zinc (Zn^{2+}) has been related to the PI3K pathway (Rintoul et al., 2003; Malaiyandi et al., 2005). In the study of inhibition of mitochondrial transport by Zn^{2+}, the finding that treatment of cortical neurons with an Akt inhibitor resulted in decreases in mitochondrial movement (Malaiyandi et al., 2005) is consistent with our observations. However, in these studies, the decrease of mitochondrial movement by Akt inhibition was much less pronounced than what we observed here. The milder effect of Akt inhibition reported previously (Malaiyandi et al., 2005) might be due to the use of a less effective inhibitor, a shorter incubation time, or a different neuronal phenotype. In our study, the strong effect of the Akt inhibitor is evident from the complete disappearance of the phosphorylated Akt band in Western blots (Fig. 4E, lane 2 and Fig. 5A, lane 5). Moreover, in the case of mitochondrial transport inhibition by Zn^{2+}, although it was shown that increased Akt activity occurred with a concomitant decrease in mitochondrial movement, Akt was not definitively identified as the downstream target of PI3K (Malaiyandi et al., 2005). The apparent paradox of a simultaneous increase in the phosphorylation of Akt and an inhibition of mitochondrial movement in that case might be explained by the non-conventional activation of GSK3β by Zn^{2+} (Min et al., 2007).

In the course of our experiments, we found that when hippocampal neurons were treated with the 5-HT1A receptor agonist, 8-OH-DPAT, mitochondrial movement along the axon segment exhibited a dramatic proximal-distal gradient in degree and rate (Fig. 2E). That is, mitochondria that were more proximal to the soma moved faster and further than those more distal to the soma. 5-HT signals might function as a regulator of axonal mitochondrial traffic flow from the axosomatic junction to the terminal end of the axon. We speculate that this may be due to the clustering of 5-HT1A receptors in the initial segment of axon, close to the soma (Azmitia et al., 1996). Another group has reported a similar gradient of fast mitochondrial transport along axons in chicken dorsal root ganglia (DRG) neurons (Miller and Sheetz, 2006). It is also noteworthy that the 5-HT1A receptor is a Gi protein-coupled receptor; and that these receptors are known to reduce the level of intracellular cyclic AMP (cAMP) and to hyperpolarize neurons (Barnes and Sharp, 1999). IBMX, a cAMP elevating reagent, was previously shown to inhibit mitochondrial movement in brainstem neurons *in vitro* (Muller et al., 2005). In preliminary experiments, we have found that the addition of IBMX drastically inhibited mitochondrial movement and decreased levels of activated Akt and inactivated GSK3β. These observations may suggest an additional link between 5-HT signaling and neuronal growth factor pathways (Cowen, 2007).

The role of 5-HT in intracellular trafficking

It is generally accepted that mitochondrial movement is regulated by interactions between mitochondria, cytoskeletal components, and motor proteins and their receptors (Hollenbeck and Saxton, 2005). But precisely how does 5-HT regulate mitochondrial transport? As we noted earlier, several lines of evidence have shown that 5-HT can affect the cytoskeleton in a manner likely to modulate intracellular trafficking. In this regard, it is notable that a significant number of GSK3β substrates are cytoskeletal proteins (Jope and Johnson, 2004), a fact consistent with the idea that the regulation of GSK3β activity is important in microtubule organization. Recently, it was reported that Akt could directly phosphorylate actin (Vandermoere et al., 2007), raising the possibility that Akt itself could induce actin remodeling in neurons. Additionally, it was shown that a pool of Akt is localized to mitochondria and that the β-subunit of ATP synthase is its substrate (Bijur and Jope, 2003). It is therefore conceivable that changes in local ATP concentration caused by Akt activity could directly modulate mitochondrial movement. It is also possible that Akt could affect receptors of motor proteins in a manner that would act to regulate mitochondrial movement. Thus, both actin filaments and microtubules could be reorganized by a modulation of the Akt-GSK3β pathway mediated via 5-HT signals. Such cytoskeletal changes would, in turn, play an important role in regulating mitochondrial movement. Apropos of this possibility, it is relevant that synaptic plasticity is correlated with 5-HT signals, which have been shown to induce actin remodeling in the amygdala (Huang and Kandel, 2007). This strongly suggests a close relationship between neuronal activity, cytoskeletal changes, and 5-HT signaling.

Akt-GSK3β pathways in depression and neurodegeneration

In the present study, we found that fluoxetine and 5-HT induce the same Akt-GSK3β signaling pathway during the modulation of mitochondrial movement in hippocampal neurons. It is notable that serotonergic activity has been reported to regulate GSK3β activity *in vivo* (Li et al., 2004a, 2007). GSK3β is a common therapeutic target

of many antidepressant drugs (Jope and Roh, 2006), and the Akt-GSK3β pathway has been implicated in schizophrenia (Kung and Roberts, 1999; Emamian et al., 2004). This suggests the possibility that aberrant 5-HT-induced mitochondrial movement may be linked to depression or similar disorders. Moreover, loss of 5-HT receptors is often observed in the hippocampi of patients suffering from depression and neurodegenerative disorders, implying a connection between depression and neurodegeneration. The Akt-GSK3β pathway has been linked to neuroprotection, and populations of 5-HT1A receptors are diminished in Alzheimer's disease patients prior to the appearance of clinical symptoms (Kepe et al., 2006). Our finding that the Akt-GSK3β pathway is involved in the regulation of mitochondrial trafficking suggests that aberrant mitochondrial trafficking may be one underlying factor in a number of 5-HT-related disorders. A deficient 5-HT signal could cause defective intracellular trafficking, which would certainly impact mitochondrial transport. This in turn could result in long-term changes in synaptic activity in certain circuits implicated in such disorders as depression and schizophrenia.

To date, few extracellular signals have been shown to regulate intracellular trafficking; this is particularly true in the case of mitochondrial transport in neurons. The findings reported here point to 5-HT as one such extracellular signal, and suggest that intracellular trafficking of mitochondria may be related to the need for localized intracellular energy in the form of ATP. This mode of transport induction may represent a more common regulatory mechanism, perhaps operating outside the hippocampus through the function of neuromodulators other than 5-HT acting via different G protein-coupled receptor types. Such a mechanism may be employed by neurons to maintain a homeostatic state in which their proper performance requires trafficking and redistribution of mitochondria to regions such as axon terminals, where energy needs may be the greatest.

Experimental methods

Primary cell culture

Primary cultures of rat hippocampal neurons were prepared according to previously described methods with some modifications (Mistry et al., 2002). Briefly, rat hippocampal cells were collected from E18 embryos and plated on poly-D-lysine- and laminin-coated 35 mm glass-bottom dishes (MatTek, Ashland, MA) in DMEM containing high glucose (25 mM) supplemented with B27, L-Asparagine, L-proline, and Vitamin B-12. This medium had been previously conditioned for 24 h on monolayers of cortical glia. Plating densities were 2800 cells/cm^2 and 1200 cell/cm^2 in conventional 35-mm dishes (for protein assays) and 35 mm glass-bottom dishes (for imaging studies), respectively. Cells were harvested at 14 DIV for protein assays or used at 17–21 DIV in time-lapse imaging studies. On the fourth day after initial plating and every 4 days thereafter, Ara-C was added to control growth of glial populations. 5-HT, 8-OH-DPAT, WAY100635, and fluoxetine were purchased from Sigma-Aldrich (St. Louis, MO); GSK3β inhibitor VII and Akt inhibitor VIII were obtained from Calbiochem (La Jolla, CA).

Infection of cell culture

To allow visualization of mitochondria during live cell imaging by fluorescence microscopy, cells were infected after 14 DIV with a Feline Immunodeficiency Virus (FIV)-based vector lentivirus, designated Flx1.8/CMVMitoEYFP, which encodes a MitoEYFP transgene (Clontech, Mountain View, CA). Pseudotyped virus was generated by co-transfecting 293T cells with the transfer vector together with plasmids encoding the vesicular stomatitis virus G-glycoprotein (VSVG) and FIV gag-pol genes (Curran et al., 2000). Viral titer was estimated by flow cytometry of infected B104 cells, and a quantity of virus was used that was sufficient to obtain an infection efficiency of 30–40%. Following infection, cells were allowed three or more days to accumulate a strong enough EYFP signal before proceeding with live imaging.

Life support for primary cultures during microscopy

To perform live-cell imaging experiments, we constructed a microscope stage-top incubator designed to enclose a 35 mm glass-bottom culture dish. This incubator both provides an atmosphere of 90% air/10% CO_2 (Airgas, Inc., San Diego, CA) and maintains ambient air and stage temperatures at 37 °C. The incubator consists of a plexiglas chamber equipped with a circular aluminum manifold for gas delivery that wraps around a heat-generating resistor attached to the outside of the chamber. The chamber has a gasket seal at its base and sits on a steel plate with an insert for a 35 mm glass-bottom culture dish. This plate is heated to 37 °C via two resistors mounted on either side. The current used to generate heat through the three resistors is provided by an external DC-regulated power supply; the current is set and monitored so as to never generate temperatures higher than 37 °C. Before placing a glass-bottom culture dish in the incubator, the lid of the dish is discarded and replaced with a clear gas permeable membrane of polytetrafluorethylene (PTFE or Gore-Tex™) stretched over a circular delrin frame. The delrin frame sits tightly around the lip of the culture dish. When the incubator chamber is placed over the 35 mm plate, the circular aluminum manifold sits over the membrane dish top and blows gas heated to 37 °C over the surface of the membrane. We have found that this incubator arrangement allows us to maintain primary neuronal cultures under near optimal conditions on the microscope stage top over many hours, or even days. Osmolarity levels of cultures grown in 35 mm dishes with PTFE membrane lids that were placed in the stage-top micro-incubator for periods exceeding 12 h were found to vary less than those of cultures grown in conventional dishes that were stored continuously in a standard cell culture incubator for similar length of time (data not shown).

Live cell imaging

Fluorescence microscopy was used to observe axonal transport of EYFP-labeled mitochondria in live hippocampal neurons. Time-lapse image series were acquired under high magnification (63× PLAN APO oil immersion objective; numerical aperture = 1.32; Leica, GmbH, Germany) using a Leica DMIRB, DMIRE, or DMI-6000B inverted fluorescence microscope (Leica GmbH, Germany) equipped with a Sutter Lamda 10-2 emission filter wheel, Sutter DG-4 xenon light source (Sutter Instruments, Novato, CA), and a Cooke Sensiscam qe™ cooled CCD camera (Cooke Corporation, Romulus, MI). Microscope control, image capture, post-processing, and particle tracking of mitochondria were all accomplished using the Slidebook™ image acquisition and analysis software package (Intelligent Imaging Innovations, Inc., Denver, CO). In each imaging session, individual frames of mitochondria within an axon segment were acquired every 30 s for a total recording time of 2 h. Cells were imaged for 2 h before and 2 h after administration of drugs. For masking purposes, each individual mitochondrion within an axon segment of interest was distinguishable by its morphology (size, shape, position, and signal intensity) and behavior (pattern of movement), due to the stability of the EYFP signal.

Image analysis

Using Slidebook™, masks of individual mitochondria were generated, and the center of area (centroid) of each mask was determined. Masks for stationary and oscillatory mitochondria were generated by Slidebook™; masks of directionally moving mitochondria were generated manually. The ID tag of each individual mitochondrion was verified by carefully reviewing

movie files frame-by-frame to ensure continuity over the course of the image series, and ambiguous labels were discarded. Manually masking was performed in a double-blind procedure. The individual carrying out this process did not know whether a given time-lapse image series was acquired before treatment or after treatment. Coordinates of the centroids of masked mitochondria were then obtained using the particle tracking module within Slidebook™. The parameters for the movement of each mitochondrion (e.g., categorized population number, displacement, speed) were calculated from recorded changes in their *X*–*Y* coordinates. The displacement plots presented in Figs. 2–4 and 6 therefore represent the mean speeds (μm/min) of each individual mitochondrion over the full duration of each imaging session. Mean speeds are shown on the abscissa, and the relative positions of individual mitochondrion along the axon segments are shown on the ordinate. Pie charts represent the proportion of each population category represented in the total pool of experiments. Speed histograms represent the mean speeds of pooled directionally moving populations from all experiments. Standard error bars represent standard errors of the mean (±SEM) calculated from the total pool of experiments; in all cases, paired Student's *t*-tests were applied to determine significance.

Immunohistochemical staining

EYFP-labeled hippocampal neurons cultured on glass-bottomed culture dishes were fixed in 4% paraformaldehyde (pH 7.4) for 20 min. The cells were then permeabilized with PBT buffer (0.01% Tween-20, 0.02% BSA in PBS) for 40 min at room temperature. Following this, the cells were first incubated with mouse monoclonal antibody (primary antibody) to phosphoneurofilament (Abcam, Cambridge, MA) for 1 h. The cells were then incubated with fluorescein-labeled goat anti-mouse IgG1 antibody (secondary antibody) for 1 h. Finally, cells were covered in ProLong Gold® antifade reagent (Invitrogen, Carlsbad, CA) before a coverslip was applied and images were taken.

Western blotting

Western blotting was performed to determine levels of kinase activation. Cells were harvested in RIPA buffer (Tris–HCl: 50 mM, pH 7.4, NP-40: 1%, Na-deoxycholate: 0.25%, NaCl: 150 mM, EDTA: 1 mM, PMSF: 1 mM, Aprotinin, leupeptin, pepstatin: 1 μg/ml each, Na_3VO_4: 1 mM, NaF: 1 mM). Samples were run on 10% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA). Western blotting was performed using primary antibodies to phospho-ser9-GSK3β, total GSK3β, phospho-ser473-Akt, and total Akt (Cell Signaling Technology, Danvers, MA). The secondary antibodies used for Western blotting were horse radish peroxidase (HRP)-conjugated (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and blots were developed using ECL (GE Healthcare, Piscataway, NJ). All secondary antibodies used for immunostaining were purchased from Invitrogen. For quantification, Western blots were scanned and intensity values were determined using Scion Image (Scion Corporation, Frederick, MD).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2007.08.004.

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