Teacher Resource for:
Sleep Drives Metabolite Clearance from the Adult Brain

Using This Teacher Resource

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GENERAL USE OF Science in the Classroom

Student Learning Goals:

Current views of science education emphasize that “one fundamental goal for K-12 science education is a scientifically literate person who can understand the nature of scientific knowledge.” (From A Framework for K-12 Science Education, National Research Council, 2012).

The U.S. National Academy of Sciences defines science as: “Any new finding requires independent testing before it is accepted as scientific knowledge; a scientist is therefore required to honestly and openly report results so that they can readily be repeated, challenged, and built upon by other scientists. Proceeding in this way over centuries, the community effort that we call science has developed an increasingly accurate understanding of how the world works. To do so, it has had to reject all dogmatic claims based on authority, insisting instead that there be reproducible evidence for any scientific claim.”

A very important student learning goal, central to any understanding of “the nature of scientific knowledge,” is to give each student an appreciation of how science is done. This includes knowing why:

- Scientists must be independent thinkers, who are free to dissent from what the majority believes.
- Science can deal only with issues for which testable evidence can be obtained.
- All scientific understandings are built on previous work
- It is to be expected that one scientist’s conclusions will sometimes contradict the conclusions of other scientists.
- Science is a never-ending venture, as the results from one study always lead to more questions to investigate.
Using This Resource

Learning Lens:

The Learning Lens tool can be found on the right sidebar of each resource and is the source of annotations. Clicking on any of the headings will result in corresponding text of the research article being highlighted. A second click on the highlighted text will produce a text box containing more information about that particular piece of text. Below is an example of the Glossary function of the Learning Lens in use.

An example of the resource with the Glossary, Previous Work, Author's Experiments, News and Policy Links, and References and Notes tools turned on. The Glossary tool is in use.
**Learning Notes:**

Learning Notes accompany each figure and are designed to help students deconstruct the methods and data analysis contained within each figure.

**References:**

The Reference section of each resource is annotated with a short statement about how or why each reference relates to the current research study.
**Suggestions for Classroom Use:**

Four alternative ways to use the SitC reading, questions, and activities:

1. Assign to small groups to complete during class

2. Assign different sections of the article to small groups to complete during class. Use class presentations or jigsaw to teach the entire class what is in the article.

3. Assign to individual students to complete during class or as homework.

4. Assign as an extra credit project.

Interactive student engagement ideas for use after reading the article:

1. Have students write answers to discussion questions (for example, those linked to the standards or those linked to the diagrams).

2. Go over the abstract, as well as information about the purpose and structure of an abstract, and have students write their own abstracts for the articles in language that could be best understood by their peers.

3. Have students edit the current version of the article, or parts of the article, to a simpler reading level.

4. Have students, working alone or in small groups, use the annotated list of references to explain how the scientists who wrote this article built on the published work of at least one independent group of scientists in making their discoveries. In the process, did they produce data that supports the findings of the earlier publication that they have cited in the text? In what way does this article support the statement that scientific knowledge is built up as a “community effort”?

5. Use the article and discussion questions linked to the standards and the diagrams for a teacher-led classroom discussion. The discussion can focus on the nature of science and scientific research, as well as on the science in the article itself.

6. Have students give a classroom presentation about the article, parts of the article, or their answers to discussion questions.
ARTICLE-SPECIFIC MATERIALS

Student Learning Goals:

Connections to the nature of science from the article

- This article nicely displays a few larger themes of science and its research. First, this paper addresses the recently discovered glymphatic system, which circulates CSF through the brain. Like so many biological systems, the glymphatic system builds on top of an existing system - the vessels which carry CSF piggyback on top of blood vessels. It is a frequently observed principle of science that complex systems are built by using new molecules and cells to add functions to previously existing structures. This “layering” of biological functions is also the reason that scientists continue to discover new features of even systems of which we’ve had a basic understanding for years.

- Scientific progress is incremental. This paper reveals an unexpected and important function of sleep. Despite the care and creativity of the authors, however, the paper explains little about the mechanism or capacity of interstitial space volume regulation. As with nearly any finding, years of work will be required to fully understand the process revealed in this article.

- Everything connects to everything. In the span of this three page article, the authors demonstrate a connection between sleep and the glymphatic system. They further connect the process with norepinephrinergic signaling, involved in arousal, activity and stress, and show a potential link to adult-onset neurodegenerative diseases. Biological systems nearly always exhibit this level of complexity, and the authors skillfully focus their efforts to clearly reveal a single component of this network of functions, while giving a sense for how their work may fit into the larger picture.

- Tools and technology are opening up exciting new scientific opportunities. A difficult and crucial part of scientific research is identifying the most important question to ask. This is partly a matter of knowledge, partly of intuition, and partly it depends on the tools available. This article uses cutting-edge imaging technology to image the brains of awake and sleeping mice. Before this tool existed, much important sleep research had been done, but we have never before had the ability to look inside the skull of an awake, behaving organism. Articles like this one advance science not just by expanding our knowledge, but by inventing new approaches to important questions.

The importance of this scientific research

- From a “basic science” perspective, this article is important. Every species which has been studied sleeps, humans spend nearly one-third of their lives sleeping, and
chronic sleep deprivation carries devastating consequences—yet we have little understanding what sleep actually does. By identifying a connection between the cerebral housekeeping system and sleep, the authors begin to explain the crucial role of sleep.

- This article is also important from a clinical perspective. Many diseases are accompanied with changes in sleep pattern, and it is unknown whether sleep is a cause or a symptom of these diseases. Furthermore, if sleep regulates the clearance of harmful proteins and metabolites, then it is likely to impact a range of diseases, such as Alzheimer’s, which involve the accumulation of neurotoxic molecules in the brain.

The actual science involved

- This paper uses advanced techniques to ask a fairly simple question—Does cerebrospinal fluid move more easily through a sleeping brain? Experimentally, this is a highly challenging topic. Awake mice are unpredictable and may experience variable levels of stress and anxiety, while sleeping mice may accidentally be awakened. Combining cutting edge microscopy technology with diffusion experiments which have been used for decades, however, the authors are able to overcome these challenges and produce very clean and compelling data.

- Microscopy is still fundamental to most biological research articles. This article uses a new iteration of the technology, but the principle is always the same—magnifying an interesting tissue as clearly as possible. In this paper, the authors use a two-photon microscope. The key attributes of this technology are: a) increased depth of focus, allowing the authors to look through the skull and into a living brain, rather than needing to remove the brain and slice it into thin sections before imaging; and b) fluorescence imaging, allowing the authors to watch a fluorescent molecule flow through the brain.

- The CSF flux and TMA+ diffusion experiments are relatively simple and direct ways to observe and explain the effect of sleep on CSF flow. Again, the experimental challenge was how to conduct these experiments in awake and sleeping mice.

- The data in Figure 4 represent an exciting clue for the mechanism controlling the impact of sleep in CSF flow. These data are convincing but incomplete. This article is not meant to fully explain the role of sleep, but rather to launch a new focus of research. Whether or not norepinephrinergic signaling is ultimately shown to regulate CSF flow during changes in wakefulness, the authors have succeeded in shedding light on what appears likely to be a fundamental function of sleep.
This paper illustrates the Science Practices standards (SP.1-SP.4). The authors take an observed phenomenon (sleep) and carefully form testable hypotheses about its function. They combine their observation that sleep is restorative and dysregulated sleep is associated with mental illness, with their knowledge of the glymphatic system. They hypothesize a connection between the two, which they carefully test in well-designed experiments. They choose powerful methods for data collection and analysis, making comparisons within individuals to minimize variability, and systematically recording data at a variety of time points and conditions to increase their probability of answering their questions. Finally, they connect their findings into a general model for how sleep, arousal, and glymphatic function could interplay in normally functioning as well as diseased brains. (page iii)

This paper further illustrates Life Science standard LS.2 “Cells as a System.” The authors reveal that different brain cell types form interlocking systems. Brain cells communicate information but rely on other cellular systems, such as the glymphatic system, for the disposal of harmful metabolites and proteins. Furthermore, this article demonstrates that the functions and roles of cells can be modulated by other forces, such as noradrenergic signaling, to change the behavior of the system. (page 58)

Next Generation Science Standards:  
Connects to Practice 2: Developing and using models

Connects to Common Core English Language Arts Standard RTS --112.4:Determine the meaning of symbols, key terms, and other domain-specific words and phrases as they are used in a specific scientific or technical context relevant to grades 11–12 texts and topics.
Summary of the Article for the Teacher:

It is recommended that this not be used by students in place of reading the article.

General Overview:
The brain is highly metabolically active and produces waste which must be removed before it accumulates and begins to harm the brain. This paper links sleep with the removal of these dangerous metabolic by-products. The authors inject fluorescent dyes into the cerebrospinal fluid (CSF) of awake, sleeping, or anesthetized mice and watch as the CSF flows through the mice’s brains. They find that sleep and anesthesia increase the space between neurons, allowing the fluid to flow more easily. They further show that hormones, such as adrenaline, may be responsible for decreasing this space during wakefulness. The authors propose that by improving the removal of harmful waste products, sleep promotes a healthier brain.

Why this Research is Important:
- From a “basic science” perspective, this article is important. Every species which has been studied sleeps, humans spend nearly one-third of their lives sleeping, and chronic sleep deprivation carries devastating consequences —yet we have little understanding what sleep actually does. By identifying a connection between the cerebral housekeeping system and sleep, the authors begin to explain the crucial role of sleep.

- This article is also important from a clinical perspective. Many diseases are accompanied with changes in sleep pattern, and it is unknown whether sleep is a cause or a symptom of these diseases. Furthermore, if sleep regulates the clearance of harmful proteins and metabolites, then it is likely to impact a range of diseases, such as Alzheimer’s, which involve the accumulation of neurotoxic molecules in the brain.

Methods used in the Research:
- This paper uses advanced techniques to ask a fairly simple question—Does cerebrospinal fluid move more easily through a sleeping brain? Experimentally, this is a highly challenging topic. Awake mice are unpredictable and may experience variable levels of stress and anxiety, while sleeping mice may accidentally be awakened. Combining cutting edge microscopy technology with diffusion experiments which have been used for decades, however, the authors are able to overcome these challenges and produce very clean and compelling data.

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focus, allowing the authors to look through the skull and into a living brain, rather than needing to remove the brain and slice it into thin sections before imaging; and b) fluorescence imaging, allowing the authors to watch a fluorescent molecule flow through the brain.

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**Conclusions:**

- Wakefulness quickly regulates CSF flow through the brain. Within the first 30 minutes of sleeping or awakening, the flow of CSF through the outer portion of the brain dramatically changes.

- These changes in CSF flow are likely caused by changes in the volume of the interstitial space. This strongly suggests that the volume of cells decreases during sleep, creating more space around them and allowing cerebrospinal fluid to flow more easily.

- Increased CSF flow during sleep affects the clearance rate of both harmful and inert proteins. This suggests that the observed impact of sleep on CSF flux could have consequences for diseases and general cell health.

- Noradrenergic signaling may be the mechanism connecting the arousal state to cell size, and therefore to CSF flow.

**Areas of Further Study:**

- How does the brain expand the interstitial space volume during sleep? Do all cells shrink in volume, or do the changes occur in a subtype of brain cells (e.g., neurons or glial cells)?

- How quickly does the interstitial space change, and afterward, how long is the process complete? Would CSF flux look substantively different after eight hours of sleep than it does at the time point used in this article, after 30 minutes?

- For what depth of tissue is this relevant? Most of this article focuses on the
outermost 100 nanometers of the mouse brain. Human brains are many centimeters thick, and so these experiments would need to be repeated in larger animals to determine whether CSF flux is increased beyond the most superficial level of the cortex.

- Do these findings have the potential to impact disease or general cell health? CSF flow is clearly increased during sleep (Figure 1), and when proteins are injected into the brain, they are cleared more rapidly in sleeping mice (Figure 3). However, 2 hours after injection, the proteins are cleared to the same level in awake, sleeping, and anesthetized mice (Figure 3A). This suggests that sleep may increase the rate of metabolite clearance, without affecting the total degree of clearance. If true, it is possible that sleep may increase cell health by promoting harmful-metabolite removal, but that it may not play a role in diseases involving a chronic buildup of proteins or metabolites, such as Alzheimer’s disease. However, this experiment only investigates clearance under a single set of conditions, and it is possible that genetic or environmental factors could interact with sleep and clearance to produce the Alzheimer’s disease pathology. Further work will be required to understand the connection between disease, metabolite clearance, and sleep.
Resources for Interactive Engagement:

1. Discussion Questions

1Q) The authors attempt to observe two arousal states (e.g., asleep and awake, or awake and anesthetized) in each mouse, whenever possible, and they confirm the arousal state using visual, ECoG, and EMG data. Why do they spend so much effort to compare different states within the same mouse, instead of simply using different mice in each group?

1A) Individual mice may vary in many ways. They may naturally have differently sized interstitial space volumes, or their interstitial fluid may be transported at different pressures. More to the point, in Figure 1 the authors measure the percentage of a cube of brain into which CSF is able to spread. The exact location of that cube in relation to arteries and the space just under the meninges is likely to strongly affect the dynamics of CSF flow. All of these sources of variability are reduced when the authors compare different arousal states within the same mouse. By analyzing the same cube of tissue in the same mouse under different states of wakefulness, the authors try to ensure that any differences they observe are due only to arousal.

2Q) To determine how easily CSF can flow through the brain, the authors infused the CSF with a fluorescent dye and then determined how deeply into a cube of brain the CSF had penetrated. After 30 minutes, CSF penetrated through about 65% of a cube located at the outermost 100 nanometers of a sleeping brain. The mouse brain is several millimeters thick. Do you think these experiments are generalizable to the entire brain? Consider how CSF is delivered to and removed from the brain.

2A) The images and graphs in Figure 1 suggest that CSF infiltration is most rapid at the outer (cortical) surface of the brain. This may limit the importance of sleep’s role in metabolite clearance to the outermost brain structures. However, there are two reasons to believe these results will matter to the entire brain. First, the authors analyzed CSF flux only during the first 30 minutes of sleep. It is possible that hours of sleep could help CSF flow deeper into the brain than would be possible in an awake mouse. Second, CSF flux is carried into the brain not via simple diffusion, but rather through vessels which run along arteries. Thus, CSF is likely to reach deep brain areas efficiently, albeit more slowly than very superficial ones. Finally, it is worth noting that the outermost layer of the brain, the cortex, is critically important to human behavior, motor control, and sensory experience. The results of this paper are therefore likely to be important even if they mainly apply to the most superficial brain structures.
3Q) The authors show that sleep and anesthesia increase CSF flux through the brain, resulting in faster clearance of metabolites and beta amyloid, which accumulates during Alzheimer’s disease. Does this suggest that Alzheimer’s disease could be treated by promoting sleep? Can you think of some ethical ways to begin testing this hypothesis?

3A) As discussed in the glossary, despite intensive research, it is not known whether beta amyloid accumulation is a cause or a symptom of Alzheimer’s disease. That is, beta amyloid plaques may form and help damage the brain, or other disease mechanisms may cause neurodegeneration and also produce amyloid plaques. It is unknown, therefore, whether attempting to reduce beta amyloid accumulation could affect the disease state. However, it seems reasonable to speculate that beta amyloid plaques relate in some way to Alzheimer's disease, and so it is possible that sleep could help treat or prevent some features of Alzheimer’s disease. One could begin testing this hypothesis by studying sleep patterns in AD patients. If more disturbed sleep is associated with larger beta amyloid plaques, or with other negative disease outcomes, this could suggest that improving sleep could be a fruitful approach for treating AD. Building on this finding, sleep-aiding drugs could be tested for possible impacts on the disease.

4Q) The authors observe that the diffusion of an injected compound, TMA+, is more rapid in sleeping mice. From this and some other calculations, they conclude that the volume of the interstitial space increases during sleep. However, the diffusion rate could increase as a result of other factors; for example, if the interstitial fluid viscosity changed with wakefulness. What mechanism do the authors propose to explain the interstitial space volume changes, and how could they test this mechanism in future studies?

4A) The authors find that CSF diffusion is faster during sleep. They note that the force which propels CSF through the glymphatic system, arterial pressure, increases during wakefulness, likely ruling out CSF transport as an explanation. The authors propose that cell bodies shrink during sleep, leaving more room for CSF flow. This hypothesis is a good one, but the results could be explained by other mechanisms; for example, if CSF viscosity changed with wakefulness (thicker, more syrupy CSF would diffuse more slowly, regardless of interstitial space volume). To test their hypothesis, the authors have at least two experimental options. The most direct would be to observe cell size in sleeping or awake mice, but this is technically challenging. Existing imaging technology is insufficient to accurately measure cell size through the skull of a living mouse. Thus, the authors would likely have to attempt to administer chemical agents to suddenly fix in place the brains of awake and sleeping mice, so that the brains could be removed and analyzed. Fixatives often distort cell shape, and it would be challenging to design this experiment in a way that eliminated distress to the mice, so this approach may be unfeasible. A second option would be to repeat the experiments used in Figures 1 and 2 of this paper, while administering drugs which are known to manipulate or interfere with cell size regulation. This is similar in principle to their experiments using norepinephrine signaling antagonists, but by using a battery of drugs which more specifically affect cell
size, the authors could build a compelling case that cell volume changes to alter the interstitial space volume with wakefulness.
2. Activities connecting to the data shown in the Article

The Activities are linked to in the red tool bar running along the bottom of the page. Activities linked to this particular resource contain raw data from the authors that the students will be able to work with directly.