Does the Sleep-Related Neurons Modulate the Sensation of Pain under the Use of GA?

Yifan Fanga*, Jiameng Zongb*, Samuel Kunesb

*aCentral Catholic High School, Lawrence, USA
bTianjin University, Tianjin, China
cHarvard University, Cambridge, USA

Email: 1907096379@qq.com

Abstract

General anesthetics (GA) has been discovered for centuries and was often used in surgeries. However, many patients are dying from the usage of GA for different reasons. Although scientists are working on to solve the problems, the mechanism of GA is still a mystery. Recently, scientists from Duke University found neurons that are active during sleep can be activated in anesthesia. These neurons are called Anesthetic Activated Neurons (AANs). This is a massive step for us to break the mystery. In this paper, we designed an experiment that aims to reveal one mechanism of GA: the relationship between sleep-related neurons and sensation of pain under the use of GA. The designed experiment involves several control groups that consist of mice with different treatments on their genes and different GA.

Keywords

General Anesthetics (GA), Anesthetic Activated Neuron (AANs), Mechanism, Sleep-Related Neurons, Pain Pathway

1. Introduction

How to manage pain has long been a question that could potentially affect every human being [1]. The discovery of anesthesia, which employed to control pain during surgical procedures, has been a considerable success in the history of medicine. General anesthesia (GA) uses a combination of intravenous drugs and inhaled gases (anesthetics) to put patients into an unconscious state [2]. Propofol, sevoflurane, sufentanil, etc. are commonly used GA in the clinics. Although GA is widely applied to surgeries, we do not fully understand the exact mechan-
isms of how GA leads patients to unconsciousness. The reason is that scientists have not determined the mechanism at the neuron and neural circuit level, and our ability to calculate levels of consciousness stays limited [3]. Thus, patients are still suffering from intraoperative awareness and other complications, which may be caused by insufficient doses of the principal anesthetic, machine malfunction, or misuse of GA delivery methods [3]. There are two dimensions of consciousness, level and content. Level of consciousness refers to the degree to which someone is conscious. The content of consciousness refers to what one subjectively experiences in a given moment, for example, dream [4]. GA can be classified into two mechanisms: bottom-up (subcortical) and top-down (cortical) mechanism by influencing those two dimensions of consciousness respectively. “Bottom-up” paradigm claims that the anesthetics inducing unconsciousness is generated by depressing the level of consciousness. This approach interprets that anesthetics modulate sleep-wake nuclei and neural circuits in the brainstem and diencephalon that have evolved to control arousal state at subcortical region of brain. “Top-down” paradigm states that anesthetics inducing unconsciousness is generated by degrading the contents of consciousness. This approach interprets that anesthetics modulate the cortical and thalamocortical circuits involved in the integration of neuronal information [4] [5] [6]. Although these two mechanisms are controversial, our hypothesis and experiments designing are based on the bottom-up mechanism. GA can interact with different voltage-gated ion channels and neurotransmitter gated ion channels on neurons that regulate synaptic transmission and membrane potentials at synaptic or extra-synaptic sites in the central nervous system [7]. Through this process, GA can activate the neurons in the supraoptic nucleus (SON), ventrolateral preoptic nucleus (VLPO), and paraSON area (the ventral preoptic area dorsal to the posterior SON). Such neurons are called Anesthetic Activated Neurons (AANs) [8]. By using GA to activate AANs, the mice will reach the ideal unconsciousness state: a reversed coma state in which they cannot feel or react to pain because related neurons are accurately controlled. Scientists have discovered that some of the AANs in the mice experiment become active during sleep [9]. This illustrates why the mice will be asleep after being anesthetized. Simultaneously, mice will not feel pain because GA will block the pain pathway, possibly due to the inhibition of the ventral posterolateral (VPL) nucleus or the blocking of axons before their activity reaches the somatosensory cortex [10]. From the above results of GA, we can summarize that the function of GA is to induce hypnosis and relieve pain.

Because AANs are activated both during sleep and GA induced unconsciousness, we suppose that there might be a relationship between sleep-related neurons and the transduction of pain. Thus, we hypothesize that sleep neurons can regulate the propagation of pain sensation. Among all the known sleep-related neurons, we will focus our study on those neurons in SON, paraSON, and VLPO area. Recently, a few studies have identified the common neuroendocrine substrate for both GA and sleep, vasopressin, dynorphin, and galanin. Nevertheless,
no research so far has identified the relationship between sleep-related neurons and transmission of pain signals [8]. Besides, related experiments usually use the larval zebrafish model. However, there are limitations because its brain is quite different from the human brain, and its cortex has not been wholly developed [11]. Compare the structure of the zebrafish brain to the mouse’s; the latter is more similar to the human brain. Therefore, our experiment will be conducted on laboratory mice, in which we will examine pain sensation via brain-wide activity measurements while manipulating the activity of sleep neurons. Through the experiment, we will reveal whether sleep neuron activity modulates the transmission of pain signals to the cortex.

2. Experiment Design

The goal of our experiment is to reveal the relationship with sleep-related neurons and the sensation of pain. Our experiment will be tested on mice. There are two sections in our experiment, and we are going to use different mice in each section. In the first part, our goal is to determine the pain stimulation activated neurons with and without GA injection by using the (Capturing Activated Neuronal Ensembles) CANE technique (Figure 1). In the second part, our goal is to determine the neuronal circuits of pain stimulation under the effect of GA.

There are one control group and four experimental groups in each part, and each group contains 6 mice for different GA. These different GA are used to judge whether it is extensive of a specific phenomenon in several GA. As for the first part, the experiment begins with the FosTVA gene knock-in mice during the embryo state. Then GA and pain stimulation are applied to the mice according to their assigned groups and GA. After the mice are sacrificed and stored at 4˚C, the mice brain will be processed with Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging-compatible Tissue-hYdrogel (CLARITY), which is a tissue clearing technology that can transform intact biological tissue into a three-dimensional transparent hydrogel-based structure [12], and will be examined through confocal microscopy. Pain activated neurons can be found and ensured after observation. In the second part, the membrane-bound Thy1-GCaMP transgenic mice will be used. After some manipulations and pain stimulation, mice brain is sliced, stained with anti-vasopressin/dynorphin antibody, and examined through confocal microscopy. The effect of AANs ablation or gene silence on the neuronal circuit of the pain pathway can be discovered (Figure 2).

There are 30 mice in the first part of the experiment. The embryos of mice are manipulated by FosTVA knock-in technique. The mice are divided into five groups, with each group containing six mice corresponding to six different GA as Table 1 shows. The trans-genetic adult male FosTVA mice at age more than eight weeks should be selected and singly housed for at least one day. Then they will be subjected to the following manipulation. First group mice experience no GA administration and no ablation of AANs. Second group mice experience GA
administration but no ablation of AANs. Third group mice experience GA administration and ablation of AANs in SON, paraSON, and VLPO by the administration of diphtheria toxin [13]. Fourth group mice experience GA administration and gene silencing of AANs in SON and paraSON by knocking out the vasopressin/dynorphin gene to remove the SON and paraSON function in the regulation of sleep [14]. Fifth group mice experience GA administration and gene silencing of AANs in VLPO by knocking out gamma-aminobutyric acid (GABA) and galanin gene [15] [16] [17] to remove the VLPO function in the regulation of sleep (Table 1). Mice should be administrated with their assigned GA, as shown in Table 2, and the administration process should follow the appropriate method with the correct dosage based on their weight. After the GA administration, all mice should be unconscious.

Figure 1. Schematic of CANE technology and phenomenon example. (a) Schematic illustration of the FosTVA mouse gene construction, and the pseudotyped viruses that are designed to infect only activated (Fos+) neurons in the CANE technology. (b) An example of image analysis. Time course of Fos (green) and dsTVA (red) expression patterns in VMHvl following social-fear experience in FosTVA mice. Sakurai, K., Zhao, S., Takatoh, J., Rodriguez, E., Lu, J., & Leavitt, A. et al. (2016) Capturing and Manipulating Activated Neuronal Ensembles with CANE Delineates a Hypothalamic Social-Fear Circuit. Neuron, 92, 739-753. https://doi.org/10.1016/j.neuron.2016.10.015
**Figure 2.** The overall image of our experiment. (a) The anesthetic activated neurons (also the sleep-related neurons) position in the brain. These neurons might be activated after the administration of GA. (b) The axon tracing image model and the possible location where the GA might block.

**Table 1.** The manipulation of five groups mice, each group consists of 6 individuals corresponded to a specific GA that will be mentioned later.

| Groups Manipulation | Pain Stimulation | AANs Ablation or Gene Silence | GA Administration |
|---------------------|------------------|-------------------------------|-------------------|
| Group 1             | Yes              | No                            | No                |
| Group 2             | Yes              | No                            | Yes               |
| Group 3             | Yes              | SON, paraSON, VLPO            | Yes               |
| Group 4             | Yes              | SON, paraSON                  | Yes               |
| Group 5             | Yes              | VLPO                          | Yes               |

**Table 2.** This table will be filled in with the amount of GA for mice in each group based on their weight. Mice in the first group will not be administrated with any GA.

| GA Groups | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 |
|-----------|---------|---------|---------|---------|---------|
| Intravenous Injection |         |         |         |         |         |
| Propofol  | None    | N/A     | N/A     | N/A     | N/A     |
| Ketamine  | None    | N/A     | N/A     | N/A     | N/A     |
| Dexametomidine | None  | N/A     | N/A     | N/A     | N/A     |
| Inhalation |         |         |         |         |         |
| Sevoflurane | None   | N/A     | N/A     | N/A     | N/A     |
| Isoflurane | None    | N/A     | N/A     | N/A     | N/A     |
| Desflurane | None    | N/A     | N/A     | N/A     | N/A     |
After the above manipulations, the mice will receive pain stimulation in the same position on their bodies. The stimulus is controlled with the same strength and duration. Then the mice will be sacrificed, and their brains will be processed by detergent. Next, CLARITY will be used to present the pain activated neurons.

We are able to figure out the position of normal pain activated neurons in mice brain from the first group. There should be red Fos’ captured by CANE method in pain activated region. In the second group, with the GA administration, it is predicted that there will be no or little amount of red Fos’ in pain activated region. We can observe whether the AANs in the corresponding area are activated by the GA as well. If the AANs are activated, but no pain neuron is activated, we can suppose that AANs are able to block the neuronal circuit of the pain pathway. In the third group, with the ablation of neurons in VLPO, SON, and paraSON area, we predict that no neuron (no Fos’ expressed) will be activated in pain activated region at cortex somatosensory part that we would find in the first group. If the result matches what we expect, we can conclude that sleep-related neurons can affect the pain pathway transduction and prevent the pain feeling reaching the cortex. After that, we want to find out which part of AANs has the most significant effect on the neuronal circuit of the pain pathway. Thus, we have different control groups in groups 4 and 5. In the fourth group, neurons in SON and paraSON areas are silenced. If Fos only appear in the thalamus but not cortex, it means VLPO neurons might prevent the transduction of pain pathway from thalamus to cortex but not SON and paraSON neurons. If Fos appear in both thalamus and cortex, but the amount is less than the expression in group 1, neurons in SON and paraSON are assumed that they play a significant role in pain releasing in both brain stem to thalamus and thalamus to cortex pathway. In the fifth group, neurons in VLPO are silenced. If Fos only appear in the thalamus but not cortex, AANs in the SON and paraSON might prevent the transduction of pain pathway from thalamus to cortex but not VLPO neurons. If Fos appear in both thalamus and cortex but less than group 1, we will conclude VLPO neurons play a significant role in pain releasing in both the brain stem to thalamus and thalamus to cortex pathway.

Therefore, from the above experiments, we will be able to discover whether sleep-related neurons play a role in pain releasing under the administration of GA. If they do, we can further find which part of neurons play the most significant role in pain releasing under the administration of GA. There are three possible results. The first possible result is neurons in the SON, paraSON are the significant part to block pain pathway. The second chance is VLPO neurons are a considerable part to block the pain pathway. The third one is SON, paraSON, VLPO neurons are indispensable and must promote each other and activated together to have the final pain releasing effect.

Fos expression has provided an extensive picture of populations of neurons activated by noxious stimuli [18] [19] [20], but there is no information about the circuits that underlie Fos activation. Although there are exceptions, the map of the pain pathway intervening circuits in the brain is almost unknown. Thus a
method is designed to trace the pain pathway neuronal circuits and find out the exact position on the axons where AANs block, the neuron membrane-bound Thy1-GCaMP. The GCaMP is a Ca$^{2+}$ indicator that can show stronger green fluorescence when the neuron is activated and weaker green fluorescence when the activated neuron is blocked.

Then, we can start the second part of the experiment. There are 30 mice in this part, and they are divided into the same groups as the mice in section 1. First of all, the neuron membrane-bound Thy1-GCaMP transgenic mice should be got. Then the mice would be manipulated using the same ablation and gene silence method mentioned before to make their characteristics match the standard for each group. After GA administration, give the pain stimulation with the same strength and duration. Then the mice will be sacrificed, and their brains should be sliced. Anti vasopressin/dynorphin/galanin antibodies will be used to stain the brain pieces and indicate the position where AANs might affect. In the first group, the group without GA administration, we can find the standard pain pathway axon circuits in mice brains. In the second group, the group without AANs ablation, anti vasopressin/dynorphin/galanin antibodies can be used to stain the brain pieces to find out whether the AANs secret these peptides. Then, these peptides can be judged by observing the position of the colored antibodies. If the peptides occur in roughly all parts of the pain pathway (which indicates the Fos expression in the group 1 from the first part), we can suppose that the AANs secreted peptides can block the pain pathway synaptic connections. The vasopressin/dynorphin/galanin position will be a criterion to judge the neurons inhibited by AANs. We should find the neurons that have an effect on the pain pathway among AANs after the experiment of group 1 and 2. After that, we can find out a more specific blocking position in this activated axon tracing and peptide location experiment. In the third, fourth, and fifth groups, we will figure out that whether AANs in the SON and paraSON area or VLPO area can block the pain pathway at some position on axons after observing the inactivated axons and peptide position compared to the first group. We suppose the group will have no peptide and almost the same axon color as group 1 because of the ablation of AANs. In the fourth group, if the axon color is the as same strength as group 1, we infer the VLPO neurons can not play a role in pain releasing. If an axon is lighter than group 1 and the peptide vasopressin/dynorphin/galanin appears, we suppose the VLPO neurons have the pain pathway blocking effect. As for group 5, the situation would be vice versa.

3. Methods

3.1. Technique Introduction

**CANE (Capturing Activated Neuronal Ensembles)**

Capturing Activated Neuronal Ensembles (CANE) with engineered mice and viruses is a technology to label, manipulate, and trans-synaptically trace neural circuits that are transiently activated with high efficiency and temporal precision.
in a behavioral situation [13]. Fos is an immediately early gene (IEG) and is widely used as a reliable marker for activated neurons in response to various sensory stimuli [21]. CANE technique can tag Fos' neurons selectively and efficiently. Researchers who developed the CANE technology have set a gene sequence called FosTVA, which is transfected into mice to present the neuron activity after stimulation. When analyzing the casual relationship, brain tissue should be infected by EnvA-coated (pseudotyped) viruses to induce the expression of dsTVA/Fos, which indicates the neuron activity. EnvA-LV and EnvA-RV are two kinds of pseudotyped viruses. EnvA-LV is a kind of non-toxic virus and can enable stable transgene expression; thus, they are ideal for introducing and persistently expressing desired transgenes in transiently Fos' neurons. Monosynaptic RVs can be used for transsynaptic tracing for presynaptic inputs onto Fos' neurons [13].

To see whether the sleep-related AANs have effects on the pain signal transduction with and without the administration of GA, we will test the Fos expression in manipulated mice. The Fos expression level can indicate the activity of neurons in a different region of the brain after pain stimulation on the trans-genetic FosTVA knock-in mice. mCherry is a kind of red fluorescence protein, and we will use the EnvA-LV-mCherry to detect Fos expression and evaluate the neuron activity [13].

**Axon tracing: Label neuronal circuits with neuron membrane-bound Thy1-GCaMP**

The sensation of pain is generated by ascending pain pathways beginning at nociceptors that are activated specifically by painful (noxious) stimuli. Then, the receptors transduce the “noxious” information into an electrical signal and transmit it from the periphery to the central nervous system along axons. In our experiment, we are going to trace axons that transmit pain through the expression of axon membrane-targeted Green Fluorescent Protein (GFP). GCaMP is created from a fusion of green fluorescent protein (GFP), calmodulin, and M13, a peptide sequence from myosin light chain kinase [22]. By applying GCaMP, the generation of action potentials in neurons can be indirectly monitored by detecting calcium influx via voltage-gated calcium channels. The sensitivity of GCaMP and its high expression levels are critical to obtaining an optimal signal-to-noise ratio and, therefore, for the successful detection of neuronal activity [22]. In our experiment, we would construct a neuron membrane-bound GCaMP gene and then transfect into adult mice so that it can show the neuronal circuits during pain stimulation within and without GA.

**CLARITY**

Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging-compatible Tissue-hYdrogel (CLARITY), tissue clearing technology, is an advanced technique that can transform intact biological tissue into a three-dimensional transparent hydrogel-based structure with all its essential structures [12]. After CLARITY operation, the membrane-bound GFP and Fos are still in place be-
cause of the hydrogel skeleton support. Therefore, we are going to use CLARITY to observe the circuit of the pain signal and expression of Fos.

3.2. Designed Procedures

Generating CANE technique needed Fos\textsuperscript{TVa} knock-in mice

Fos\textsuperscript{TVa} knock-in mice will be generated by inserting a 2A sequence, followed by a destabilized nuclear CFP (nCFP fusing a PEST sequence), the second 2A sequence, followed by destabilized TVA (TVA combining a PEST sequence), bGH polyA, and an Frt-flanked PGK-neomycin resistance cassette immediately before the stop codon of Fos coding sequence by homologous recombination. Then feed trans-genetic mice to the adult state [13].

CANE captured activated neurons

After generating the Fos\textsuperscript{TVa} knock-in mice, five groups of mice’s AANs will be manipulated by some ablation. Recent research [15] [16] [17] found VLPO secreted gamma-aminobutyric acid (GABA) and galanin are neuroendocrine substrates that related to sleep. SON, paraSON neurons secreted vasopressin, or dynorphin can promote sleep [14]. Therefore the ablation of some Neurons can be reached by deletion of the functional neuroendocrine substrate gene using homologous knockout.

Trans-genetic adult male Fos\textsuperscript{TVa} mice at ages more than eight weeks will be singly housed for at least one day and then subjected to the manipulation, just as Table 1 shows. We are going to administrate six different kinds of GA (propofol, ketamine, dexmedetomidine, sevoflurane, isoflurane, desflurane) to the mice in each group. Propofol, ketamine, and dexmedetomidine are intravenous GA, while sevoflurane, isoflurane, and desflurane are inhaled GA. Each mouse will be administrated with a certain amount of GA based on their weight to ensure that the effect of GA on them is the same (Table 2, depending on the real data). Then the virus EnvA-LVs-mCherry should be produced or purchased. Two hours after giving the pain stimulation to these five groups’ mice by needle acupuncture, the EnvA-LVs-mCherry will be introduced into the brain of Fos\textsuperscript{TVa} mice to capture pain activated neurons.

CLARITY observation

Five minutes after pain stimulation, the mice will be sacrificed and stored at 4°C. Hydrogel polymers have to be grown from inside the tissue to support the mice’s brain’s structure and molecular content. This can be done with the infusing of 4°C hydrogel monomers cocktail, formaldehyde, and thermally triggered initiators into the tissue [12]. Formaldehyde serves the dual purposes of cross-linking amine-containing tissue components to each other and covalently binding the hydrogel monomers to native biomolecules including proteins, nucleic acids, and other small molecules but not a membrane. Then, the hydrogel polymerization is triggered by the heat of 37°C [12]. Because of the formaldehyde binding effect, lipids can now be extracted from the brain without destroying important components of the brain. This can be achieved by using
strong ionic detergent-based clearing solution [borate-buffered 4% (wt/vol) SDS] at 37˚C [12]. After the brain has been cleared, it will be immersed in the refractive index (RI) homogenization solution [12]. Followed by constructing the three-dimensional brain model is the visualization of the brain using confocal microscopy at a high resolution. The previously labeled pain circuitry and the neurons which can express Fos can be detected.

**Thy1-GCaMP transgenic mice**

GCaMP2.2c gene will be generated by changing the second arginine to valine and serine at 118 to cysteine of GCaMP2.0. All in vitro expression constructs of GCaMPs were connected with the coding sequence of tdTomato via a 2A peptide (P2A) sequence and subcloned into a modified pBluescript plasmid, which contained the CAG promoter (a combination of the cytomegalovirus early enhancer element and chicken beta-actin promoter). When generating the Thy1-GCaMP transgenic mouse, the GCaMP2.2c and GCaMP3 coding sequences should be cloned into a Thy1 transgenic construct. Afterward, all constructs have to be verified by sequencing. Embryos need to be obtained by mating F1 hybrids in advance. Then, the prepared embryos will be injected with gel-purified DNA using standard techniques to generate Thy1-GCaMP transgenic mice [23] [24].

4. Data Analysis

4.1. Section One

By using CLARITY, the brain graphs of each mice group with red Fos+ expression can be showed. First of all, through the comparison between group 1 and group 2, whether or not AANs are effective at blocking the neuronal circuit of the pain pathway will be revealed. If the graphs of group 1 and group 2 suggest that AANs can block the neuronal circuitry of the pain pathway, the area of red Fos+ expression will be measured and calculated by integral. The results will be compared afterward. According to these results, the effectiveness of the area (VLPO), or the combination of areas (VLPO, SON, paraSON/SON, paraSON), at blocking the neuronal circuit of the pain pathway can be ranked.

4.2. Section Two

The images of antibody staining in group 1 and group 2 will be recorded. The comparison between these two groups can demonstrate whether AANs can produce certain neurotransmitters during pain relief. If AANs are proved to be effective at relieving pain, we can continue the analysis for groups 3, 4, and 5. The color strength of GFP in the last three groups will be recorded by the software Adobe Kular. Then, the images of the experimental groups will be presented. By comparing to the difference between the photos before (which is the image of group 1) and after the change (which are the images of groups 3, 4, and 5), the sites of the pain-sensation pathway can be found. The GFP color intensity of each graph will be calculated by the computer. The data will be recorded, and
a bar graph will be created based on the data. These data can be compared and analyzed through the bar graph, and the effectiveness of AANs at secreting neurotransmitters during pain relief can be found.

The above analysis should be repeated for every kind of GA. Compare the final result of the effectiveness of AANs under each GA. From the comparison, analyze whether the effects of AANs are universal or are different with different GA.

5. Discussion

Due to the limit of equipment, the above is only a proposal for the experiment. Scientists will have to choose a type of mice for the experiment based on their resources. However, all the mice used in the experiment need to be male, and elder than eight weeks old. In the rest of this section, we are going to discuss the ideal results of the experiment.

Through the first part of the experiment, whether sleep-related neurons activity can or cannot block the pain pathway should be found. The ideal result of this experiment is that sleep-related neurons activity can block the pain pathway, then the next part of the experiment can be performed. In the ideal condition, the blocking position of pain transduction and its preliminary mechanism can be found. However, the designed experiment is a theoretical model, and some details need to be paid attention during operation. When performing the Fos\textsuperscript{TVA} knockin technique, we should ensure the Fos\textsuperscript{TVA} gene is successfully inserted into mice embryo. After the mice grow up, we need to check the Fos expression screening before the total validation experiments. It is a theoretical design that the target neuron silencing can be reached by knocking out the neuron functional peptides gene. We are aware that knocking out genes might also change other essential metabolisms. Thus, after knocking out the functional peptides genes, we should add some behavior tests to check out the mice’s activity and to ensure whether they have abnormal mortality and activities except sleep-related activities. If there is a large number of deaths or mice have mental disorders, we shall change the gene knocking out into neuron ablation by accessing articles and finding the agent that only ablates VLPO or SON/para son neurons. In the process of doing the CLARITY technique, we should ensure the thermal trigger procedure would not inactivate the red fluorescent protein in the CANE experiment by controlling the temperature. In the second part of the experiment, the transgenic Thy1-GCaMP mice needs to be screened as well. We would observe the axon green fluorescence strength and compare it to the control group. Thus the high-resolution microscopy is required to guarantee the distinguishability of green fluorescence intensity. At the step to perform antibody staining, three peptide vasopressin/dynorphin/galanin antibodies should all be used since we are not sure yet which peptide plays a role in pain pathway blocking under the different neuron ablation or gene silencing conditions.

If the ideal results appear after doing all the designed experiments, the fol-
allowing further researches could be done. Scientists can test more GA by using this protocol to find more relationships between sleep-related neurons and the transduction of pain signals. Surgeons then can choose GA base on the experiment results and apply different GA to different scenarios. Also, this experiment may lead to an understanding of the mechanism of GA to a deeper level. Based on the relationship of sleep-related neurons and transduction of pain signals, scientists can improve current GA and invent new GA. Scientists can look at GA complications and sequelae that relate to sleep and pain, and may find out a way to prevent these from happening.

6. Conclusion

Our experiment is designed to test the hypothesis that the sleep neuron activity can modulate the transmission of pain signals to the cortex. By corresponding trans-genetic mice, our experiment is aimed to show the activated neurons and the activated neuronal circuits. In the first part of our experiment, the pain activated neurons can be found. The part of AANs or the combination of multiple parts that has the most significant effect on blocking the pain signal transduction can also be discovered. In the second part of our experiment, we would figure out how and where the AANs block the pain signal transduction. If vasopressin/dynorphin/galanin is detected at pain activated regions, and there is no signal in the mice brain cortex, we can suppose that sleep neurons are activated by GA and that they can secrete peptides which action at pain pathway. After that, by activating axon tracing, the lighter and weaker GFP on axons can tell us what point on the axon where the pain signal disappears. In the end, we can evaluate our hypothesis and find out the significant pain pathway blocking neurons and their preliminary mechanism.

Acknowledgements

We thank the Cathaypath Institute of Science for allowing us to meet with the distinguished professor and cutting edge technology, which helped us to finish and improve the paper.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Robinson, D. and Toledo, A. (2012) Historical Development of Modern Anesthesia. Journal of Investigative Surgery, 25, 141-149. https://doi.org/10.3109/08941939.2012.690328
[2] Mayo Clinic (2019) General Anesthesia. http://www.mayoclinic.org/tests-procedures/anesthesia/about/pac-203845683
[3] Alkire, M., Hudetz, A. and Tononi, G. (2008) Consciousness and Anesthesia. Science, 322, 876-880. https://doi.org/10.1126/science.1149213
[4] Lydic, R. and Biebuyck, J. (1994) EDITORIAL II Sleep Neurobiology: Relevance for Mechanistic Studies of Anaesthesia. British Journal of Anaesthesia, 72, 506-508. https://doi.org/10.1093/bja/72.5.506

[5] Nelson, L., Guo, T., Lu, J., Saper, C., Franks, N. and Maze, M. (2002) The Sedative Component of Anesthesia Is Mediated by GABA<sub>A</sub> Receptors in an Endogenous Sleep Pathway. Nature Neuroscience, 5, 979-984. https://doi.org/10.1038/nn913

[6] Franks, N. (2008) General Anaesthesia: From Molecular Targets to Neuronal Pathways of Sleep and Arousal. Nature Reviews Neuroscience, 9, 370-386. https://doi.org/10.1038/nrn2372

[7] Rudolph, U. and Antkowiak, B. (2004) Molecular and Neuronal Substrates for General Anaesthetics. Nature Reviews Neuroscience, 5, 709-720. https://doi.org/10.1038/nrn1496

[8] Jiang-Xie, L., Yin, L., Zhao, S., Prevosto, V., Han, B., Dzirasa, K. and Wang, F. (2019) A Common Neuroendocrine Substrate for Diverse General Anesthetics and Sleep. Neuron, 102, 1053-1065.E4. https://doi.org/10.1016/j.neuron.2019.03.033

[9] Wigren, H. and Porkka-Heiskanen, T. (2018) Novel Concepts in Sleep Regulation. Acta Physiologica, 222, e13017. https://doi.org/10.1111/apha.13017

[10] Physiology Plus (2019) Pain Pathway Physiology. http://physiologyplus.com/pain-pathway-physiology/

[11] Bercier, V., Rosello, M., Del Bene, F. and Revenu, C. (2019) Zebrafish as a Model for the Study of Live in Vivo Processive Transport in Neurons. Frontiers in Cell and Developmental Biology, 7, 17. https://doi.org/10.3389/fcell.2019.00017

[12] Liu, A., Lai, H., Chang, R. and Gentleman, S. (2017) Free of Acrylamide Sodium Dodecyl Sulphate (SDS)-Based Tissue Clearing (FASTClear): A Novel Protocol of Tissue Clearing for Three-Dimensional Visualization of Human Brain Tissues. Neuropathology and Applied Neurobiology, 43, 346-351. https://doi.org/10.1111/nan.12361

[13] Sakurai, K., Zhao, S., Takatoh, J., Rodriguez, E., Lu, J., Leavitt, A., et al. (2016) Capturing and Manipulating Activated Neuronal Ensembles with CANE Delineates a Hypothalamic Social-Fear Circuit. Neuron, 92, 739-753. https://doi.org/10.1016/j.neuron.2016.10.015

[14] Sherin, J., Elmquist, J., Torrealba, F. and Saper, C. (1998) Innervation of Histaminergic Tubero mamillary Neurons by GABAergic and Galaninergic Neurons in the Ventrolateral Preoptic Nucleus of the Rat. The Journal of Neuroscience, 18, 4705-4721. https://doi.org/10.1523/JNEUROSCI.18-12-04705.1998

[15] Li, X., Zhao, X., Fang, Y., Jiang, X., Duong, T., Fan, C., et al. (1998) Generation of Destabilized Green Fluorescent Protein as a Transcription Reporter. Journal of Biological Chemistry, 273, 34970-34975. https://doi.org/10.1074/jbc.273.52.34970

[16] Kroeger, D., Absi, G., Gagliardi, C., Bandaru, S., Madara, J., Ferrari, L., et al. (2018) Galanin Neurons in the Ventrolateral Preoptic Area Promote Sleep and Heat Loss in Mice. Nature Communications, 9, Article No. 4129. https://doi.org/10.1038/s41467-018-06590-7

[17] Gaus, S., Strecker, R., Tate, B., Parker, R. and Saper, C. (2002) Ventrolateral Preoptic Nucleus Contains Sleep-Active, Galaninergic Neurons in Multiple Mammalian Species. Neuroscience, 115, 285-294. https://doi.org/10.1016/S0306-4522(02)00308-1

[18] Menétrey, D., Gannon, A., Levine, J. and Basbaum, A. (1989) Expression of c-fos Protein in Interneurons and Projection Neurons of the Rat Spinal Cord in Response to Noxious Somatic, Articular, and Visceral Stimulation. Journal of Comparative
Neurology, 285, 177-195. https://doi.org/10.1002/cne.902850203

[19] Abbadie, C., Taylor, B., Peterson, A. and Basbaum, A. (1997) Differential Contribution of the Two Phases of the Formalin Test to the Pattern of c-fos Expression in the Rat Spinal Cord: Studies with Remifentanil and Lidocaine. Pain, 69, 101-110. https://doi.org/10.1016/S0304-3959(96)03285-X

[20] Neumann, S., Braz, J., Skinner, K., Llewellyn-Smith, I. and Basbaum, A. (2008) Innocuous, Not Noxious, Input Activates PKC Interneurons of the Spinal Dorsal Horn via Myelinated Afferent Fibers. Journal of Neuroscience, 28, 7936-7944. https://doi.org/10.1523/JNEUROSCI.1259-08.2008

[21] Johnson, Z., Revis, A., Burdick, M. and Rhodes, J. (2010) A Similar Pattern of Neuronal Fos Activation in 10 Brain Regions Following Exposure to Reward- or Aversion-Associated Contextual Cues in Mice. Physiology & Behavior, 99, 412-418. https://doi.org/10.1016/j.physbeh.2009.12.013

[22] Nakai, J., Ohkura, M. and Imoto, K. (2001) A High Signal-to-Noise Ca2+ Probe Composed of a Single Green Fluorescent Protein. Nature Biotechnology, 19, 137-141. https://doi.org/10.1038/84397

[23] Feng, G., Lu, J. and Gross, J. (2004) Generation of Transgenic Mice. In: Luo, Z.D., Ed., Pain Research. Methods in Molecular Medicine, Humana Press, New York, 255-267. https://doi.org/10.1385/1-59259-770-X:155

[24] Chen, Q., Cichon, J., Wang, W., Qiu, L., Lee, S., Campbell, N., et al. (2012) Imaging Neural Activity Using Thy1-GCaMP Transgenic Mice. Neuron, 76, 297-308. https://doi.org/10.1016/j.neuron.2012.07.011