Qualitative investigation of μ- and κ-opioid receptor distribution in the brains of budgerigars (*Melopsittacus undulatus*)

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OBJECTIVE
To perform a qualitative analysis of the distribution of μ- and κ-opioid receptor mRNA in the forebrain and midbrain of budgerigars (*Melopsittacus undulatus*).

SAMPLE
8 brains of male budgerigars.

PROCEDURES
Custom-made RNA hybridization probes (RNAscope; Advanced Cell Diagnostics Inc) were used for fluorescent in situ hybridization (FISH) assays performed on selected fresh frozen prepared sections of brain tissue to identify μ- and κ-opioid receptor mRNA.

RESULTS
There was κ-opioid receptor mRNA present in the nucleus dorsomedialis posterior thalami, lateral striatum, mesopallium, tractus corticohabenularis et corticoseptalis, griseum et fibrosum, stratum griseum centrale, medial striatum, and area parahippocampalis. There was μ-opioid receptor mRNA present in the stratum griseum centrale, stratum opticum, dorsomedialis posterior thalami, area parahippocampalis, medial striatum, and nidopallium intermedium.

CLINICAL RELEVANCE
Consistent with previous studies in pigeons and domestic chicks, κ-opioid receptors were more abundant than μ-opioid receptors in the samples of the present study. The results of this study may also help explain the hyperexcitability or lack of response that can occur with administration of pure μ-opioid receptor agonists, but not κ-opioid receptor agonists. This study was not quantitative, so further research should endeavor to compare the various regions of the brain using FISH technology.
μ-opioid receptor 1 (OPRM1) than κ-opioid receptor 1 (OPRK1) reported in the brains of both species. Multiple autoradiographic studies\textsuperscript{15–17} have been performed in the junco (Junco hyemalis), examining opioid receptor abundance in the vocal centers and the hypothalamus. Zebra finch (Taenopygia guttata) song control regions have been examined using PCR, in situ hybridization, immunoblotting, and immunohistochemistry to determine the relative abundance of μ and δ receptors.\textsuperscript{18} In that study,\textsuperscript{18} there was higher expression of μ-opioid receptor mRNA than δ-opioid receptor mRNA in the lateral magnocellular nucleus of the anterior nidopallium, Area X, medial striatum, hyperstriatum ventrale, and robust nucleus of the arcopallium. The genetic structures of μ-, κ-, and δ-opioid receptors have also been published\textsuperscript{21} for the peregrine falcon (Falco peregrinus), snowy owl (Bubo scandiacus), and blue-fronted Amazon parrot (Amazona aestiva). To the author’s knowledge, there have been no published reports investigating the distribution of opioid receptors in the central nervous system of a psittacine species. 

Budgerigars (Melopsittacus undulatus) are the third most popular pet in the world after the dog and cat and have been domesticated for approximately 150 years. They are also one of the few psittacine species with a completely sequenced genome and brain atlas, both of which are publicly available.\textsuperscript{20,21} Budgerigars are used as a model for vocal learning, so many researchers are familiar with their nervous system, and they are therefore a useful model for studying the central nervous system in birds.\textsuperscript{22}

Fluorescent in situ hybridization (FISH) is an older technology that has seen a resurgence in recent years with the advent of highly sensitive and specific probes that can target mRNA, DNA, and proteins. Historically, FISH provided inconsistent results and was highly dependent on experimental conditions and viewer interpretation.\textsuperscript{23} Autoradiography, a technique using radiographic film to visualize molecules that have been radioactively labeled, was instead used to quantify the number of opioid receptors within nervous tissue using radioactive probes.\textsuperscript{11} These radionuclide probes are effective but may be time consuming, carcinogenic, and pose unnecessary health risks to operators.\textsuperscript{23} The use of RNA hybridization probes (RNAscope; Advanced Cell Diagnostics Inc) is a novel method by which single molecules in individual cells can be observed using hybridization-based signal amplification. Frozen or fixed tissue can be used to identify mRNA molecules within cells. Messenger RNA-specific probes are developed or selected from a catalog that attaches to the strands of mRNA of interest. These probes then bind to fluorophores, which can then be visualized using a fluorescent microscope.\textsuperscript{24} This method strengthens signals produced by fluorescent probes while simultaneously suppressing background noise. Multiple DNA, RNA, or protein targets may be visualized during the same assay. This method also provides greater sensitivity and specificity than previous FISH methods; this is achieved through the use of 2 independent probes that hybridize on the same molecule to achieve signal amplification.\textsuperscript{24} This method of in situ hybridization has been utilized in zebra finches previously to investigate a potassium-chloride cotransporter in the vocal control center.\textsuperscript{25} The purpose of the study reported here is to identify μ- and κ-opioid receptor distribution within selected areas of the forebrain and midbrain of the budgerigar using FISH.

**Materials and Methods**

Twenty-three budgerigars housed at a zoological institution died in an acute mortality event. The animals were housed in a barn with exposure to a portable propane heater. They consumed ad libitum seed and pelleted mixture diet. Necropsies revealed no clinically important findings other than pulmonary hemorrhage, consistent with the highly suspected carbon monoxide toxicosis. Brains were collected from 8 adult males of varying ages within 48 hours after death. They were immediately frozen on dry ice at –78°C and were stored at –80°C as per a published protocol,\textsuperscript{26} until the study could be performed. Brains were equilibrated to –20°C for 1 hour to create 6 section blocks per brain with a No. 10 scalpel. They were placed in optimal cutting temperature media and immediately frozen with liquid nitrogen. Blocks were stored at –80°C until sectioned and were once again equilibrated to −20°C. Sixteen-micrometer-thick coronal sections were produced with a cryostat (Microm Microtome Cryostat HM505e; Carl Zeiss Jena GmbH) and placed onto slides (Fisherbrand Superfrost Plus; Fisher Scientific), which were stored at −80°C (Figure 1). The FISH assays were performed on all 8 brains within 6 months of initial brain sampling.

The FISH protocol was carried out per the manufacturer’s instructions (RNAscope Multiplex Fluorescent Reagent Kit v2 Assay 2019; Advanced Cell Diagnostics Inc). Sections were treated with hydrogen peroxide and protease and then incubated with 2 sets of 20 custom-designed RNA-based double Z target probe pairs. One set of probe pairs targeted the adjacent 18 to 25 base regions within the target sequence 2-1063 of XM_005150073.1 against the budgerigar μ-opioid receptor mRNA, or OPRM1 (Melopsittacus undulatus-LOC101868267; Advanced Cell Diagnostics). The other set of probe pairs targeted the adjacent 18 to 25 base regions within the target sequence 113 to 1332 of XM_005152549.2 against the budgerigar κ-opioid receptor mRNA, or OPRK1 (Melopsittacus undulatus-LOC101880522-C2; Advanced Cell Diagnostics). A positive control probe was designed for the purposes of this study, which targeted the FOXP2 gene in the medial striatum. The 20 RNA-based double Z target probe pairs targeted the adjacent 18 to 25 base regions within the target sequence 1286 to 2406 of NM_001281546.1 against FOXP2 (Melopsittacus undulatus-FOX2; Advanced Cell Diagnostics). The expression of FOXP2 mRNA in budgerigars is known and documented in multiple in situ hybridization studies.\textsuperscript{27,28}
For the FISH study, sections were incubated in probe solution for 2 h at 40°C and then washed and incubated (40°C each time) in sequence with 4 additional amplifying solutions per manufacturer’s instructions. A pretreatment reagent (Protease Plus; Advanced Cell Diagnostics Inc) was utilized to digest the cell material. After initial results revealed extensive digestion, the manufacturer’s recommended digestion time was reduced by 5 minutes. By reducing protease digestion time, visualization of cell architecture and opioid receptor distribution was improved. Slides were tested for the integrity of their RNA using the probe specific to FOXP2 mRNA. The negative control was determined using a probe specific to the bacterial DapB gene from the Bacillus subtilis strain SMY, a soil bacterium. There was < 1 dot of label per cell when stained with the negative control probe.

Dyes (Opal 520 and Opal 570; PerkinElmer Inc) were used as the fluorophores for µ- and κ-opioid receptor RNA, respectively. The fluorophore dye for µ-opioid receptor RNA fluoresces with a wavelength of 494 to 525 nm, producing a green emission, whereas the fluorophore dye for κ-opioid receptor RNA fluoresces with a wavelength of 550 to 570 nm, producing an orange emission. These colors are easily distinguishable in fluorescent microscopy, so misinterpretation is unlikely. A nuclear counterstain, 4’,6-diamidino-2-phenylindole, was used to facilitate visualization of landmarks on the slides, as it stains nuclear DNA and emits blue light at wavelengths of 358 to 461 nm. Slides were kept in the dark at 4°C until they could be imaged. Imaging of fluorescently labeled tissue was performed between 8 hours and 2 weeks after conclusion of the assay, consistent with manufacturer’s recommendations, using a camera (Olympus Q-color camera; Olympus Corp) with a fluorescent microscope (BX51 Olympus microscope; Olympus Corp). Images were collected at 40X, 100X, and 400X magnification using image-capturing software (Image ProPremier; Media Cybernetics Inc). Histology was performed on normal budgerigar brains to compare anatomic locations of µ- and κ-opioid receptors. All images were obtained and

Figure 1—Photograph of a representative 16-µm thick coronal cryosection of brain tissue from 1 of 8 adult male budgerigars (Melopsittacus undulatus) sampled from among 23 that had been housed at a zoological institution and died in an acute mortality event. Cb = Cerebellum. Te = Telencephalon. Scale bar = 5 mm.

Figure 2—Photomicrographs of a select region of the forebrain of one of the adult male budgerigars described in Figure 1, showing the distribution of κ-opioid receptor mRNA (A) or µ-opioid receptor mRNA (B) tagged with fluorophores after RNA hybridization probes were used for fluorescent in situ hybridization assays. Each signal (dot) represents a molecule of mRNA. A—Fluorophore dye (Opal 570; PerkinElmer Inc); bar = 50 μm. B—Fluorophore dye (Opal 520; PerkinElmer Inc); bar = 50 μm.
reviewed by the same person (P.A.S.) to ensure consistency in interpretation.

**Results**

The use of mRNA probes created and utilized with FISH assays for determination of µ- and κ-opioid receptor distribution worked as expected and permitted visualization using a standard fluorescent microscope (Figures 2–5). The positive and negative control probes also worked as expected. Locations within the budgerigar brain were compared with images reported by the Brauth Lab at the University of Maryland. Structures of the avian telencephalon and diencephalon were labeled and described using updated nomenclature. The probes utilized for this study had different affinities for each receptor type, so the signal intensity of µ- and κ-opioid receptors can only be compared to themselves and not to each other (Figure 2). Each dot, or signal, is a visualization of a molecule of mRNA. The brains examined did show a very similar distribution pattern of µ- and κ-opioid receptors. However, due to financial and sampling constraints, only a limited number of assays could be performed, making this a purely qualitative study.

There was κ-opioid receptor mRNA present in the lateral nucleus dorsomedialis posterior thalami, lateral striatum, mesopallium, tractus corticohabenularis et corticoseptalis, griseum et fibrosum, stratum griseum centrale, medial striatum, and area parahippocampalis (Figures 3–5).

There was µ-opioid receptor mRNA present in the stratum griseum centrale, stratum opticum, stratum griseum centrale, stratum opticum,

![Figure 3](image-url)

**Figure 3**—Photomicrographs of a select region of the forebrain, including the lateral nucleus dorsomedialis posterior thalami (DMP), globus pallidus (GP), lateral striatum (LSt), and tractus corticohabenularis et corticoseptalis (CHCS), of an adult male budgerigar described in Figure 1, showing the distribution of κ-opioid receptor mRNA (A) or µ-opioid receptor mRNA (B) tagged as described in Figure 2. A—Fluorophore dye (Opal 570, PerkinElmer Inc); bar = 0.05 mm. B—Fluorophore dye (Opal 520, PerkinElmer Inc); bar = 0.5 mm.

![Figure 4](image-url)

**Figure 4**—Photomicrographs of a select region of the forebrain, including the area parahippocampalis (APH), cerebellum (Cb), mesopallium (m), nidopallium intermedium (NI), of an adult male budgerigar described in Figure 1, showing the distribution of κ-opioid receptor mRNA (A) or µ-opioid receptor mRNA (B) tagged as described in Figure 2. A—Fluorophore dye (Opal 570; PerkinElmer Inc); bar = 0.05 mm. B—Fluorophore dye (Opal 520; PerkinElmer Inc); bar = 0.5 mm.
nucleus dorsomedialis posterior thalami, area parahippocampalis, medial striatum, nidopallium intermedium, lateral striatum, and tractus corticohabenularis et cortico-septalis (Figures 3–5).

Discussion

To the authors’ knowledge, this was the first published use of FISH assays to characterize µ- and κ-opioid receptors of selected areas of any psittacine forebrain and midbrain. The data support the clinical impression that many psittacine birds have an analgesic response to κ-opioid receptor agonist and µ-opioid antagonist drugs, which is contrary to what is observed in most mammalian species. The findings reported herein are most consistent with those observed in earlier pigeon studies, that there is a higher relative predominance of κ-over µ-opioid receptors in the forebrain but no relative predominance in the midbrain. Conversely, in the day-old chick forebrain and midbrain, µ-opioid receptors predominate over κ-opioid receptors. Semi-quantitative analysis can be performed using the RNA hybridization probes and FISH assay protocol but was beyond the scope of this investigation.

This study was facilitated by the publication of the budgerigar genome. This information allowed for the development of highly sensitive and specific probes for µ- and κ-opioid receptor mRNA specific for this species. There are known variations in the mRNA sequence of opioid receptors in multiple species. However, the method of FISH used in this study limits the risk of both false positive and false negative results, ensuring that the signals observed actually represent κ- and µ-opioid receptor mRNA.

The nomenclature for the avian brain has changed since the early opioid distribution studies to reflect the more appropriate embryonic origins of the avian brain. Recent research has shown that the avian brain has a very unique organizational pattern, consisting of compartmentalized nuclear groupings. The striatum is composed predominantly of inhibitory GABAergic neurons that project to GABAergic neurons in the pallium. The µ- and κ-opioid receptor distribution of the striatum was examined in this study. The medial striatum is partially responsible for the processing of sensory-motor information for the production and integration of auditory, visual, and singing behaviors. It is also partially responsible for food, water, sex-related reward, and motivation of appetite. The lateral striatum participates in visual information processing. Other regions of the avian brain are still being better characterized.

The avian brain is unique when compared to the mammalian brain, especially in regards to the cerebrum. The avian cerebrum is composed of a nuclear pallium, while the mammalian brain is arranged as a laminar cortex. Homologies with birds are still uncertain for most of the telencephalic pallium in birds, so a direct one-to-one comparison is not possible. Even among mammals, there may be substantial differences in opioid receptor mRNA distribution. Studies of human (Homo sapiens) and rat (Rattus norvegicus), µ- and κ-opioid receptor mRNA distribution showed similar but not equivalent distribution, suggesting caution with regard to direct comparison even between related species.

A positive control probe was designed specifically for this study, as one was not readily available for psittacine birds using RNA hybridization probe and FISH assay technology. Housekeeping genes are usually utilized for this purpose, as they are present in most tissues being studied, such as the GAD2 gene in zebra finches. The FOXP2 gene has been linked to human speech and vocal plasticity. This gene has been reported as being present in the medial striatum of the budgerigar and was therefore chosen as the control for this study. Unfortunately, due to its location only within the medial striatum, obtaining a representative coronal section
including the medial striatum proved difficult. Future FISH investigations should utilize a housekeeping gene that is more readily distributed throughout the budgerigar brain.

Previously published in situ hybridization protocols report that the interval between death and freezing of tissues should be just minutes. Brains were collected within 48 h of death. The cause of death in the birds that were used in this study was consistent with carbon monoxide poisoning. Carbon monoxide is an odorless, colorless gas that is not flammable or explosive at concentrations < 12% and readily causes a fatal hypoxemia with prolonged exposure. Carbon monoxide has been deemed an appropriate method of euthanasia if proper safety measures occur to prevent human exposure. The mass mortality situation that occurred is too uncontrolled to be an appropriate method of euthanasia to perform in a repeat scenario. In a study comparing poultry euthanasia methods during an avian influenza epidemic, all birds died within 2 h when exposed to 1.5% to 2% carbon monoxide. This noxious gas induces a loss of consciousness without pain and with minimal discernible discomfort due to its insidious nature. Death should occur rapidly if birds are exposed to concentrations of 4% to 6%. Recent research suggests that carbon monoxide gas may be useful as a local antinociception agent. In mammals, multiple studies have shown endogenous carbon monoxide inhibits inflammation, acute nociception, and neuropathic pain. However, due to the short agonal interval and high doses of carbon monoxide, gene expression is unlikely to be affected.

Despite RNA remaining stable for only a few hours in a live specimen, RNA appears to become more stable in the postmortem period. A number of mechanisms have been hypothesized for this profound difference, including reduced RNase activity, increased longevity of endogenous RNase inhibitors, and a decreased susceptibility to degradation of RNA when translation stops. There is progressive RNA degradation over time with no genes in the human shown to have significantly increased expression with an increase in postmortem interval. The means with which death occurred can also cause substantial alterations in brain pH and stability of RNA. An agonal scoring system has been developed in humans to help determine how much physiologic change may have occurred in the postmortem brain but has not been applied to animal species. Ribonucleases (RNases) can become more active in agonal states, breaking down RNA, which would otherwise be stable in the patient postmortem. Total RNA within the brain has been reported as being stable in multiple species for 24 to 48 hours, including humans, mice, rats, and pigs. The stability of brain tissue for use in FISH studies has also been shown to remain intact for as long as 33 months after the initial freezing without loss of mRNA. The spinal cord, by contrast, should be dissected and frozen rapidly due to the high concentration of RNases present within the tissue. For further determination of mRNA stability, pH can be measured. A lower pH would facilitate RNA degradation and is most commonly observed in agonal patients.

Male specimens were chosen for this study to limit variability by comparing opioid receptor mRNA differences between sexes. Also, budgerigars are sexually dimorphic, and males can be easily identified by their light blue cere. One large difference between the sexes is the act of courtship whereby males utilize song to attract a female mate. Endogenous opioids modulate motivated behaviors, and methionine-enkephalin and leucine-enkephalin have been found in some regions of the song control pathway of zebra finches (Taenopygia guttata), such as Area X. Area X is not an area present in the budgerigar brain, and the vocal pathways of budgerigars and songbirds are only superficially similar. Further studies comparing the male and female budgerigar brain would be necessary to determine if sex plays a role in opioid receptor mRNA distribution.

The distribution of the δ-opioid receptor was not included in the scope of this study. The function of the δ-opioid receptor in avian species is still unclear, but its distinction from the µ-opioid receptor is important. The δ-opioid receptor is widely expressed in the central and peripheral nervous system, usually found in different cell types than the µ-opioid receptor. It may also be more efficacious in inflammatory, neuropathic, and chronic pain than µ-opioid receptors, which are more effective in acute pain. Animal models have also shown anxiolytic and antidepressant effects with δ-opioid receptor stimulation that have not been observed with µ-opioid receptors. Multiple drugs have been developed targeting the δ-opioid receptor specifically, but none are commercially available at present.

This study helps reaffirm the knowledge that the opioid distribution of avian species is very different from mammals. Utilizing FISH to identify opioid receptor distribution in the forebrain and midbrain is both safe and effective. Further studies in multiple avian species can clarify differences in physiologic response to opioid administration.

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