Researchers overcome winking, napping pigs to prove brain test works

July 8 2021, by Lauren Quinn

Adam Jones, research coordinator for the Piglet Nutrition and Cognition Laboratory at the University of Illinois, settles piglet into a custom-built hammock. Credit: University of Illinois at Urbana-Champaign

If you've ever been to an eye doctor, there's a good chance you've felt the sudden puff of air to the eye that constitutes a traditional test for glaucoma. It's no one's favorite experience, but the puff is non-invasive and harmless.

Scientists use a similar method to test learning and memory in animals and humans. Like Pavlov's classic experiments linking a neutral stimulus with a physiological response, the eyeblink test pairs a light or sound with a quick puff of air to the eye. With repetition, the animal learns to close its eye, or blink, in response to the light or sound only. It's called associative learning, and the response is ruled by a brain region known as the cerebellum.

While the eyeblink test has been around since 1922, it had never been attempted in pigs until now. In a new study in *Frontiers in Behavioral Neuroscience*, researchers prove the eyeblink test works in 3-week-old pigs, a model species for nutritional neuroscience research in human infants.

"The idea is, if we can improve structural development in the brain through nutritional interventions, it should take pigs fewer trials to learn the rule. We're in the process of assessing the nutrition piece now, but we had to get the test to work first," says Ryan Dilger, professor in the Department of Animal Sciences at the University of Illinois and co-author on the study.

Dilger specializes in the effects of nutrition on the developing brain, with much of his work feeding directly into the infant formula industry. He uses neonatal pigs because, unlike rodents, their brain anatomy and structure, gut physiology, and nutritional requirements are strikingly similar to human infants.

Dilger's team typically studies pig brain response to new ingredients through magnetic resonance imaging, MRI, which focuses on the structure and size of various brain regions. They also rely on well-validated behavioral tasks, such as novel object recognition, that reflect activity in the hippocampus and striatum, some of the brain regions related to learning and memory.

But Dilger wanted a tool to specifically assess cognitive processing in the cerebellum. That's when he turned to Henk-Jan Boele and Sebastiaan Koekkoek, eyeblink specialists and neuroscientists at Erasmus Medical Center in the Netherlands.

Although humans and all sorts of animals have undergone the eyeblink test, the behavioral paradigm had never been validated for pigs.

"For human and mouse eyeblink conditioning, we use completely different systems," says Boele, a postdoctoral researcher with dual appointments at Erasmus and Princeton University. "Humans are easy to instruct, usually are very cooperative, and sit still during the experiment, which makes it easy to deliver the puff and measure the eyelid. Mice are mostly head-fixed during the experiment, which makes it easy to deliver the air puff and measure the eyelid. Pigs, instead, were a challenge because we did not want to fixate their head. It was really difficult to reliably deliver the air puff and measure the eyelid responses.

"We tried video cameras, diodes, and all kinds of things, to capture the blink. And we had to use a piece of equipment to deliver the air puff that was very close to the eye to avoid any delays. We need really sharp, short puffs that are not invasive for the animal but are still very precisely timed. So that was a challenge," he says.

The solution was taping a short piece of air tubing next to one eye, and pasting magnetic sensors on the forehead and eyelid to record the blink. The system measured the eyeblink down to the millisecond.

And yes, Boele puffed himself in the eye to test out the system. It worked. "Oh yes, I blinked," he laughs.

To ensure the pigs had free head movement but didn't get up and run around, the researchers placed them in a custom-built sound-dampening

box fitted with a pig-sized hammock.

It was apparently very comfy.

Sangyun Joung, a doctoral student in the Neuroscience Program at Illinois and co-author on the study, says, "Each pig had five days of training to habituate them to the hammock and the testing environment. By day three, they were very relaxed, to the point where some of them were literally falling asleep. That was a little challenge for us during the analysis, because that definitely influenced their responses. But it also told us this whole behavior paradigm is not stressful for them. For us, it was interesting and frustrating at the same time."

Once the pigs were used to the setup, the real work began. The pigs did a series of eight tests in a row. The first was the air puff only, to measure the eyeblink reflex. The next six tests paired a small blue LED light with the air puff. The light came on for half a second, 500 milliseconds, and right at the end, with the light still on, the air puff was delivered. That last part, with both the light on and the air puffing, lasted a tiny 50 milliseconds. The final test was the light only—no puff.

The researchers repeated each set of eight tests five times on each of five consecutive days. The time between tests varied a bit to keep the pigs guessing.

"It wasn't just on and off, once per second. The system waits until the eye is in a stable place, and then repeats the test at random times so the pigs can't anticipate the puff," Dilger says.

But they did learn, over the course of the five-day experiment, to anticipate the puff. Pretty soon, pigs were closing their eyes at exactly the 500-millisecond mark during the eighth trial—the one with a light but no puff.

"The timing is perfect. If you look at the conditioned eyelid responses, you can see that the eyelid is closed exactly at the moment the puff would have been delivered," Boele says. "Just perfect motor timing, down to the millisecond. That's beautiful."

The research team learned something else about pigs they hadn't known before.

"We learned that pigs can just close one eye at a time; they can wink. We weren't sure about that, actually," Dilger says. "But because they are pigs, they will frustrate you all day long. Some pigs would just lay there with their one eye closed, which meant we couldn't use that that particular subject. They're smart creatures."

The eyeblink test specifically targets activity in the cerebellum, the part of the brain responsible for making quick, unconscious predictions. These predictions relate to motor responses, such as where to step while you're walking; and cognitive processes, such as predicting what someone is going to say next in a conversation.

"The cerebellum is making short-term predictions all the time, continuously. It's essential to interact with our environment. When we think about learning and memory, often we think about really complex cognitive things, but most of our daily life behavior is just smooth, automatic interactions with our environment," Boele says. "In eyeblink conditioning, your cerebellum basically solves the problem for you. You don't have to think about it. It's making this short-term prediction, and that's what we are studying."

Pigs are born with a more developed cerebellum than human babies. That's clear from eyeblink conditioning tests Boele has done with six-to-eight-month-old infants; they don't typically learn the task at that age.

Unlike humans, pigs need to be able to make motor and cognitive predictions right away, since they can get up and walk around minutes after birth. When Dilger studies the pig cerebellum using MRI, which focuses mostly on structure, he typically doesn't see much change due to nutritional interventions. That's because the cerebellum is more developed at birth in the pig, which makes them a precocial species. But nutritional changes might alter the functioning of the brain region. That is what the eyeblink test will tell him in future studies.

"Often, function follows structure, but not always. Having a nutritional deficiency may show a deficit in eyeblink conditioning, this associative learning task," he says. "We want to be able to use a nutritional intervention as a relatively non-invasive way of understanding cerebellar development here."

More information: Henk-Jan Boele et al, Young Domestic Pigs (Sus scrofa) Can Perform Pavlovian Eyeblink Conditioning, *Frontiers in Behavioral Neuroscience* (2021). DOI: 10.3389/fnbeh.2021.690019

Provided by University of Illinois at Urbana-Champaign