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ACTIVATION OF STATE-REGULATING NEUROCHEMICAL SYSTEMS IN NEWBORN AND EMBRYONIC CHICKS

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Abstract—Coordinated activity in different sets of widely-projecting neurochemical systems characterize waking (W) and sleep (S). How and when this coordination is achieved during development is not known. We used embryos and newborns of a precocial bird species (chickens) to assess developmental activation in different neurochemical systems using cFos expression, which has been extensively employed to examine cellular activation during S and W in adult mammals. Similarly to adult mammals, newborn awake chicks showed significantly higher cFos expression in W-active hypocretin/orexin (H/O), serotonergic Dorsal Raphe, noradrenergic Locus Coeruleus and cholinergic Laterodorsal and Pedunculopontine Tegmental (Ch-LDT/PT) neurons when compared to sleeping chicks. cFos expression was significantly correlated both between these systems, and with the amount of W. S-active melanin-concentrating hormone (MCH) neurons showed very low cFos expression with no difference between sleeping and awake chicks, possibly due to the very short duration of S episodes. In embryonic chicks, cFos expression was low or absent across all five systems at embryonic day (E) 12. Unexpectedly, a strong activation was seen at E16 in H/O neurons. The highest activation of Ch-LDT/PT (also S-active) and MCH neurons was seen at E20. These data suggest that maturation of arousal systems is achieved soon after hatching, while S-control networks are active in late chick embryos.

Key words: chick embryo, newborn chick, sleep, waking, widely-projecting neurochemical systems, cFos.

INTRODUCTION

Three distinct physiological brain states can be distinguished in adult mammals: waking (W), rapid eye movement sleep (REMS), and non-REMS (NREMS). Each state is thought to be triggered and maintained by activity in different combinations of neurochemical systems, as proposed by the reciprocal interaction and flip-flop models of sleep (S) control (Saper et al., 2010). Several widely-projecting neurochemical systems cooperate to produce arousal, including hypocretin/orexin (H/O), serotonergic Dorsal Raphe (5HT-DR), noradrenergic Locus Coeruleus (NA-LC), and cholinergic Laterodorsal and Pedunculopontine Tegmental (Ch-LDT/PT) neurons (Jones, 2011). S is promoted by various GABAergic populations, including those of the anterior hypothalamus and adjacent basal forebrain (Jones, 2011; Luppi et al., 2016). The most important non-GABAergic neurons supporting REMS include hypothalamic melanin-concentrating hormone (MCH) neurons (Verret et al., 2003; Jego et al., 2013; Konadhode et al., 2013) and Ch-LDT/PT neurons (Datta and Siwek, 1997). H/O, 5HT-DR and NA-LC neurons show maximal firing rate (Jones, 2011; McCarley, 2011) and increased expression of the cFos transcription factor during W (Tononi et al., 1994; Maloney et al., 1999; España et al., 2003; Modirrousta et al., 2005). MCH neurons display reciprocal firing patterns with respect to W-active neurons (Hassani et al., 2009) and show increased cFos expression during S recovery/rebound (SR) following S deprivation (SD; Verret et al., 2003; Modirrousta et al., 2005). Ch-LDT/PT neurons appear to be W/REMS active (Boucetta et al., 2014) and show highest cFos expression during SR conditions (Maloney et al., 1999).

Two previously-articulated S ontogeny theories differ in their interpretations of existing data. According to the 'presleep theory', REMS and NREMS emerge from a common precursor 'presleep' state at the time of electroencephalogram (EEG) differentiation (Frank and Heller, 2003, 2005). In the pre-EEG period, S and W regulatory systems are very immature and elements of both

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Abbreviations: 5HT, serotonergic; ANOVA, analysis of variance; BS, blocking solution; cFos-ir, cFos immunoreactivity; Ch, cholinergic; ChAT, Choline Acetyltransferase; cPBS, chicken embryology PBS; DR, Dorsal Raphe (nucleus); E, embryonic day; EDTA, ethylenediaminetetraacetic acid; EEG, electroencephalogram; H/O, hypocretin/orexin; LC, Locus Coeruleus; LDT, Laterodorsal Tegmental (nucleus); MCH, melanin-concentrating hormone; NA, noradrenergic; NREMS, non-rapid eye movement sleep; P, post hatch/natal day; PBS, Phosphate-buffered saline; PBS-T, PBS containing 0.1% Tween 20; PT, Pedunculopontine Tegmental (nucleus) (in mammals) and Peduculotegmental (nucleus) (in chickens); REMS, rapid eye movement sleep; S, sleep; SD, sleep deprivation; SDS, sodium dodecyl sulfate; SR, sleep recovery/rebound; TH, Tyrosine Hydroxylase; TrpH, Tryptophan Hydroxylase; W, waking.

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REMS and NREMS may coexist (Frank and Heller, 2003, 2005). The 'precursor theory' instead posits that two different, behaviorally-distinguishable S states exist before EEG maturation (Blumberg et al., 2005). In order to understand S ontogeny, it is necessary to better understand various aspects of the activation of S and W regulatory systems during development, including what the characteristic activity patterns of regulatory systems look like at different times, when coordination among them is first achieved, how regulatory system activity is related to behavioral phenotypes in developing organisms, and how they compare with adult patterns. The answers to these questions would help us to better define 'immature' brain states and relate them to adult ones.

Developmental studies are difficult in both altricial and precocial mammals given their physiological dependence on the mother. Precocial birds like chickens represent a convenient alternative for studying the development of mechanisms controlling W and S, because birds present the same three behavioral states as mammals (Mellor and Diesch, 2007; Rattenborg et al., 2009). Chick embryos appear to develop S-like EEG patterns a few days before hatching, and chicks display mature and differentiated behavioral states just a few hours after hatching (Cusick and Peters, 1974; Schlehuber et al., 1974; Mellor and Diesch, 2007). This suggests that much of the maturation of behavioral state control may occur *in ovo*, when the developing embryo is easily accessible and lives within a controlled environment.

Although cFos induction is not always associated with increased electrical activity in neurons, it does indicate the recent occurrence of sufficient amounts of neuronal activation (depolarization combined with Ca^{2+} entry and intracellular signaling pathway activation via metabotropic receptors) to affect intracellular gene expression, normally part of a pathway leading to changes in synaptic morphology and plasticity (Cirelli and Tononi, 2000). cFos is used here as a marker for this type of neuronal activation (and not general electrical activity). In order to explore whether newborn chicks and adult mammals share similar activation signatures for the five neurochemical systems during S and W, we characterized cFos expression patterns in post hatch day (P) 1 chicks that were kept awake for 2 h by gentle handling (SD group) and chicks that were sleeping for 2 h either spontaneously (S group) or after 2 h of SD (SR group). We used double-labeling immunofluorescence to study cFos expression in five relevant neuronal populations: H/O, 5HT-DR, NA-LC, Ch-LDT/PT and MCH neurons. We then extended our observations to chick embryos at embryonic days (E) 12, E16 and E20 (hatching is after 20–21 days of incubation) in order to survey the prenatal patterns of activation in these five neurochemical systems.

EXPERIMENTAL PROCEDURES

Ethics statement

The experimental use and care of P1 chicks was approved by the McGill University Animal Care Committee (protocol number: 2012-7284). The experimental protocol for the use of chick embryos was submitted to the McGill University Animal Compliance Office, which, after consultation with the McGill University Animal Care Committee, issued a written waiver stating that according to Canadian and McGill University animal care guidelines, no formal approval was necessary to perform these experiments. Nevertheless, McGill Standard Operating Procedures were followed to minimize any possible suffering by embryos.

Tissue collection for embryos and P1 chicks

Fertilized chicken eggs (Bovan Brown, *Gallus gallus*, Couvoir Simetin, Mirabel, QC, Canada) were incubated under constant conditions (37 °C, 60% relative humidity) in a commercial egg incubator with an automatic egg rotator (Octagon, Brinsea Products Inc., Titusville, FL, USA). Incubator and incubation conditions were the same for all eggs. Egg incubations were all started at 10 AM. Embryos of different ages were obtained from each batch of eggs. Embryonic sacrifices were all carried out between 2 PM and 4 PM. At E12 ($n = 6$), E16 ($n = 5$), or E20 ($n = 13$), embryos were anesthetized by exposure to isoflurane vapor *in ovo* (0.5 mL/0.5 L, for 30 s), then intracardially perfused with Howard Ringer's Solution (123 mM NaCl, 1.5 mM $CaCl_2$, 5 mM KCl; chemicals from Sigma–Aldrich, St. Louis, MO, USA) for 2 min followed by 10% formalin/chicken embryology phosphate-buffered saline (cPBS; 150 mM NaCl, 28 mM KH_2PO_4, 72 mM K_2HPO_4, pH 7.4; chemicals from Sigma–Aldrich) for 10 min using a peristaltic pump (at 2, 4.5 and 6 mL/min, respectively). P1 chicks (total $n = 21$) were deeply anesthetized with isoflurane vapors (15 mL/5L, for 2–3 min) at the end of the behavioral experiment (see below), and then perfused as described above using a peristaltic pump (at 7.5 mL/min).

For both embryos and P1 chicks, heads were removed and kept in a 10% formalin/cPBS solution at 4 °C overnight. The next day, the brains were extracted from the skull, cryoprotected in 10% and 30% sucrose/cPBS at 4 °C, and then frozen in Tissue-Tek O.C.T. (Sakura Finetek USA Inc., Torrance, CA, USA) at −65 °C. All brains were cut into coronal sections on a cryostat (Leica CM3050S; Leica Microsystem Canada, Richmond Hill, ON, Canada) following a chick brain atlas (Puelles et al., 2007). P1 and embryo brains were cut at 20 μm (all sections were collected) and 10 μm (every other section was collected), respectively. Sections were deposited on SuperFrost Plus glass slides (Fisher Scientific, Ottawa, ON, Canada) in series of six and were kept at −65 °C until use.

P1 behavioral protocol

After 20 days of incubation, pairs of eggs were moved into a brooder (37 °C with autoregulated humidity settings; Brinsea Products Inc.) held inside a lightproof, sound-insulated chamber. A GoPro camera (GoPro Inc., San Mateo, CA, USA) was set up and left overnight to take an image every 10 s in order to record the time of hatching for each chick. On the following day, newborn