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Elevated aggressive behavior in male mice with thyroid-specific *Prkar1a* and global *Epac1* gene deletion

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ABSTRACT

Alterations in circulating thyroid hormone concentrations are associated with several psychological and behavioral disorders. In humans, behavioral disorders such as anxiety, depression, and attention-deficit hyperactivity disorder can be associated with thyroid disease. The *Tpo-Cre;Prkar1a*$^{flox/flox}$*;Epac1*$^{-/-}$ (R1A-Epac1KO) mice, originally bred to investigate the role of exchange protein directly activated by cAMP (Epac1) in follicular thyroid cancer, displayed self-mutilating and aggressive behaviors during casual observation. To assess these atypical responses, behavioral testing was conducted with the R1A-Epac1KO mice, as well as their single knockout counterparts, the thyroid-specific *Prkar1a*$^{-/-}$ and global *Epac1*$^{-/-}$ mice. Mice of all three genotypes demonstrated increased aggressive behavior against an intruder mouse. In addition, *Epac1*$^{-/-}$ mice increased response to an auditory stimulus, and the *Prkar1a*$^{-/-}$ and R1A-Epac1KO mice increased swimming behavior in the Porsolt forced swim test. Both *Prkar1a*$^{-/-}$ mice and R1A-Epac1KO mice have increased circulating thyroxine and corticosterone concentrations. Although hyperthyroidism has not been previously associated with aggression, increased thyroid hormone signaling might contribute to the increased aggressive response to the intruder mouse, as well as the increased swimming response. Mice with a genetic background of *Tpo-Cre;Prkar1a*$^{flox/flox}$*;Epac1*$^{-/-}$ are aggressive, and both the thyroid-specific knockout of *Prkar1a* and global knockout of *Epac1* likely contribute to this aggressive behavior. This study supports the hypothesis that altered thyroid signaling and aggressive behavior are linked.

1. Introduction

Hyperthyroidism has long been associated with psychiatric symptoms. In humans with hyperthyroidism, comorbidity with affective disorders, including anxiety, depression, and attention-deficit hyperactivity disorder (ADHD) is often reported (Thomsen and Kessing, 2005; Grabe et al., 2005). Thyrotoxicosis induced by consumption of ground beef contaminated by bovine thyroid tissue induced aggression and fire-setting in a 4 year-old boy (Bhatara et al., 2009). Thyroid receptors are expressed throughout the adult brain, including regions involved in affective behavior such as the prefrontal cortex and the amygdala (Lechan et al., 1993). Therefore, alterations in thyroid hormone signaling are likely to affect behavior. Indeed, pharmacological induction of hyperthyroidism in rodents can cause an array of behavioral effects, including changes in locomotor activity, muscle strength, motor coordination, learning, memory, and affective behaviors (Yu et al., 2015; Bitiktas et al., 2016; Rakov et al., 2016). Behavioral effects differ based on the specific pharmacological or genetic induction of elevated thyroid function. Thyroid-specific deletion of *Prkar1a* causes hyperthyroidism in mice Pringle et al., 2012), but their behavior remains unspecified.

The thyroid-specific *Prkar1a*$^{-/-}$ mouse (R1A) was developed to investigate the tumorigenic role of *PRKAR1A* in the thyroid gland, a cAMP-responsive tissue (Pringle et al., 2012). *PRKAR1A* encodes the type1a regulatory subunit of protein kinase A (PKA), and mutations of this gene have been implicated in both inherited and sporadic cases of thyroid cancer (Sandrini et al., 2002). Over 40% of R1A mice develop follicular thyroid cancer (FTC) by one year of age, and R1A mice have hyperthyroidism (Pringle et al., 2012). Increased thyroid stimulating hormone (TSH) concentrations are associated with human thyroid cancer. PKA signals downstream of TSH through the production of cAMP (Hargadine et al., 1970; Haymart et al., 2008), however, because of the pervasive effects of cAMP, therapies attempting to target cAMP signaling can cause a large number of side effects (Saunders et al., 1997;

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Propper et al., 1999). Therefore, increasing focus has been on understanding downstream targets of cAMP signaling and targeting those pathways instead (Pringle et al., 2014).

Exchange protein directly activated by cAMP (Epac) presents a potential alternative therapeutic target. Epac is an intracellular sensor, in addition to PKA, that mediates the effects of cAMP to activate downstream Rap1 (Kawasaki et al., 1998; de Rooij et al., 1998), in many tissues including the thyroid (Ribeiro-Neto et al., 2004). Epac regulates Rap activity, both in concert with PKA and independently, and the effects, whether stimulatory or inhibitory, seem to be dependent on cell type and context, as well as the type of stimuli (Almahariq et al., 2013; Cheng et al., 2008; de Rooij et al., 1998; Tsygankova et al., 2001). Epac plays an important role in cell migration and invasion in other cancers (Grandoch et al., 2009). *Epac1*$^{-/-}$ mice are viable and fertile, with no obvious thyroid gland defects. Therefore, the R1A mice were crossed with *Epac1*$^{-/-}$ mice, generating *Tpo-Cre;Prkar1a*$^{flox/flox}$;*Epac1*$^{-/-}$ (R1A-Epac1KO) mice to investigate the role of *Epac1* in FTC.

Epac genes are expressed in both developing and adult brains; however, *Epac1* is more highly expressed during development whereas *Epac2* is more prevalent in adults (Kawasaki et al., 1998; Murray and Shewan, 2008). Developmental expression of EPAC proteins promotes cAMP-dependent axon growth and regeneration (Murray and Shewan, 2008), and are located throughout the hippocampus, the prefrontal cortex, cerebellum, and the suprachiasmatic nucleus of the hypothalamus (Nikolaev et al., 2004; Dwivedi et al., 2006; O'Neill et al., 2008). EPAC proteins have also been linked with behavioral changes. *Epac2* deletion, but not *Epac1* deletion, in mice altered anxiety-like behaviors, hyperactivity, and depressive-like responses (Zhou et al., 2016). Both *Epac1*$^{-/-}$ and *Epac2*$^{-/-}$ mice display normal cognitive functions such as LTP and spatial learning, whereas mice lacking both isoforms display deficits in these tasks (Yang et al., 2012), suggesting a redundant role for the proteins. Epac1 has been implicated in causing deficits in sensorimotor gating (Kelly et al., 2009), which is associated with many psychiatric disorders (Geyer, 2006).

Inadvertent behavioral alterations are often induced by genetic modifications of mice generated for other purposes. While working with the R1A-Epac1KO mice, behavioral abnormalities were observed that warranted further behavioral phenotypic investigation. This mouse model presents a unique opportunity to investigate the roles of *Prkar1a* and *Epac1* in behavior, as well as the reciprocal effect of the double KO. Considering the *Prkar1a* knockout is thyroid-specific, behavioral alterations are likely the result of downstream effects of the deletion, such as alterations in thyroid hormone signaling. Here, we characterize the behavioral phenotype of all three genotypes, R1A, Epac1KO, and R1A-Epac1KO mice. We investigated sensorimotor function, memory, locomotor function, affective behaviors, and aggressive behavior to provide a relatively complete behavioral assessment. All three genotypes showed a dramatic increase in aggression toward an intruder mouse along with other alterations in affective behavior. This study is, to our knowledge, the first to implicate hyperthyroidism in murine aggressive-like behaviors, and provides insight into additional roles for *Prkar1a* and *Epac1* in affective and aggressive behaviors.

2. Methods

2.1. Animals

Male adult mice comprising four genotypes were maintained in a sterile environment under a 12-hour light/dark cycle with access to ad libitum food (Harlan Teklad 8640; Madison, WI, USA) and filtered tap water, except when noted below. Prior to behavioral testing, all mice were group housed with littermates. Mice selected for behavioral testing were then separated and single-housed, allowing 3 weeks to acclimate prior to the initiation of the first test. Cages were kept on ventilated racks in a temperature- and humidity-controlled vivarium at The Ohio State University. *TPO-cre*; *Prkar1a*$^{loxP/loxP}$ (Pringle et al., 2012) were mated with *Epac1* KO mice (Suzuki et al., 2010), provided kindly by the Ishikawa group at Yokohama City University, Japan, to produce a double knockout, R1A-Epac1KO. All experiments were performed in a C57BL/6 CBA and FVB mixed background. All experimental mice were derived from the same parents of origin and inbred to produce each of the four genetic models: *TPO-cre; Prkar1a*$^{loxp/loxp}$ (R1A), *TPO-cre; Prkar1a*$^{loxp/loxp}$; *Epac1*$^{-/-}$ (R1A-Epac1KO), Prkar1a$^{loxp/loxp}$; *Epac1*$^{-/-}$ (Epac1KO), and *Prkar1a*$^{loxp;loxp}$ (WT Control). Twelve animals from each of the four genotypes were included in the behavioral study. Animals were between 6 and 10 months of age at the onset of testing, and were coded with random experimental numbers to ensure the experimenter remained uninformed of the experimental groups. All behavioral testing was conducted during the animal's light phase, between the hours of 1000 and 1800 h EST, and mice were acclimatized to the room for 30 min prior to testing. The rooms used for behavioral testing and acclimatization were at approximately the same light level as in the animal housing room. Behavioral testing was conducted in the order of least to most stressful over 40 days, with at least one day between tests (see Table 1). For tests which required multiple days, mice were divided into two groups, with each genotype equally represented in both groups. Testing was not conducted on days in which cage changes occurred. All experiments were conducted with approval from the Ohio State University Institutional Animals Care and Use Committee and were consistent with the NIH regulations.

Table 1
Order and timing of behavioral tests.

Day	Behavioral test
1	Initial assessment
3	Visual placing, contact placing, limb clasping, and auditory function tests, grip strength test
5–6	Find the hidden cookie test
8	Open field and elevated plus maze
11–12	Marble burying test
17	Rotorod test
19–23	Sucrose anhedonia test
25	Y-maze
28	Digging test
30	Porsolt forced swim test
33–34	Aggression against and intruder test
39	Hot plate test

2.2. Initial assessment

An initial assessment of each mouse was conducted to ensure behavioral differences did not arise from gross deficits. Body weight was measured and length was measured from the tip of the nose to the base of the tail. Vibrissae were visually assessed and assigned a score from 0 to 4; mice with all whiskers present and un-barbered were assigned a score of 0, mice with no whiskers were given a score of 4, and scores of 1, 2, and 3 were assigned for mice with conditions between the two extremes. Eye appearance was assessed visually for discrepancies in size, shape, and condition, with a score of 0 assigned for normal, a score of 1 for mild abnormalities, and a score of 2 was given for mice with severe abnormalities. Muscle tone was assessed by handling each animal, allowing him to climb on the examiner's fingers, using the 0–2 scale described for eye appearance. Ear appearance was scored using a 0–2 scale where 0 indicated both ears were normal, 1 indicated one ear pinna was malformed or missing, and a 2 indicated both ear pinna were malformed or missing.

2.3. Sensorimotor tests

Several sensorimotor tests were conducted to detect any deficits that could potentially affect other behaviors. The *visual placing test* was used to detect visual impairment. Each mouse was held by the tail and