Neuroglobin deficiency increases seizure susceptibility but does not affect basal behavior in mice

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Abstract
Neuroglobin (Ngb) is found in the neurones of several different brain areas and is known to bind oxygen and other gaseous molecules and reactive oxygen species (ROS) in vitro, but it does not seem to act as a respiratory molecule for neurones. Using male and female Ngb-knockout (KO) mice, we addressed the role of Ngb in neuronal brain activity using behavioral tests but found no differences in general behaviors, memory processes, and anxiety/depression-like behaviors. Oxidative stress and ROS play key roles in epileptogenesis, and oxidative injury produced by an excessive production of free radicals is involved in the initiation and progression of epilepsy. The ROS binding properties led us to hypothesize that lack of Ngb could affect central coping with excitatory stimuli. We consequently explored whether exposure to the excitatory molecule kainate (KA) would increase severity of seizures in mice lacking Ngb. We found that the duration and severity of seizures were increased, while the latency time to develop seizures was shortened in Ngb-KO compared to wildtype adult female mice. Consistently, c-fos expression after KA was significantly increased in Ngb-KO mice in the amygdala and piriform cortex, regions rich in Ngb and known to be centrally involved in seizure generation. Moreover, the measured c-fos expression levels were correlated with seizure susceptibility. With these new findings combined with previous studies we propose that Ngb could constitute an intrinsic defense mechanism against neuronal hyperexcitability and oxidative stress by buffering of ROS in amygdala and other Ngb-containing brain regions.

KEYWORDS
behavior, c-fos, cognition, kainate, kainic acid, neuroglobin, oxidative stress, reactive oxygen species.
Members of the globin protein family are found in almost all organisms, and eight members are found in vertebrates, but the exact number of all the different globin genes is unknown due to wide variety of groups where they occur (e.g., the Caenorhabditis elegans genome contains approximately 33 globins) (Vinogradov et al., 2006). Globins are characterized by having a globin fold, which can harbor a hem-ring. Two of these globins, hemoglobin and myoglobin, are involved in transport and release of oxygen (respiratory proteins), whereas the endogenous function for the other globins is unknown. Neuroglobin (Ngb) was discovered in 2000 (Burmester et al., 2000) and was thought at that time to be the respiratory molecule of the brain. Later studies have, however, shown that lack of Ngb does not lead to an altered phenotype in terms of increased overall susceptibility to hypoxia or cerebral ischemia (Hundahl et al., 2011; Kelsen et al., 2008; Raida et al., 2013; Raida, Hundahl, et al., 2012; Raida, Reimets, et al., 2012), indicating that the endogenous function of Ngb is not as a respiratory protein in classical terms like hemoglobin and myoglobin. However, deprivation of Ngb exacerbated hypoxia-dependent neuronal excitability via up-regulation of Hif1A and c-fos and impacted the transcriptional response of genes involved in the glycolytic pathway (Hundahl et al., 2011). Hypoxia and altered glycolytic activity are known to generate reactive oxygen species (ROS) (Chandel et al., 1998; Liemburg-Apers et al., 2015), and it may therefore not be the lack of oxygen per se but generation of ROS which triggers an altered gene response in Ngb-knockout (KO) mice exposed to hypoxia. Moreover, hypoxia has also been shown to increase c-fos expression more in Ngb-KO mice as compared to wildtype (WT) littermates (Hundahl et al., 2011). Ngb besides oxygen can also interact with ROS and nitric oxide (NO) species (Li et al., 2008; Tiso et al., 2011; Watanabe et al., 2012), and since oxidative stress and ROS are believed to be crucially involved in hyperexcitability and epileptogenesis (Pikkarainen et al., 1999), we decided to test if Ngb-KO mice have increased seizure susceptibility in a kainate (KA)-induced acute seizure model, which has also been shown to involve oxidative stress as an underlying pathological mechanism (Liang et al., 2007; Shin et al., 2008). KA is an excitatory amino acid agonist at glutamatergic KA receptors that can act as a neurotoxic substrate at high doses, and it is known to induce hyperexcitability developing into convulsive status epilepticus (SE) when injected into rodents (Epsztain et al., 2010; Lerma & Marques, 2013). Induction of prolonged epileptic discharges leads to various cellular events and results in neuronal damage or death, and unbalanced oxidative stress is one of the early cellular events and a critical factor determining the fate of neurones in epilepsy (Rusina et al., 2021). Both exogenous and endogenous antioxidants counteract oxidative damage (Lin et al., 2020). In the mouse brain, Ngb is found in several brain areas connected to the hippocampal formation and relevant for development of seizures (i.e., piriform cortex, medial amygdala, lateral septum, posterior hypothalamus among others), but is not found in the hippocampus itself (Hundahl et al., 2010). Thus, we analyzed if lack of Ngb would make mice more susceptible to KA-induced seizures than WT mice, and we studied c-fos activation in the brain of these mice after KA to potentially identify brain regions activated by Ngb deficiency. We also conducted the first comprehensive study of whether general behavior, memory, or anxiety- and depression-like behaviors were modified, as an altered behavior in the Ngb-KO mice might confound the seizure results.

2 | EXPERIMENTAL PROCEDURES

2.1 | Animals

Ngb deficient mice were generated in C129 and backcrossed to C57BL/6 as previously described (Hundahl et al., 2011), and they have a genomic deletion of Ngb exons 2 and 3 resulting in lack of Ngb protein. All animal experiments followed the ARRIVE guidelines, and were performed in accordance with Danish Animal Welfare Agency guidelines under directive BEK 88 and the European Union directive 2010/63/EU (22nd of September, 2010) on the protection of animals used for scientific purposes. The study was approved by Dyreforstegstilsynet, Ministry of Justice, Denmark (under license numbers 2008/561-1542; 2010/561-1834; AT nr.: 231-0112108-7160). All animals were group-housed under standard conditions (23°C, 50% humidity, 12/12 h light/dark cycle) with wood shavings, bedding, a shelter, free access to food and water. Every effort was made to minimize the number of animals used and to minimize any distress. General health and wellbeing of the animals were assessed regularly by veterinarians and trained caretakers. Animals were monitored daily, and if general health status of an animal was significantly worsened, the animal would be terminated by an overdose of CO2 and decapitated instantly. Definitions of acceptable endpoints included: no spontaneous movements and inability to drink or eat in a 24-h observation period, loss of body weight greater than 20% from start, massive bleeding, missing anatomy, and inability to right itself in 30 s period. No animals met the criteria for these humane endpoints. All researchers involved in animal experiments...
were trained and certified with required FELASA (Federation of European Laboratory Animal Science Association) licenses, in line with the EU directive 2010/63, article 23 and the EC guidelines on education. Animals were randomized to treatment groups within blocks to avoid introduction of bias influencing the results, considering nuisance variables to avoid bias (e.g., due to cage location, housing conditions, experimental time of day, and investigators). All experiments were performed by persons blinded to the treatment groups. Power analyses were not performed, rather the number of animals was selected based on prior experience with the used experimental setups and models.

At the time of the experiment the age of the mice was 16–21 weeks. Ten WT (five males and five females) and nine Ngb-KO (five males and four females) mice were used in the SHIRPA experiments in a 1-day experimental period; 23 WT (15 males and 8 females) and 28 Ngb-KO (13 males and 15 females) mice were used for testing in behavioral paradigms (open field, elevated plus maze, novel object recognition, forced swim) over 3-week period. Nine WT and 11 Ngb-KO female mice were used for evaluation of KA-induced seizures in a 1-day experimental period, and additionally 12 females WT and 12 females Ngb-KO were allocated for evaluation of the c-fos response to KA-induced seizures after 75 and 120 min, in a 1-day experimental period. Of these, one Ngb-KO mouse developed severe seizures and died before 75 min, and one Ngb-KO and one WT mouse developed severe seizures and died before 120 min. Thus, 11 WT and 10 Ngb-KO mice were evaluated for c-fos response. If not otherwise stated, animals were sacrificed humanely at the end of the experiments by appropriately trained and experienced persons by means of decapitation.

### 2.2 | Behavioral phenotype assessment, primary screen (SHIRPA)

Behavioral and physiologic characterizations of experimentally naive mice were assessed using the SHIRPA primary screen procedure (Rogers et al., 1999) with minor adjustments essentially as previously described (Schmidt et al., 2011). Briefly, body weight and body length were measured. Then the mice were placed in a cylindrical clear Perspex viewing jar (14 x 10 cm in diameter), and the undisturbed behavior (body position, spontaneous activity, respiration, and tremor) was observed and noted. Then the mice were transferred to an arena (55 x 35 x 18 cm) for observation of transfer arousal, palpebral closure, ploioception, startle response, gait, pelvic elevation, tail elevation, touch escape, and positional passivity. Subsequently, the mice were held by the tail, and trunk curl and limb grasping were rated. The animal was then lowered toward a grid, and the visual acuity was evaluated. The mouse was allowed to grip the grid, and a gentle backwards pull was applied; and grip strength, body tone, and reflexes of the ears, eyes, and limbs were scored. Then the mice were placed hanging in their forelegs on a wire (15 cm long, 1 mm in diameter), and behavior was scored. Skin color, heart rate, limb tone, abdominal tone, lacrimation, salivation, and provoked biting of the mice were recorded. Finally, the righting reflex, contact righting reflex, and negative geotaxis were also evaluated. Throughout the procedure, incidence of abnormal behavior, fear, irritability, aggression, or vocalization were recorded. Basal locomotor activity was measured in an arena (25 x 42 x 40 cm) with a scant lining of fresh wood-chip bedding. A video camera (Logitech QuickCam Ultra Vision, Lausanne, CH) recorded the activity of the animals for 30 min and locomotion was analyzed using Noldus EthoVision versus 3.1 software (Wagening, NL, RRID:SCR_000441).

### 2.3 | Open field test

To further analyze locomotor as well as anxiety-like behavior, mice were tested in a larger open field arena (40 x 40 x 80 cm) placed in a dimly lit room (Schmidt et al., 2011). Above the open field a camera (Logitech Quickcam Ultra Vision, Lausanne, CH) connected to a computer-assisted monitoring system (Ethovision, Noldus, Wagening, NL, RRID:SCR_000441) recorded the whereabouts of the mouse for 1 h. Total distance moved, time in the center zone (34 x 34 cm with center in the center of the open field) and time in the rim zone (6 cm wide from the edge) were recorded for 30-min periods using the Ethovision video tracking software. The relationship between time spent in center and rim zones was analyzed as a measure of anxiolytic-like behavior.

### 2.4 | Elevated plus maze test

This test was performed in a setup consisting of an elevated (50 cm above the floor) cross-shaped arena, with two opposing open arms (27 x 7 cm), two opposing closed arms (27 x 7 x 13.5 cm), and a center zone (7 x 7 cm) as previously described (Schmidt et al., 2011). The maze was positioned in the center of a dimly lit room, and each mouse was placed in the center zone facing a closed arm and tested for 10 min. The ratio between entries into and time spent in the presumed anxiety-provoking open arms and entries and time spent in the presumed safe and closed arms are believed to reflect the level of anxiety-like behavior. Time spent in open arms and entries into open or closed arms from the center zone were recorded and tracked by the video system mentioned above. The Ethovision tracking system recorded movements and location, and the precise location of the mouse was defined by the midpoint of the mouse body as to define an entry into the arms and center area.

### 2.5 | Novel object recognition test

The mice were habituated to the Perspex testing arena (40 x 40 x 80 cm) for 1 h 1 day prior to the training trial. Two identical objects were placed in adjacent corners of the testing arena and mice were allowed to freely explore for 10 min. Following a 1-h intertrial period the mice were reintroduced to the testing arena...
for a new 10-min test, but this time one of the original objects was replaced with a different and clearly distinguishable novel object. Each trial was recorded, and the time spent interacting with the objects as well as distance moved during the trials were analyzed using EthoVision. Exploration of an object was defined as the mouse facing the object and with the nose being within 2 cm of the object. Novel object recognition index was calculated as the time spent exploring the novel object divided by the time spent exploring both objects.

2.6 | Forced swim test

The forced swim test was initiated by placing each mouse into a glass cylinder (diameter: 10 cm, height: 25 cm) filled with 10 cm of temperature maintained as previously described (Schmidt et al., 2011). Each trial consisted of a 6-min swimming period. The mice were considered immobile when floating in an upright position, only making small movements to keep their heads above water. Mouse activity was recorded (Logitech QuickCam Ultra Vision, Lausanne, Switzerland) and an observer blinded to treatment scored the total duration of immobility and latency to first immobility period.

2.7 | KA-induced seizures

The KA-induced seizure model was performed essentially as previously described (Olesen et al., 2012; Woldbye et al., 2005). Mice were injected subcutaneously with 35mg/kg KA (K2389, Sigma-Aldrich, Brondby, Denmark; RRID:SCR_008988) dissolved in isotonic saline and adjusted to pH 7.4. Pilot studies showed that 35 mg/kg of KA was necessary to produce motor seizures in WT mice. The mice were placed individually in transparent Plexiglas boxes (10×10×10 cm) and observed for motor seizures for 2 h by an observer unaware of the genotype. Latencies to first motor seizure and SE and percentage time spent in motor seizures were determined. Motor seizures were defined as continuous forelimb clonic convulsions lasting for at least 5 s, and SE was defined as minimum 10 min of continuous motor seizure activity. Finally, seizure severity was scored accumulatively according to a modified rating scale of Marsh et al. (1999): grade 0 (no motor seizure occurrence), grade 1 (starring or facial movements), grade 2 (head nodding or isolated twitches), grade 3 (motor seizure with limb clonus), grade 4 (motor seizure with rearing), grade 5 (motor seizure with loss of posture or SE), and grade 6 (death). In an additional subset of animals (12 WT and 12 Ngb-KO females) the mice were sacrificed at time points 75 or 120 min after KA injection for visualization of Ngb and c-fos immunostaining and in situ hybridization for regional c-fos mRNA levels at these time points. Animals were sacrificed by an overdose of i.p. pentobarbital and perfusion fixed transcardially with phosphate buffered saline (PBS) followed by phosphate buffered 4% formaldehyde (PFA). Brains were removed and postfixed in PFA for at least 24 h at 4°C and used for histology.

2.8 | Histology

The brains from the KA-treated mice were cryopreserved in 30% sucrose in PBS for 5 days and the sectioned on a freezing stage microtome in four series of 40μm sections; thus, one series represents one fourth of the whole brain. Sections were stored in PBS plus 0.1% sodium azide at 4°C until immunostaining or in situ hybridization.

2.8.1 | Immunostaining

For an overview of primary antibodies used, see Table S1. One series was immunostained with a rabbit polyclonal anti-Ngb antibody against purified recombinant mouse Ngb (Dewilde et al., 2001) (University of Copenhagen, #4836). The anti-Ngb antibody was previously characterized (Hundahl et al., 2011), and specificity for binding to Ngb was evidenced using Western blotting and immunohistochemical staining in tissue from mice with or deprived of Ngb (Ngb-null) mice as control (Hundahl et al., 2012). In addition, Ngb immunohistochemical staining and mRNA in situ hybridization showed identical and overlapping expression patterns (Hundahl et al., 2010). The antibody was diluted 1:100,000 in PBS+1% human serum albumin (hSA)+0.1% sodium azide and 0.1% Triton X-100 (hSA-PTA). Another series was stained with a rabbit anti-c-Fos antibody (cat # H-125, Santa Cruz Bio Technology, CA, USA; RRID:AB_2106765), with an epitope corresponding to amino acids 210-335 mapping the c-Fos c-terminus. The anti-c-fos antibody was diluted 1:3000 in hSA-PTA. In brief, the whole mount immunostaining protocol was as follows: Sections were rinsed in PBS for 10 min, incubated in 1% H2O2 in PBS for 15 min. Rinsed in three times PBS for 30 min and preincubated in hSA-PTA for 1 h before adding the primary antibody. Sections were incubated in primary antibody (i.e., rb-anti-neuroglobin or rb-anti-c-Fos) over night at 4°C, and then rinsed three times in hSA-PTA for 30 min before being incubated with the secondary donkey-anti-rabbit Fab2 antibody (cat #711-066-152 Jackson ImmunoResearch Laboratories, Baltimore, PA, USA) diluted 1:2500 in hSA-PTA for 1 h at RT. All incubations were conducted on a shaking table. Sections were then washed three times in hSA-PTA and incubated for 1 h at RT in avidin–biotin complex (Vector Elite ABCComplex [Vector Labs, Burlingame, CA, USA]) diluted 1:50 in PBS. Then rinsed three times in PBS and incubated for 15 min in 0.05% dianisobenzindine (DAB) (Sigma)+0.0001% H2O2. Chromogene reaction was stopped by washing 5 min in PBS+1% H2O2 followed by rinse in Milli-Q water. Finally, sections were transferred to a bath containing chromogelatine at RT and mounted on object slides.

2.8.2 | In situ hybridization

One series was processed for free-floating in situ hybridization. An antisense 48’mer DNA oligonucleotide probe, complementary to the rat c-fos mRNA (GeneBank accession number NM_022197.2) bases 156-203, 5’GCAGCGGGGA
GGATGACGCCTCGTAGTCCGCGTTGAAACCCGAGAACAT-3′ was labeled with 35S-dATP (Amersham # SJ1334/PerkinElmer) by use of terminal transferase (Sigma-Aldrich, Europe). Free-floating 40μm PFA fixed sections were acetylated and then incubated in 4 ml hybridization (tRNA 1 mg/ml, 50% formamide, 12.5% dextran sulfate, 12.5% SALTS, 10 mM DTT) buffer for 1 h at 38°C. Fifteen microliter of the labeled probe (0.3 pmol) were added to 1 ml of hybridization buffer and applied to the sections. Incubations were conducted on a shaking table at 38°C O/N. Sections were then washed 4 x 15 min at 55°C in 1x SSC followed by 2 x 15 min in 0.1x SSC at RT, rinsed briefly in Milli-Q water, mounted to glass slides, and exposed on a Kodak MR x-ray film for 2 and 8 weeks at 4°C for analyzing intensely and less intensely labeled regions on the films, respectively. All analysis and comparisons were conducted between images of the same procedure and x-ray film exposure duration.

2.8.3 | Image analysis

Computer-assisted autoradiographic image analysis of the x-ray films, was performed using Scion Image (National Institute of Health, USA; RRID:SCR_008673), essentially as previously described (Gotzsche et al., 2012). For the quantitative determination of mRNA levels, optical densities (kBq/g) based on calibration curves were obtained with 14C-microscales (Amersham Biosciences). Measurements were conducted by an observer blinded to animal genotype, in three adjacent sections per animal in the brain regions: hippocampal CA1 and CA3 (pyramidal cell layer), dentate gyrus (hilus), and subiculum, basolateral, medial, and central amygdala, cingular cortex areas 1 and 2, lateral and medial septum, and piriform cortex. Right and left side values were averaged per section and per animal. Background measurements were subtracted from each image in each measurement before further analysis.

2.9 | Data analysis

To analyze for phenotypic differences between WT and Ngb-KO mice in the SHIRPA procedure the continuous data including body weight, body length, and spontaneous locomotor activity were analyzed using two-tailed unpaired Student’s t-tests. For the remaining discrete parameters of the SHIRPA procedure the Mann–Whitney U-test was used. To analyze for phenotypic differences between WT and Ngb-KO in data obtained in the OF, EP, NOR, and FS multiple unpaired Student’s t-test adjusted for false discovery rate (Q = 1%) was used for comparisons. In the KA seizure model two-tailed unpaired Student’s t-test was used to analyze for genotypic differences in latency time and seizure duration, two-tailed unpaired Mann–Whitney U-test was used to analyze for genotypic differences in SE and mortality rates, and two-way ANOVA followed by Bonferroni post-hoc analysis for multiple comparisons in seizure score. Number of animals included was based upon the empiric-based experiences from previous experiments conducted in the laboratory. Data are presented as mean±SD if not otherwise stated. A value of p < .05 was considered statistically significant. Statistical analysis and graphical illustrations were performed using GraphPad Software, Inc. Vs. 6.0 (San Diego, CA, US; RRID:SCR_002798).

3 | RESULTS

3.1 | Behavioral and physical characterization

To examine if lack of Ngb affects basic behavioral and physical functions, we assessed composite basal behavior (SHIRPA), locomotion (open field test), anxiety-like behavior (open field, elevated plus maze test), learning and memory (novel object recognition test), and depression-like behavior (forced swim test) in mice with normal Ngb levels (WT) and Ngb-KO mice. For analysis female and male mice were pooled since no significant sex-specific differences were observed. The behavioral and physical characterization revealed no other phenotypic differences between WT and Ngb-KO mice (Tables S2 and S3) besides a small difference in body weight in male mice, with the Ngb-KO being slightly lighter than their age-matched WT counterparts (not shown; Student’s t-test), t(8) = 4.876, p = .0012. No genotypic statistic significant differences were observed between Ngb-KO and WT in (1) the open field test (total distance moved (t,FDR(138) = 0.37159, p = .7108), duration in center (t,FDR(138) = 0.06284, p = .9500), and entries into center (t,FDR(138) = 0.03014, p = .9760)), (2) elevated plus maze test (duration in open arms (t,FDR(49) = 1.441, p = .01558)) and entries in open arms (t,FDR(49) = 0.8378, p = .4062)), (3) novel object recognition test (training trial (t,FDR(49) = 0.0076, p = .9939) and test trial (t,FDR(49) = 0.2898, p = .7732)), and (4) forced swim test (latency to first immobility (t,FDR(49) = 0.5963, p = .5537) and immobility (t,FDR(49) = 1.051, p = .2984)).

3.2 | KA-induced seizures

We hypothesized that a Ngb deficit would be more likely to manifest itself under stressful conditions as seen when challenged by hyperexcitability during seizures. We therefore subjected both WT and Ngb-KO mice to the KA seizure model. Indeed, Ngb-KO mice developed seizures faster than WT mice as shown by shorter latencies to first motor seizure (52 min vs. 84 min, respectively; Student’s t-test, t(18) = 2.623, p = .0173) and SE (52 min vs. 91 min, respectively; Student’s t-test, t(18) = 2.954, p = .0085) (Figure 1a) and spent longer time in motor seizures (46% vs. 24%, respectively; Student’s t-test, t(18) = 2.209, p = .0404) (Figure 1b). Overall, the Ngb-KO mice also developed more severe seizures as revealed by significantly higher cumulative seizure scores than the WT controls during the 2-h observation period (Figure 1c: two-way ANOVA, F(1, 18) = 7.757, p = .0122). In addition, in comparison between Ngb-KO and WT animals, SE development rates were
91% versus 56% (Mann–Whitney U = 82, p = .0617) and mortality rates were 27% versus 0% (Mann–Whitney U = 106, p = .3382), respectively.

### 3.3 | C-fos expression

Since we observed a more seizure susceptible phenotype in the Ngb deficient mice, we subjected another subset of animals to the KA-induced seizure model and collected the brains at time points 75 and 120 min after the KA injection, with the objective to analyze which brain areas were activated during seizure development. C-fos mRNA levels were measured in the following brain areas: hippocampal CA1, CA3 and dentate gyrus (DG), subiculum (sub), basolateral (BLA), central (CeA), and medial amygdala (MeA), cingulate cortex areas 1 and 2 (CCA1 and 2), lateral and medial septum (LS and MS), and piriform cortex (PC) (see Table 1 and Figure 2). After 75 min, c-fos mRNA expression was significantly higher in the medial, basolateral, and central amygdala in the Ngb-KO mice compared to WT mice (Table 1). All other measured areas showed no differences in c-fos mRNA expression levels. After 120 min, significantly higher c-fos mRNA expression was found in the hippocampal CA1, CA3, DG and subiculum as well as in the medial, basolateral, and central amygdala, and lateral septum of Ngb-KO mice versus controls (Table 1). A similar tendency was observed in the cingulate cortex areas 1 and 2, and in the piriform cortex, but did not reach significance. Similarly, c-fos protein expression as revealed by immunohistochemistry (IHC) showed intense labeling of the medial, basolateral, and central amygdala, in Ngb-KO mice both 75 and 120 min after the KA injection compared to WT (Figure 3a,e,i,m). Even though c-fos mRNA did not reach significantly increased levels in the piriform cortex, still intense labeling was observed at 75 min post-KA in Ngb-KO compared to WT, and after 120 min this difference seemed to be reduced again (Figure 3d,h,l,p). Lateral septum showed high c-fos protein expression 75 min after the KA injection in both Ngb-KO and WT mice, and the high c-fos protein expression in Ngb-KO remained at 120 min (Figure 2c,g,k,o). In the hippocampal formation, c-fos protein expression in the CA1, CA3, dentate gyrus (DG), and subiculum appeared equal in intensity in the WT and Ngb-KO mice after 75 min, in accordance with the mRNA expression. However, after 120 min, hippocampal c-fos protein expression in the WT was reduced compared to 75 min expression levels and compared with the Ngb-KO, whereas hippocampal c-fos protein expression in the Ngb-KO was more intense than after 75 min, and more intense than in the WT (Figure 3b,f,j,n).

We utilized the expression of c-fos mRNA as indicator of neuronal activation (Ceccatelli et al., 1989; Curran & Morgan, 1995; Hale et al., 2008; Hunt et al., 1987; Kovacs, 1998; Sonnenberg et al., 1989; Woldbye et al., 1996) to provide a potential rationale for the observed increased seizure susceptibility in Ngb-KO animals compared to WT animals. In the subset of animals used for c-fos analysis, we observed a similar increase in seizure score at 75 min (and a tendency at 120 min) in Ngb-KO animals compared to WT (Figure S1). A Spearman's rank coefficient correlation analysis revealed a correlation between increases in c-fos mRNA levels in basolateral, central, and medial amygdala, and piriform cortex and a higher seizure score reached at 75 min (Figure S2), and a correlation between increased c-fos mRNA levels in CA1, CA3, subiculum, cingulate cortex areas 1, and lateral septum and higher seizure score reached at 120 min (Figure S3).
TABLE 1  C-fos mRNA levels 75 or 120 min after KA injection

| Time after KA | 75 min | 120 min |
|---------------|--------|---------|
|               | WT     | Ngb-KO  | WT     | Ngb-KO |
| Brain region  |        |         |        |        |
| Hippocampal CA1 | 250.8 ± 44.2 | 240.7 ± 71.1 | 162.7 ± 36.5 | 281.5 ± 90.5 |
| Dentate gyrus  | 191.9 ± 48.1 | 184.8 ± 35.9 | 162.2 ± 36.8 | 246.1 ± 55.5 |
| Subiculum      | 418.7 ± 103.9 | 399.0 ± 116.4 | 112.1 ± 14.3 | 342.7 ± 177.6 |
| Basolateral amygdala | 146.6 ± 52.4 | 136.2 ± 30.4 | 105.0 ± 15.5 | 178.5 ± 64.6 |
| Central amygdala | 61.0 ± 16.1 | 91.7 ± 15.0 | 51.4 ± 10.9 | 87.9 ± 22.7 |
| Medial amygdala | 3.4 ± 12.9 | 124.0 ± 55.1 | 59.6 ± 23.6 | 125.1 ± 66.0 |
| Cingular cortex area 1 | 59.6 ± 14.5 | 80.4 ± 41.2 | 46.7 ± 5.2 | 75.8 ± 29.6 |
| Cingular cortex area 2 | 91.4 ± 22.2 | 105.3 ± 56.7 | 53.6 ± 6.6 | 87.1 ± 37.2 |
| Lateral septum | 107.3 ± 13.2 | 106.1 ± 26.6 | 64.3 ± 10.7 | 98.8 ± 20.1 |
| Medial septum | 53.4 ± 6.7 | 49.5 ± 6.7 | 43.5 ± 5.4 | 51.4 ± 6.9 |
| Piriform cortex | 60.8 ± 17.4 | 110.4 ± 45.4 | 62.2 ± 11.9 | 99.1 ± 52.3 |

Note: C-fos mRNA levels 75 or 120 min after KA injection. After 75 min, c-fos mRNA expression was significantly higher in the medial, basolateral, and central amygdala in the Ngb-KO mice compared to WT mice. After 120 min, significantly higher c-fos mRNA expression was found in the hippocampal CA1, CA3, DG, and subiculum as well as in the medial, basolateral, and central amygdala, and lateral septum of Ngb-KO mice versus controls (Table 1). Two-way ANOVA analysis (CA1: Interaction F(1, 17) = 5.40, p = .0328/Time F(1, 17) = 0.7292, p = .4050/Genotype F(1, 17) = 3.849, p = .0664; CA3: Interaction F(1, 17) = 5.323, p = .0339/Time F(1, 17) = 0.6441, p = .4333/Genotype F(1, 17) = 3.796, p = .0681; DG: Interaction F(1, 17) = 5.905, p = .0265/Time F(1, 17) = 12.43, p = .0026/Genotype F(1, 17) = 4.195, p = .0563; Sub: Interaction F(1, 17) = 4.439, p = .0503/Time F(1, 17) = 0.0003370, p = .9856/Genotype F(1, 17) = 2.517, p = .1311; BLA: Interaction F(1, 17) = 0.1595, p = .6946/Time F(1, 17) = 0.8454, p = .3707/Genotype F(1, 17) = 21.17, p = .0003; CeMA: Interaction F(1, 17) = 0.5298, p = .4766/Time F(1, 17) = 0.02582, p = .8742/Genotype F(1, 17) = 25.06, p = .0001; MeA: Interaction F(1, 17) = 0.0190, p = .8920/Time F(1, 17) = 0.03688, p = .8500/Genotype F(1, 17) = 12.63, p = .0024; CCA1: Interaction F(1, 17) = 0.1334, p = .7194/Time F(1, 17) = 0.5898, p = .4530/Genotype F(1, 17) = 4.828, p = .0421; CCA1: Interaction F(1, 17) = 0.4032, p = .5339/Time F(1, 17) = 3.308, p = .0866/Genotype F(1, 17) = 2.376, p = .1416; LS: Interaction F(1, 17) = 4.914, p = .0406/Time F(1, 17) = 9.758, p = .0062/Genotype F(1, 17) = 4.259, p = .0546; MS: Interaction F(1, 17) = 4.139, p = .0532/Time F(1, 17) = 1.959, p = .1796/Genotype F(1, 17) = 0.4691, p = .5027; PC: Interaction F(1, 17) = 0.1637, p = .6908/Time F(1, 17) = 0.09773, p = .7584/Genotype F(1, 17) = 7.756, p = .0127) followed by Bonferroni post-hoc analysis for multiple comparisons. Data are presented as means ± SD.

*p < .05; **p < .01.

3.4 Comparison of c-fos and Ngb expression

To investigate if brain areas in WT mice harboring Ngb containing neurones were more responsive to c-fos expression, we compared c-fos expression in WT and Ngb-KO with Ngb localization in WT mice. This showed that brain areas with increased c-fos response after KA injection in the Ngb-KO mice appeared to overlap with those brain areas that in WT harbor Ngb expressing neurones such as lateral septum, piriform cortex, and amygdala among others (Figures 2 and 3q-t). One exception was the hippocampal formation where all brain regions showed higher levels at 120 min (i.e., CA1, CA3, dentate gyrus, and subiculum) in Ngb-KO compared to WT mice. Only very few and scattered c-fos positive cells were observed in non-exposed mice (data not shown), and we therefore regard the c-fos expression as predominantly a response to KA injection.

4 DISCUSSION

This is the first comprehensive assessment of the Ngb-KO mouse phenotype. The circadian behavior and response to hypoxia were previously studied (Hundahl et al., 2011, 2012). Our data show that lack of Ngb does not alter basal behavioral and physical characteristics of Ngb deficient mice. We therefore do not think that effects observed post-KA derive from a predisposed difference in behavior but rather could be due to a deficit in coping with neuronal hyperexcitation.

Systemically induced KA seizures typically start in the hippocampus and later spread to other brain regions (Sperk, 1994). Consistent with this view, we found higher levels of c-fos expression in the hippocampus than in other regions at the early time point of 75 min in both mutant and WT mice. C-fos mRNA is known to appear in activated neurones approximately 30 min after stimulation and to remain for at least 30 min (see Kovacs, 1998 for review) (Kovacs, 1998). Time points were chosen based on the time course of the seizure development, for example, seizure scores 2–3 appear approximately 45 min after the KA injection. Thus, the observation time point at 75 min should reveal c-fos induction in neurones activated approximately 30 min after these non-generalized seizures occur. The 120 min observation time point should reveal c-fos activation in neurones activated by more severe and generalized seizures (scores 4–5). At 75 min, levels of c-fos expression did not differ...
between WT and Ngb-KO, consistent with the absence of Ngb in the hippocampus (Hundahl et al., 2010). However, brain areas harboring a high number Ngb-containing neurones, that is, the amygdala (medial, basomedial, and central divisions), piriform cortex, and lateral septum (Hundahl et al., 2010) were observed to display significantly elevated c-fos mRNA levels at the early time point 75 min in Ngb-KO mice. This suggests that increased excitatory activity in amygdala and piriform cortex may be important since they are known to be sensitive brain regions for seizure generation (Borowicz-Reutt & Czuczwar, 2020). Piriform cortex contains available KA receptors (Patel et al., 1986) and might become stimulated directly by KA. Whether amygdala is also stimulated directly by systemic KA, or its c-fos activation at 75 min is a down-stream effect is unknown. Thus, although KA may initiate seizures first in the hippocampus in both Ngb-KO and WT mice, either direct activation by KA of piriform cortex/amygdala or increased sensitivity of these brain regions to excitatory input in the Ngb-KO mice, could be related to the earlier occurrence of motor seizures in Ngb-KO mice.

The lateral septal nucleus is also available for systemically administered KA, and the high levels of c-fos in the lateral septal nuclei after 75 min in WT and Ngb-KO mice might indicate that KA fully activates this area and that lack of Ngb does not further increase this effect at 75 min but on the other hand does extend the stimulation to the 120-min time point.

Thus elevated c-fos expression in Ngb-KO mice at the later time point (120 min) in hippocampal regions may be the result of seizure spread from Ngb-deficient brain regions (e.g., amygdala, piriform cortex) that either provide direct or indirect input particularly via entorhinal cortex to the hippocampus (Pikkarainen et al., 1999). There are hippocampal projections to lateral septum and amygdala (mainly the basal part) (Witter et al., 1989; Witter & Amaral, 1991, 2021). These latter (hippocampal target) regions have many neurones containing Ngb, and thus altered function of these neurones either provoked by KA itself, or by increased input from the neurones of the hippocampal formation could affect the feedback loop to the hippocampal formation and thus increase the susceptibility to KA-induced seizures in Ngb-KO mice.

The increased susceptibility to the KA-induced seizures in Ngb-deficient mice could therefore both result from a dysfunction of target neurones receiving projections from the hippocampal formation after response to excitatory stimuli and from excessive seizure activity being generated in extrahippocampal regions (i.e., amygdala, piriform cortex) that participate in spreading seizure activity to hippocampus and other regions. This is in line with seizure development being a consequence of dysregulations generated either by reducing inhibitory (γ-aminobutyric acid; GABAergic) or increasing excitatory (glutamatergic) signaling (Paz & Huguenard, 2015). Reducing energy capacity in glutamatergic neurones by conditional KO of the mitochondrial pyruvate carrier 1 (Mpc1) in glutamatergic but not GABAergic neurones can lead to increased seizures after

FIGURE 2 C-fos mRNA expression. (a–l) Show c-fos mRNA expression in WT mice after 75 min (a–c) and 120 min (g–i) after KA injection, and Ngb-KO mice 75 min (d–f) and 120 min (j–l) after KA injection. (a,d,g,j) Show the c-fos mRNA levels at all brain levels, and (b,e,h,k) show details of c-fos expression in the lateral septal region (arrow) and piriform cortex (double arrow), and (c,f,i,l) show details of c-fos expression in hippocampus (arrow) and amygdala (double arrow).
GABA receptor antagonism by pentylenetetrazol or by KA-induced glutamatergic stimulation (De La Rossa et al., 2022). Also reducing metabolism in glutamate and GABA neurones by using brain-specific pyruvate dehydrogenase E1 subunit alpha 1 (Pdha1) KO mice can lead to increased susceptibility to seizures due to dysfunctional excitability in glutamatergic neurones (Jakkamsetti et al., 2019).

The reason why lack of Ngb leads to increased seizure susceptibility and severity remains to be determined. However, oxidative stress and ROS are believed to play key roles in hyperexcitability and epileptogenesis (Pikkarainen et al., 1999; Shekh-Ahmad et al., 2019) and, consequently, the hypothesized role of Ngb as a scavenger of ROS could be an explanation for the observed increased seizure susceptibility in Ngb-KO mice in line with increased susceptibility to hypoxia induced c-fos expression (Hundahl et al., 2011). The fact that a similar increased c-fos response is seen in KO-mice exposed to hypoxia and KA supports that Ngb may be involved in ROS signaling and/or regulation rather than oxygen transport. Ngb, unlike hemoglobin and myoglobin, has three cysteine residues where two at position CD7 and CD5 form an internal disulfide bond and breakage of the bond decreases ligand affinity by an order of magnitude. This thereby links functional properties of Ngb to the redox state of the cell (Hamdane et al., 2003). Ngb has been shown to interact with NO₂ and H₂O₂, and the reactivity depends on the presence of a disulfide bond (Nicolis et al., 2007). Similarly, Ngb nitrate reductase activity to NO was shown to highly depend on the presence of the disulfide bond between CD7 and CD5 in the neuroglobin molecule (Tiso et al., 2011). Ngb-KO mice may therefore have a reduced capacity to sense changes in the redox state of cell, which affects not only the cell that would have expressed Ngb but interestingly also makes non-Ngb expressing cells more susceptible as judged by the increased c-fos expression reported in this study and a previous study (Hundahl et al., 2011). In addition, it could be speculated that other neural cell types, such as the glial cells, could be involved in the observed regulations, however, we have previously shown that Ngb is not expressed in glial cells (Raida et al., 2013), and we therefore suspect the effects to be attributed to the neurones.

One important limitation of this study is that while lack of Ngb was shown to lead to increased seizure susceptibility in adult female mice, no male mice were subjected to KA-induced seizures, and therefore the existence of sex-specific differences cannot be
categorically excluded. Previously, sex and hormonal differences have been reported, from pre- and clinical studies, as potential influence-
ners of development of seizures and epilepsy (Christensen et al., 2005; Scharfman & MacLusky, 2014). This difference has primarily been as-
cribed to effects induced by the hormones estrogen, progesterin, and androgens (Kight & McCarthy, 2014). In addition, circadian rhythms in other hormones, particularly the glucocorticoids, have also been sug-
gested to play a role in differential seizure susceptibility (Scharfman & MacLusky, 2014). In experimental epilepsy, it has previously been re-
ported that adult female rats were more resistant to pilocarpine-
induced SE and mortality than their male counterparts (Scharfman & MacLusky, 2014). However, this was not the case in the KA-induced seizure model where no sex differences were observed on develop-
ment of SE and mortality rates (Scharfman & MacLusky, 2014). Coherently, pilocarpine-induced SE and mortality rates were also not differ-
ent in adult male and female mice (Oliveira et al., 2015). In gen-
eral, the KA-induced seizure model in mice used in this study has been re-
ported as a robust and powerful model with few or only indistin-
guishable sex-specific effects on seizure development and mortality
between male and female subjects (Scharfman & MacLusky, 2014).
Potentially, KA induces such severe motor seizures and SE, that they
cannot be sufficiently countered or prevented by the sex hormonal
fluctuations, while more subtle seizures could go unnoticed while still
being modulated (Scharfman et al., 2005). Therefore, the stable ex-
perimental conditions (e.g., experimental time of day, age and body-
weight of animals, subject matching and housing/diet), together with
the selected seizure model, and the fact that the same result could be
replicated in the present study two times in the KA-induced seizure
model provide further support for the overall relevance of the finding
and could be extrapolated to adult male mice.

In a set of previous studies (Hundahl et al., 2011; Raida et al., 2012)
where we investigated the neuroprotective effect of neuroglobin, we
have shown that exposure to mild hypoxia or cerebral infarction by
middle cerebral artery (MCA) occlusion do not affect Ngb-KO com-
pared to WT mice, and thus that Ngb is not a respiratory molecule per
se. As both exposure to hypoxia and infarction are oxidative stress
situations, these studies seem to conflict with the present study.
However, hypoxia will affect all parts of the brain, but as Ngb is only
found in a few percent of neurons, the lack of ROS scavenging ef-
fact in this experimental situation could be small. As for the cerebral
infarction, the cortical areas supplied by MCA are almost devoid of
Ngb, again the ROS scavenging effect of lack of Ngb could be small.

Therefore, the more specific targeting of Ngb-containing neurons by
KA in this study could make the Ngb-KO mice more susceptible to ox-
idative stress due to lack of adequate ROS scavenging. In conclusion,
we suspect that lack of Ngb reduces the ability of neurons to cope
with increased excitation and the following increased metabolism and
oxidative stress. This, in turn, results in the faster development of sei-
zures and SE after exposure to the excitatory agent KA.

AUTHOR CONTRIBUTIONS
All authors had full access to all the data in the study and take responsi-
bility for the integrity of the data and the accuracy of the data analysis.

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CONFLICT OF INTEREST
DPWD is co-founder and consultant of CombiGene AB (Lund, Sweden). All other authors declare no potential conflict of interest.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available in the
supplementary material of this article.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**FIGURE S1** KA-induced seizures in WT and Ngb-KO female mice. The Ngb-KO mice (n = 6 per group) displayed increased sensitivity to seizures compared with the WT controls (n = 6 per group). An observed increased cumulative seizure score indicative of more severe seizures was observed for Ngb-KO mice as compared to WT 75 min (Student’s t-test, t(10) = 4.616, p = .0010) after KA injection (whereas it did not reach significant levels after 120 min; Student’s t-test, t(10) = 1.112, p = .2920). 75 min was the first time point selected for c-fos mRNA measurement. *p < .05, Student’s t-test.*

Data are mean ± SD with each replicate shown in scatter plots

**TABLE S1** Primary antibodies

**TABLE S2** Behavioural phenotype assessment, primary screen (SHIRPA)

**TABLE S3** Behavioural phenotype assessment of locomotion, anxiety-like behaviour, learning and memory, and depression-like behaviour

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