Disruption of the expression of the proprotein convertase PC7 reduces BDNF production and affects learning and memory in mice

William C. Wetsel[a,b,c,d], Ramona M. Rodriguiz[a,d,1], Johann Guillemot[e,1], Estelle Rousselet[e], Rachid Essalmani[e], Il Hwan Kim[a,d], Jesse C. Bryant[a,d], Jadwiga Marcinkiewicz[e], Roxane Desjardins[f], Robert Day[f], Daniel B. Constam[g], Annik Prat[e], and Nabil G. Seidah[e,2]

Departments of [a]Psychiatry and Behavioral Sciences, [b]Cell Biology, and [c]Neurobiology, [d]Mouse Behavioral and Neuroendocrine Analysis Core Facility, Duke University Medical Center, Durham, NC 27710; [e]Laboratory of Biochemical Neuroendocrinology, Clinical Research Institute of Montreal, affiliated with the University of Montreal, Montreal, QC, Canada H2W 1R7; [f]Institut de Pharmacologie de Sherbrooke, Département de Chirurgie/Urologie, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, QC, Canada J1H 5N4; and [g]Swiss Institute for Experimental Cancer Research, School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland

Edited* by Donald F. Steiner, The University of Chicago, Chicago, IL, and approved September 13, 2013 (received for review August 5, 2013)

PC7 belongs to the proprotein convertase family, whose members are implicated in the cleavage of secretory precursors. The in vivo function of PC7 is unknown. Herein, we find that the precursor proBDNF is processed into mature BDNF in COS-1 cells coexpressing proBDNF with either PC7 or Furin. Conversely, the processing of proBDNF into BDNF is markedly reduced in the absence of either Furin or PC7 in mouse primary hepatocytes. In vivo we observe that BDNF and PC7 mRNAs are colocalized in mouse hippocampus and amygdala and that mature BDNF protein levels are reduced in these brain areas in PC7 KO mice but not in the hippocampus of PC1/3 KO mice. Various behavioral tests reveal that in PC7 KO mice spatial memory is intact and plasticity of responding is mildly abnormal. Episodic and emotional memories are severely impaired, but both are rescued with the tyrosine receptor kinase B agonist 7,8-dihydroxyflavone. Altogether, these results support an in vivo role for PC7 in the regulation of certain types of cognitive performance, in part via proBDNF processing. Because polymorphic variants of human PC7 are being characterized, it will be important in future studies to determine their effects on additional physiological and behavioral processes.

gene knockout | fear conditioning | BDNF processing | PC7 substrates | brain phenotypes

Nine secretory proprotein convertases (PCs) play major roles in regulating multiple cellular and extracellular processes both in health and disease states (reviewed in refs. 1 and 2). The convertases PC1/3, PC2, Furin, PC4, PC5/6, PACE4, and PC7 cleave their substrates after single or pairs of basic amino acids, SKI-1/S1P processes protein precursors after nonbasic residues, and PCSK9 has no known substrates other than itself (1). Studies that have analyzed tissue expression, levels, regulation, ontogeny, phenotypes of model animals lacking one or more PCs, and human/mouse natural mutations are starting to provide clues as to the specific roles of these enzymes in cells and whole-animal physiological and pathological processes.

The type I membrane-bound PC7 is the most ancient member of the mammalian basic amino acid-specific PC family, and it exhibits the closest homology to yeast kexin (3). Human PC7 is synthesized as an *N*-glycosylated zymogen (proPC7) that, like most other PCs, undergoes autocatalytic cleavage in the endoplasmic reticulum at $\mathbf{RAKR}_{141}\downarrow$SV. Mature PC7 can reach the cell surface by an unconventional route from the endoplasmic reticulum (4), but it also accumulates in the trans Golgi network (TGN) and can cycle between the cell surface and TGN via endosomes, in part by virtue of a Pro-Leu-Cys_{726} motif in its cytosolic tail (5). The tail also contains two cysteine residues, Cys_{699} and Cys_{704}, which are palmitoylated (3, 4, 6), that may assist in this process. Confocal and electron microscopy studies have revealed that PC7 localizes to vesicles located immediately beneath the plasma membrane (4, 7). No soluble shed forms of PC7 have been detected. Enzymatic activity assays using only the soluble luminal/extracellular domain of PC7 (sol.PC7) and fluorogenic substrates have indicated that this Ca^{2+}-dependent enzyme exhibits a neutral pH optimum and a cleavage specificity similar to that of Furin, cleaving within the general motif $(\mathbf{R}/\mathbf{K})\text{-}2X_n\text{-}\mathbf{R}\downarrow$ where $n = 0$–2 (8, 9).

Only the membrane-bound PC7 induces the processing of pro-epidermal growth factor into a ∼115-kDa transmembrane form (10). PC7 is abundant in neurons (11) but is also expressed in microglia (12). It has been shown that PC7 exerts an important function in MHC class I-mediated antigen presentation (7), befitting its high expression within the immune system (3). Finally, PC7 is unique because it is able to shed the human transferrin receptor 1 (TfR1) into a soluble form by cleavage at $\underline{\mathbf{K}}$TECE$\underline{\mathbf{R}}_{100}\downarrow$LA within endosomes (13).

The physiological importance of the PCs is illustrated by the early death or major phenotypes observed in mice lacking one or more convertase (1, 14). Although generated several years ago, deletion of PC7 is the only PC KO mouse for which no overt phenotype(s) has been described (15). In contrast, PC7 knockdown in *Xenopus* is embryonic lethal. These embryos lack eyes and brain and exhibit abnormal anterior neural development (16). Unless

Significance

The 7-membered proprotein convertase (PC) family of basic amino acid-specific proteases is implicated in the cleavage-activation of secretory precursor proteins. Although the in vivo functions of most members have been well studied, those of the seventh member PC7 are largely unknown. Herein, we find that PC7 participates in the generation of mature BDNF both in cells and in specific brain areas such as hippocampus and amygdala. Indeed, PC7 exerts a critical role in the brain: The results of various behavioral tests in normal mice compared with those lacking PC7 expression support an in vivo role for PC7 in the regulation of certain types of cognitive performance, such as emotional memories, in part via BDNF activation.

Author contributions: W.C.W., J.G., A.P., and N.G.S. designed research; W.C.W., R.M.R., J.G., E.R., R.E., I.H.K., J.C.B., J.M., R. Desjardins, and R. Day performed research; W.C.W., R.M.R., J.G., E.R., R.E., I.H.K., J.C.B., J.M., R. Desjardins, R. Day, D.B.C., A.P., and N.G.S. contributed new reagents/analytic tools; W.C.W., R.M.R., J.G., A.P., and N.G.S. analyzed data; and W.C.W., R.M.R., J.G., D.B.C., A.P., and N.G.S. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

[1]R.M.R. and J.G. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: seidahn@ircm.qc.ca.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1314698110/-/DCSupplemental.

this knockdown is due to an off-target effect, amphibian PC7 seems to fulfill essential neuronal functions that, in mammals, may be either nonessential or redundantly assumed by other PCs. Notably, the regulation of the PC7 gene (*PCSK7*) has not been examined (3, 17).

In the present work we describe behavioral alterations in mice lacking PC7. Our results show that PC7 KO mice have lower levels of BDNF in the hippocampus and amygdala than WT mice and exhibit learning and memory impairments. Reduced BDNF levels are likely responsible for some of these deficits, because they are rescued by an agonist to the BDNF receptor tyrosine receptor kinase B (TrkB). Therefore, PC7 seems to play unique roles in the CNS, at least in part, through regulating levels of BDNF.

Results

BDNF Levels Are Reduced in PC7 KO Mice. In a recent publication (18), we found that PC7 KO mice display anxiolytic-like behavior. Because PC7 is expressed in the hippocampus and amygdala (Fig. 1 and Fig. S1), and because these brain structures are involved in anxiety responses (19), it may be the case that PC7 plays some functional role in these areas. Although a number of peptides are present in these brain regions, transgenic BDNF overexpression in the hippocampus and amygdala leads to increased anxiogenesis in mice (20). These relationships prompted us to examine the status of BDNF in PC7 mice.

In situ hybridization revealed that PC7 and BDNF mRNAs were colocalized in hippocampus and amygdala (Fig. 1). Using quantitative PCR (Table S1), we determined that the levels of BDNF mRNA in both brain areas were similar between PC7 KO and WT mice (Table S2). Western blot analyses from brain tissue extracts revealed that the levels of mature BDNF protein were significantly (~40%) lower in PC7 KO than in WT hippocampus ($P < 0.05$) and amygdala ($P < 0.01$) (Fig. 2). In agreement with a previous report showing that the precursor proBDNF is present in very small quantities in brain (21), a longer exposure of the Western blot film revealed that proBDNF levels did not significantly vary between the WT and KO mice (Fig. 2). This difference between proBDNF and mature BDNF levels suggested that the precursor may be selectively destabilized in PC7 KO tissues. Mature Sortilin has been reported to participate in the stabilization of proBDNF and to protect it from degradation (22). Sortilin is a type-1 membrane-bound receptor expressed in the central and peripheral nervous systems, which is synthesized as a precursor (proSortilin) that is converted to the mature receptor in vitro at the conserved $\mathbf{RWRR}_{77}\downarrow\mathbf{SA}$ site by Furin (23). Because this motif does not exclude the participation of other PCs, we tested whether proSortilin could also be a substrate for PC7. Indeed, in HEK293 cells cotransfection of a cDNA coding for proSortilin with cDNAs for either Furin or PC7 revealed that both convertases can completely process proSortilin and likely activate Sortilin (Fig. S2*A*). Hence, when PC7 is absent, proSortilin would not be processed sufficiently such that lower levels of proBDNF would be detected. This effect may explain why proBDNF levels were similar between WT and PC7 KO mice despite the ~40% decrease of BDNF in the PC7 KO mice (Fig. 2).

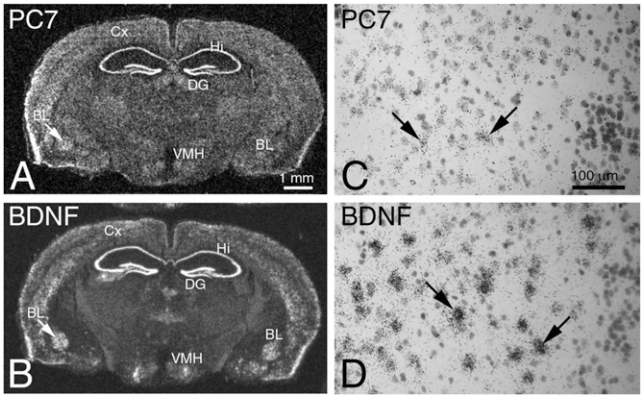

Fig. 1. Comparative distributions of BDNF and PC7 mRNA. Three adjacent sections of an adult mouse brain at the level of the basolateral (BL) amygdaloid nucleus were submitted to (*A*) PC7 and (*B*) BDNF in situ hybridization (dark-field illumination). Cx, cerebral cortex; DG, dentate gyrus; Hi, hippocampus; VMH, ventromedial hypothalamic nucleus. Emulsion autoradiography of (*C*) PC7 and (*D*) BDNF expression in pyramidal neurons (arrows) of the BL amygdaloid nucleus, followed by staining with cresyl violet.

Our data suggest that proBDNF processing is reduced in PC7 KO mice. Because the enzymatic conditions and cleavage specificities are similar for Furin and PC7 (8, 9), we overexpressed human proBDNF in primary hepatocytes isolated from livers of Furin KO or PC7 KO mice. We observed reductions in secretion of mature BDNF by ~65% and ~35% in absence of Furin and PC7, respectively (Fig. S2*B*). Besides these two convertases, PC1/3 has been reported to process proBDNF in vitro (24). However, in our hands the levels of hippocampal BDNF in WT and PC1/3 KO mice were indistinguishable (Fig. S2*C*).

To examine the roles of Furin and PC7 in processing proBDNF in greater detail, we used an in vitro system. In COS-1 cells coexpressing proBDNF and PC7, we found that PC7, like Furin, induced an increase in mature BDNF levels in cell extracts and media (Fig. S2*D*). However, mostly membrane-bound PC7 was active in processing proBDNF, compared with Furin, whereas soluble Furin (sol.Furin) generated more secreted BDNF than its membrane-bound form. This requirement for full-length PC7 was also observed for the shedding of human TfR1 (13), suggesting here also that proBDNF cleavage by PC7 occurs at the cell surface and/or in endosomes. Importantly, mRNA levels of other PC family members (24) or members of the tissue plasminogen activator (tPA)/plasmin pathway (25) were not downregulated in the hippocampus and amygdala of the two genotypes (Table S2), demonstrating that they are not responsible for the decrease in BDNF protein levels in these brain areas of PC7 KO mice.

PC7 KO Mice Are Deficient in Episodic Memory and a TrkB Agonist Rescues the Response. Because PC7 KO mice do not exhibit visible abnormalities (15) and because PC7 is expressed in the hippocampus (Fig. 1), we examined cognition. WT and PC7 KO mice were indistinguishable on tests of gross sensory and motor performance (Table S3). Episodic memory, recollection of past events or experiences, was examined with the social transmission of food preference (STFP) and the novel object recognition memory (NORM) tests (26). To monitor STFP, a demonstrator mouse that ate a flavored diet was returned to its home cage to interact with its cage-mates. The cage-mates were tested 20 min later (short-term memory, STM) and 24 h later (long-term memory, LTM) for their preference between the demonstrator and a novel-flavored diet. This choice depends on the ability of tester mice to detect and remember olfactory cues on the breath and whiskers of the demonstrator during their social interaction in the home-cage. A score of zero means no preference for either diet, whereas a positive score reflects a preference for the demonstrator diet. WT control mice strongly preferred the demonstrator diet at both time points, whereas this preference was significantly decreased in the PC7 KO animals (Fig. 3*A*, *Left*). Importantly, these reduced preferences were not due to alterations in social interactions between the demonstrator and tester mice (nose-to-nose contacts per tester-mouse, WT: 136 ± 11, PC7 KO: 151 ± 9; duration of contacts, WT: 46 ± 6, PC7 KO: 62 ± 8 s per tester-mouse), the amount of food eaten, time spent with the diets, bowl contacts, or latency to first bowl contact (Fig. S3 *A–D*).

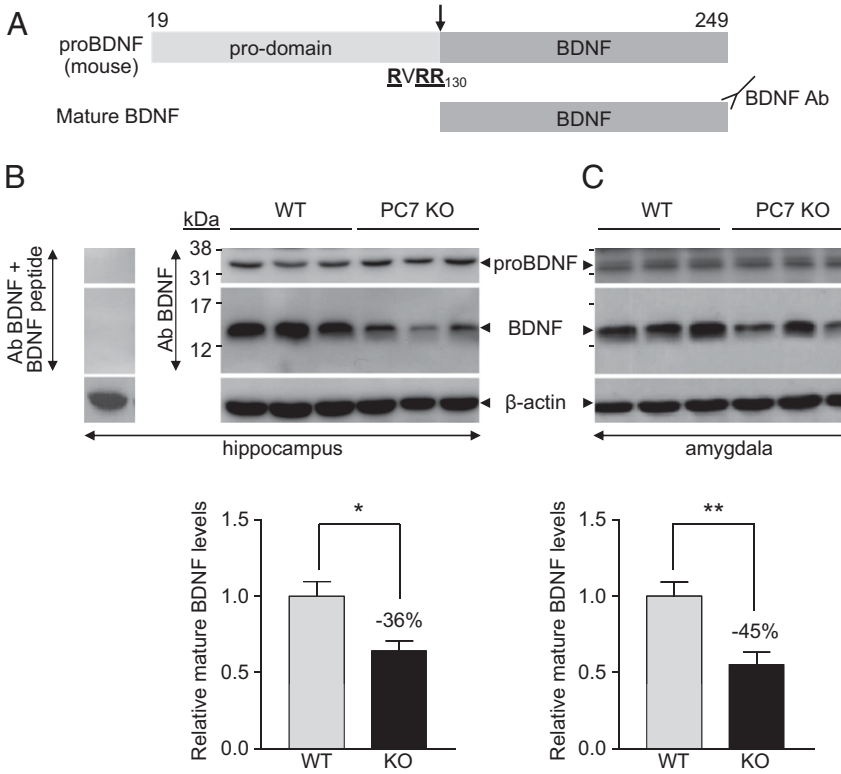

Fig. 2. The hippocampus and amygdala of PC7 KO mice contain lower levels of mature BDNF protein. (*A*) Schematic representation of the BDNF precursor and its PC cleavage site (arrow). Representative Western blot analysis of protein extracts from the (*B*) hippocampus and (*C*) amygdala of WT and PC7 KO mice, using a BDNF-specific antibody. The preabsorption control (left lane) demonstrates the specificity of the antibody. Normalized relative band intensities with respect to β-actin revealed a significant decrease in mature BDNF levels in both tissues ($n = 11$ mice/brain area/genotype); Student t test probability: $^{*}P < 0.05$; $^{**}P < 0.01$.

On day 1 of NORM testing, mice were presented with two identical objects. Twenty minutes (STM) and 24 h (LTM) later mice were presented with the now familiar and a novel object (27). If mice spend more time examining the novel object, it signifies that they recognize and remember the familiar one. Preference for the novel object in the two memory tests was significantly lower for PC7 KO than for WT mice (Fig. 3*A*, *Right*). This reduced preference for the novel object was not related to the number of object contacts or the latency to the first object contact (Fig. S3 *E* and *F*). Because LTM in this test was especially impaired and because BDNF levels were reduced in the PC7 KO mice, we injected (i.p.) another cohort of mice 1 h before training with 0.7 mg/kg 7,8-dihydroxyflavone (DHF), a brain-penetrable BDNF receptor (TrkB) agonist (28, 29). When tested 24 h after training, performance of the PC7 KO mice was virtually identical to that of the WT controls (Fig. 3*A*, *Right*). Together, the results from the episodic memory tests indicate that hippocampal function is abnormal in PC7 KO mice and that DHF can rescue the response.

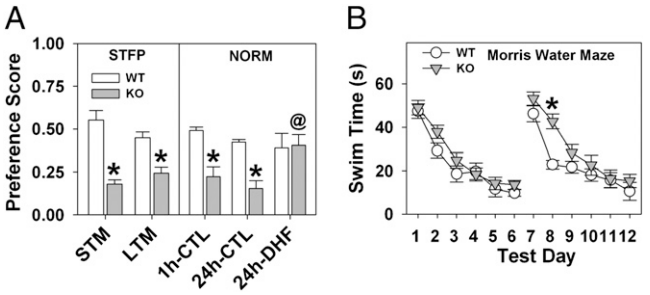

Fig. 3. PC7 KO mice are deficient in episodic memory. (*A*) Preference scores in the STFP and NORM tests for STM and LTM. $n = 11$ mice/genotype; $^{*}P < 0.05$, WT vs. PC7 KO mice. (*B*) Time to locate the hidden platform in the Morris water maze. $n = 10$ mice/genotype; $^{*}P < 0.05$, WT vs. PC7 KO mice; $^{@}P < 0.05$, within genotype CTL vs. DHF at the 24 h test time.

Mice were next evaluated in the Morris water maze (27). Swim times in the visible platform test were similar in WT and PC7 KO mice (Fig. S4*A*), demonstrating that motor performance, visual abilities, and motivation were similar between genotypes. In acquisition testing with the hidden platform, swim times (Fig. 3*B*, *Left*) and swim distances (Fig. S4 *B* and *C*) were similar between genotypes. However, when the location of the platform was moved from one quadrant to another, swim time and swim distance were initially prolonged for the PC7 KO mice (Fig. 3*B*, *Right*). The probe trial results confirmed these findings (Fig. S4 *D* and *E*). These data indicate that although spatial memory appears intact, plasticity of responding is mildly deficient in PC7 KO mice.

PC7 KO Mice Are Impaired in Emotional Memory, and a TrkB Agonist Rescues the Response. Emotional learning and memory processes were examined by fear conditioning and fear-potentiated startle (FPS) (27). In fear conditioning, responses are measured by freezing, which can be elicited by associating a neutral cue (e.g., environmental context or tone) with an aversive event (e.g., electric foot-shock) (30, 31). Because tissue levels of BDNF were reduced in PC7 KO mice, we conducted these tests with animals injected (i.p.) with saline (vehicle) or DHF and conditioned the mice 1 h later. When the vehicle-treated mice were evaluated 1 h (STM) or 24 h (LTM) after conditioning, we found that freezing was enhanced in the context test at both time points in WT mice,

whereas freezing was reduced in PC7 KO animals (Fig. 4*A*, *Left* and *Center*). In the cued test before presentation of the conditioned stimulus (CS), freezing was very low in both genotypes (Fig. 4*B*, *Left* and *Center*). However, we observed that freezing to the CS was increased at 1 h and 24 h in the WT vehicle-controls, whereas freezing in PC7 KO animals was not different between the pre-CS and CS intervals at either time point. We tested the DHF-treated mice 24 h after conditioning, when responses were most robust. We found that DHF enhanced freezing in the PC7 KO mice in both the contextual and cued tests to levels that were similar to those of the WT controls (Fig. 4 *A* and *B*, *Center* and *Right*). Importantly, sensitivity to foot-shock was not distinguished by genotype or treatment condition (Fig. S5 *A* and *B*). Hence, PC7 KO mice are impaired in both STM and LTM in contextual and cued fear conditioning, and these responses are rescued by the TrkB agonist.

Because deficiencies in contextual and cued fear conditioning indicate amygdala dysfunction (30, 32), mice were evaluated in FPS, an amygdala test (27). On day 1, baseline startle responses were higher in PC7 KO mice than in WT animals (Fig. S5*C*). On day 2, no genotypic differences in startle potentiation were observed to these same acoustic stimuli (Fig. S5*D*). On the next day, mice were injected with vehicle or 0.7 mg/kg DHF (i.p.) and conditioned 1 h later. When FPS was examined 48 h later in vehicle-treated mice, we observed that the 0.2-mA shock was without effect in either genotype (Fig. 4*C*, *Left*). Despite this fact, only vehicle-treated WT mice showed FPS after conditioning to the 0.4- or 0.6-mA shock (Fig. 4*C*, *Center*). Because 0.4 mA was a moderate level of shock in this test (27), we evaluated the effects of DHF at this intensity. We found that the levels of FPS in DHF-treated PC7 KO mice were indistinguishable from those of the WT mice given the vehicle or DHF (Fig. 4*C*, *Center Left* and *Right*). Collectively, these findings indicate that PC7 KO mice are deficient in fear memories and that a TrkB agonist can normalize these responses in the mutant mice.

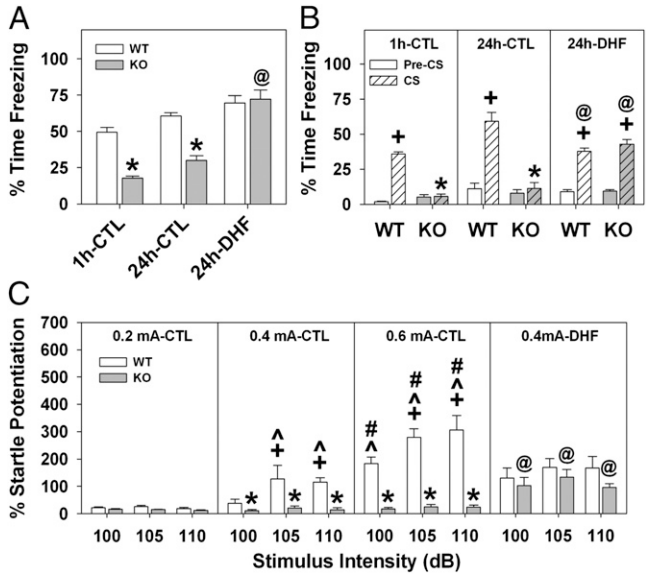

Fig. 4. PC7 KO mice are deficient in fear memories. (*A*) Percent time spent freezing in contextual fear conditioning at 1 h, or at 24 h after treatment (i.p.) with vehicle (CTL) or 0.7 mg/kg DHF. $n = 9$–10 mice/genotype/condition; $^{*}P < 0.05$, WT vs. PC7 KO mice; $^{@}P < 0.05$, within genotype CTL vs. DHF at the 24 h test time. (*B*) Percent time spent freezing in cued fear conditioning at 1 h, or at 24 h after CTL or DHF treatment. $n = 9$–10 mice/genotype/condition; $^{*}P < 0.05$, WT vs. PC7 KO mice; $^{+}P < 0.05$, pre-CS vs. CS phase within each test; $^{@}P < 0.05$, within genotype CTL vs. DHF during the CS phase at 24 h. (*C*) FPS in PC7 mice. $n = 8$–11 mice/genotype/treatment; $^{*}P < 0.05$, WT vs. PC7 KO mice; $^{+}P < 0.05$, compared with the 100-dB response within each CTL group; $^{\wedge}P < 0.05$, the 0.2-mA vs. the 0.4- and 0.6-mA responses within the CTL groups; $^{\#}P < 0.05$, the 0.4-mA vs. the 0.6-mA response; $^{@}P < 0.05$, CTL vs. DHF in the 0.4-mA shock condition.

Discussion

In the present investigation we report that BDNF and PC7 are colocalized in mouse hippocampus and amygdala and that PC7 KO mice have ∼40% reduced levels of mature BDNF protein in these same brain areas, with no effect on BDNF mRNA. Additionally, we demonstrate that when proBDNF and PC7 are coexpressed in COS-1 cells, this precursor is processed by PC7 into mature BDNF and that the levels of secreted BDNF from primary hepatocytes lacking PC7 are reduced by ∼35%. Behaviorally, we find that spatial memory is intact in PC7 KO mice, whereas plasticity of responding is mildly abnormal. Both episodic and emotional memories are impaired in these mutants, but they are rescued with the TrkB agonist DHF. Collectively these findings indicate that PC7 is a proBDNF processing enzyme in vivo and that deletion of PC7 in mice is deleterious to certain types of cognitive performance.

Although we examined many potential substrates for PC7 (10), we finally focused on BDNF because of our behavioral results. BDNF is known to regulate neuronal development, differentiation, and survival of neurons and to mediate synaptic plasticity (33). Because proBDNF can be processed intracellularly by several PC family members (24) and extracellularly by the tPA/plasmin cascade (25) and because PC7 was cloned after these studies were published (3), proBDNF processing by PC7 was not tested in our original report (24). PC7 is expressed in many tissues, including the hippocampus and amygdala, which are known to synthesize BDNF (3). We find that PC7 can process proBDNF into its mature form and that its absence from primary hepatocytes reduces proBDNF processing. These findings agree with our in vivo observations, which demonstrate that BDNF levels are lower in the PC7 KO than in WT hippocampus and amygdala. Together these results suggest that decreased BDNF levels in PC7 KO mice are directly related to reduced proBDNF processing. Interestingly, whereas mature BDNF levels were significantly reduced in PC7 KO mice, those of the proBDNF were not. One possible explanation for these findings is that PC7 affects proBDNF via two separate mechanisms. Processing of proSortilin would be reduced in PC7 KO mice, and this should increase degradation of proBDNF (22). By contrast, decreased processing of proBDNF may be expected to increase its levels. The net effect would be no change in proBDNF in PC7 KO mice, and these concentrations would be indistinguishable from the WT control. We also noted that the lack of PC1/3 does not significantly affect BDNF levels in the hippocampus. Furthermore, the mRNA levels of other proteases implicated in proBDNF processing (i.e., certain PC family members and the tPA/plasmin cascade) are not down-regulated in PC7 KO mice. Hence, it does not seem that these other proteases undergo compensatory regulation in the PC7 KO hippocampus and amygdala.

Alterations in BDNF levels in vivo can affect cognitive performance. For instance, virally induced BDNF deletion in the hippocampus disrupts NORM performance (34). Similarly, we find that PC7 KO mice are impaired not only in NORM but also in STFP, another test of episodic memory. However, hippocampal function is not completely lost, because Morris water maze performance is normal. Nevertheless, PC7 KO mice are mildly deficient in response plasticity on this test, suggestive of some hippocampal abnormalities. Other investigators have noted that water maze performance is perturbed in forebrain-deleted BDNF mice (35) and in mice with hippocampal virally induced BDNF ablation (34), whereas transgenic overexpression of BDNF in the cerebral cortex and hippocampus facilitates performance (36). The basis for the discrepancy between these and our

results is unclear, but it may be related to differing genetic backgrounds and brain areas affected, different behavioral protocols, and/or activities of other PC7 substrates not yet identified in the various brain regions that subserve spatial memories (37, 38). Regardless, our data indicate that PC7 KO mice have some hippocampal dysfunction and that hippocampal–cortical circuits may be abnormal (39).

Emotional memory is also modulated by BDNF. For example, fear conditioning selectively increases BDNF mRNA in the amygdala, whereas mRNAs of other neurotrophins and trophic factors are unaffected (40). BDNF protein levels and TrkB phosphorylation are also enhanced by fear conditioning (41, 42). In our studies, the concentrations of BDNF protein are reduced in the PC7 KO hippocampus and amygdala. Moreover, these mutants are impaired not only in contextual and cued fear conditioning but also in FPS. Other investigators have reported that $BDNF^{+/-}$, $BDNF^{Met/Met}$, and forebrain-inducible BDNF-deleted mice are impaired in contextual fear conditioning, whereas cued conditioning is retained (35, 43–45). Cued fear conditioning, however, can be deficient in older $BDNF^{+/-}$ animals (46). By contrast, transgenic overexpression of TrkB in cortical and hippocampal subfields augments contextual fear conditioning and taste aversion (36); the latter is a test of amygdala function. Our results in fear conditioning are fully compatible with these findings, because together with our FPS results they indicate that emotional memories are impaired in the PC7 KO hippocampus and amygdala. It is noteworthy that these effects cannot be attributed to the acquisition of fear conditioning, because the sensitivities to foot-shock and the freezing responses during the pretone (WT: 7 ± 0.9 s; KO: 8 ± 1.0 s) and posttone (WT: 23 ± 2.3 s; KO: 23 ± 2.5 s) conditioning intervals were not different between genotypes.

Several different approaches have been taken to rescue cognitive performance in BDNF-deficient mice. For instance, infusion of BDNF protein into $BDNF^{+/-}$ hippocampus can partially normalize fear responses (43). Interestingly, more complete rescue is achieved with DHF (47). This TrkB agonist is also reported to enhance acquisition and prolong extinction of fear conditioning in C57BL/6 mice (29) and to reverse some of the Alzheimer's disease-associated memory loss (48). It should, however, be emphasized that our DHF results are based on a single pharmacological approach, and that although DHF is efficacious in activating TrkB in cells (29), the specificity of this compound in vivo has not been fully established. Despite this possible limitation in our own studies, we find that DHF is efficacious in rescuing not only episodic LTM but also fear LTM in PC7 KO mice. The ability to rescue these differential memories provides additional support that PC7 is a proBDNF processing enzyme in vivo and that its disruption in mice has untoward behavioral consequences. Because analysis of polymorphic variants of the human PCSK7 (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=9159) have revealed that some of the variants have functional consequences [e.g., in iron metabolism (13)], it will be important in future studies to determine not only the effects of these mutations on proBDNF processing but also to ascertain their possible functional effects on other physiological and behavioral processes.

Materials and Methods

In Situ Hybridization. Ten-micrometer-thick cryosections were prepared from brains of 3-mo-old mice, fixed in 4% formaldehyde, and hybridized as previously described (49) with mouse sense (negative control) and antisense cRNA probes. The latter probes corresponded to the mouse PC7 or mouse BDNF coding regions for residues 1–213 or residues 25–117, respectively, and were synthesized using ^{35}S-UTP (PerkinElmer).

Cell Culture and cDNA Transfections. COS-1 and HEK293 cells were grown in DMEM with 10% FBS (Invitrogen) and were maintained at 37 °C under 5% CO_2. Using Lipofectamine (Invitrogen), 80–90% confluent COS-1 cells were cotransfected with pcDNA3 recombinants of human proBDNF with a C-terminal V5 tag together with either full-length or soluble human Furin (Furin, sol.Furin), full-length or soluble rat PC7 (PC7, sol.PC7), or an empty pIRES plasmid. Using jetPRIME (Polyplus), 80–90% confluent HEK293 cells were cotransfected with pcDNA3 recombinants of human proSortilin with a C-terminal c-Myc tag together with either full-length human Furin, rat PC7, or an empty pIRES plasmid. Twenty-four hours after transfection, the cells were washed and incubated in serum-free medium for an additional 20 h before medium collection and cell lysis.

Behavioral Tests. The procedures for the neurophysiological screen, STFP, NORM, Morris water maze, fear conditioning, and FPS have been described previously (26, 27). In pharmacology studies, mice were injected (i.p.) with saline or DHF (29) 1 h before behavioral testing.

Additional materials and methods can be found in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank all members of the Seidah laboratory for helpful discussions; Brigitte Mary for editorial assistance; Donald F. Steiner (The University of Chicago) for providing the PC1/3 KO mice; and Jiechun Zhou, Carolyn Kim, Chris Means, and Theodore Rhodes for assisting with the behavioral testing. This research was supported by Canadian Institutes of Health Research Grants MOP-36496 and CTP-82946 (to N.G.S.), a Canada Chair #201652, and unrestricted funds (to W.C.W.). J.C.B. was supported by the National Science Foundation, Award NSF0851905, and I.H.K. was supported by Mental Health MH082441. Some of the behavioral experiments were conducted with equipment purchased from the North Carolina Biotechnology Center. J.G. is a postdoctoral fellow supported by the Fonds de Recherche du Québec-Fonds de la Recherche en Santé du Québec.

1. Seidah NG, Prat A (2012) The biology and therapeutic targeting of the proprotein convertases. *Nat Rev Drug Discov* 11(5):367–383.
2. Steiner DF (1998) The proprotein convertases. *Curr Opin Chem Biol* 2(1):31–39.
3. Seidah NG, et al. (1996) cDNA structure, tissue distribution, and chromosomal localization of rat PC7, a novel mammalian proprotein convertase closest to yeast kexin-like proteinases. *Proc Natl Acad Sci USA* 93(8):3388–3393.
4. Rousselet E, Benjannet S, Hamelin J, Canuel M, Seidah NG (2011) The proprotein convertase PC7: Unique zymogen activation and trafficking pathways. *J Biol Chem* 286(4):2728–2738.
5. Declercq J, Meulemans S, Plets E, Creemers JW (2012) Internalization of proprotein convertase PC7 from plasma membrane is mediated by a novel motif. *J Biol Chem* 287(12):9052–9060.
6. van de Loo JW, et al. (1997) Biosynthesis, distinct post-translational modifications, and functional characterization of lymphoma proprotein convertase. *J Biol Chem* 272(43): 27116–27123.
7. Leonhardt RM, et al. (2010) Post-endoplasmic reticulum rescue of unstable MHC class I requires proprotein convertase PC7. *J Immunol* 184(6):2985–2998.
8. Seidah NG, Chrétien M (1997) Eukaryotic protein processing: Endoproteolysis of precursor proteins. *Curr Opin Biotechnol* 8(5):602–607.
9. Seidah NG, Chrétien M (1999) Proprotein and prohormone convertases: A family of subtilases generating diverse bioactive polypeptides. *Brain Res* 848(1-2):45–62.
10. Rousselet E, et al. (2011) Proprotein convertase PC7 enhances the activation of the EGF receptor pathway through processing of the EGF precursor. *J Biol Chem* 286(11): 9185–9195.
11. Dong W, et al. (1997) Cellular localization of the prohormone convertases in the hypothalamic paraventricular and supraoptic nuclei: Selective regulation of PC1 in corticotrophin-releasing hormone parvocellular neurons mediated by glucocorticoids. *J Neurosci* 17(2):563–575.
12. Marcinkiewicz M, et al. (1999) Nerve growth factor and proprotein convertases furin and PC7 in transected sciatic nerves and in nerve segments cultured in conditioned media: Their presence in Schwann cells, macrophages, and smooth muscle cells. *J Comp Neurol* 403(4):471–485.
13. Guillemot J, Canuel M, Essalmani R, Prat A, Seidah NG (2013) Implication of the proprotein convertases in iron homeostasis: Proprotein convertase 7 sheds human transferrin receptor 1 and furin activates hepcidin. *Hepatology* 57(6):2514–2524.
14. Creemers JW, Khatib AM (2008) Knock-out mouse models of proprotein convertases: Unique functions or redundancy? *Front Biosci* 13(5):4960–4971.
15. Villeneuve P, et al. (2002) Altered processing of the neurotensin/neuromedin N precursor in PC2 knock down mice: A biochemical and immunohistochemical study. *J Neurochem* 82(4):783–793.
16. Senturker S, Thomas JT, Mateshaytis J, Moos M, Jr. (2012) A homolog of Subtilisin-like Proprotein Convertase 7 is essential to anterior neural development in *Xenopus*. *PLoS ONE* 7(6):e39380.
17. Meerabux J, et al. (1996) A new member of the proprotein convertase gene family (LPC) is located at a chromosome translocation breakpoint in lymphomas. *Cancer Res* 56(3):448–451.
18. Besnard J, et al. (2012) Automated design of ligands to polypharmacological profiles. *Nature* 492(7428):215–220.

19. Pardon MC (2010) Role of neurotrophic factors in behavioral processes: Implications for the treatment of psychiatric and neurodegenerative disorders. *Vitam Horm* 82:185–200.
20. Govindarajan A, et al. (2006) Transgenic brain-derived neurotrophic factor expression causes both anxiogenic and antidepressant effects. *Proc Natl Acad Sci USA* 103(35): 13208–13213.
21. Matsumoto T, et al. (2008) Biosynthesis and processing of endogenous BDNF: CNS neurons store and secrete BDNF, not pro-BDNF. *Nat Neurosci* 11(2):131–133.
22. Yang M, Lim Y, Li X, Zhong JH, Zhou XF (2011) Precursor of brain-derived neurotrophic factor (proBDNF) forms a complex with Huntingtin-associated protein-1 (HAP1) and sortilin that modulates proBDNF trafficking, degradation, and processing. *J Biol Chem* 286(18):16272–16284.
23. Munck Petersen C, et al. (1999) Propeptide cleavage conditions sortilin/neurotensin receptor-3 for ligand binding. *EMBO J* 18(3):595–604.
24. Seidah NG, Benjannet S, Pareek S, Chrétien M, Murphy RA (1996) Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. *FEBS Lett* 379(3):247–250.
25. Pang PT, et al. (2004) Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. *Science* 306(5695):487–491.
26. Rodriguiz RM, et al. (2008) Animals lacking endothelin-converting enzyme-2 are deficient in learning and memory. *Genes Brain Behav* 7(4):418–426.
27. Porton B, et al. (2010) Mice lacking synapsin III show abnormalities in explicit memory and conditioned fear. *Genes Brain Behav* 9(3):257–268.
28. Jang SW, et al. (2010) A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone. *Proc Natl Acad Sci USA* 107(6):2687–2692.
29. Andero R, et al. (2011) Effect of 7,8-dihydroxyflavone, a small-molecule TrkB agonist, on emotional learning. *Am J Psychiatry* 168(2):163–172.
30. Phillips RG, LeDoux JE (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav Neurosci* 106(2):274–285.
31. LeDoux JE (2000) Emotion circuits in the brain. *Annu Rev Neurosci* 23:155–184.
32. Fendt M, Fanselow MS (1999) The neuroanatomical and neurochemical basis of conditioned fear. *Neurosci Biobehav Rev* 23(5):743–760.
33. Lessmann V, Brigadski T (2009) Mechanisms, locations, and kinetics of synaptic BDNF secretion: An update. *Neurosci Res* 65(1):11–22.
34. Heldt SA, Stanek L, Chhatwal JP, Ressler KJ (2007) Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. *Mol Psychiatry* 12(7):656–670.
35. Gorski JA, Balogh SA, Wehner JM, Jones KR (2003) Learning deficits in forebrain-restricted brain-derived neurotrophic factor mutant mice. *Neuroscience* 121(2):341–354.
36. Koponen E, et al. (2004) Transgenic mice overexpressing the full-length neurotrophin receptor trkB exhibit increased activation of the trkB-PLCgamma pathway, reduced anxiety, and facilitated learning. *Mol Cell Neurosci* 26(1):166–181.
37. Mumby DG, Gaskin S, Glenn MJ, Schramek TE, Lehmann H (2002) Hippocampal damage and exploratory preferences in rats: Memory for objects, places, and contexts. *Learn Mem* 9(2):49–57.
38. Kogan JH, Frankland PW, Silva AJ (2000) Long-term memory underlying hippocampus-dependent social recognition in mice. *Hippocampus* 10(1):47–56.
39. Dere E, Kart-Teke E, Huston JP, De Souza Silva MA (2006) The case for episodic memory in animals. *Neurosci Biobehav Rev* 30(8):1206–1224.
40. Rattiner LM, Davis M, French CT, Ressler KJ (2004) Brain-derived neurotrophic factor and tyrosine kinase receptor B involvement in amygdala-dependent fear conditioning. *J Neurosci* 24(20):4796–4806.
41. Rattiner LM, Davis M, Ressler KJ (2004) Differential regulation of brain-derived neurotrophic factor transcripts during the consolidation of fear learning. *Learn Mem* 11(6):727–731.
42. Ou LC, Gean PW (2006) Regulation of amygdala-dependent learning by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol-3-kinase. *Neuropsychopharmacology* 31(2):287–296.
43. Liu IY, Lyons WE, Mamounas LA, Thompson RF (2004) Brain-derived neurotrophic factor plays a critical role in contextual fear conditioning. *J Neurosci* 24(36):7958–7963.
44. Monteggia LM, et al. (2004) Essential role of brain-derived neurotrophic factor in adult hippocampal function. *Proc Natl Acad Sci USA* 101(29):10827–10832.
45. Chen ZY, et al. (2006) Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science* 314(5796):140–143.
46. Endres T, Lessmann V (2012) Age-dependent deficits in fear learning in heterozygous BDNF knock-out mice. *Learn Mem* 19(12):561–570.
47. Choi DC, et al. (2010) Prelimbic cortical BDNF is required for memory of learned fear but not extinction or innate fear. *Proc Natl Acad Sci USA* 107(6):2675–2680.
48. Devi L, Ohno M (2012) 7,8-Dihydroxyflavone, a small-molecule TrkB agonist, reverses memory deficits and BACE1 elevation in a mouse model of Alzheimer's disease. *Neuropsychopharmacology* 37(2):434–444.
49. Seidah NG, et al. (2003) The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): Liver regeneration and neuronal differentiation. *Proc Natl Acad Sci USA* 100(3):928–933.