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Knockout of spinophilin, an endogenous antagonist of arrestin-dependent α_2 -adrenoceptor functions, enhances receptor-mediated antinociception yet does not eliminate sex-related differences

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Abstract

We have previously shown gonadal steroid-dependent, gender specific modulation of nociception by α_2 -adrenoceptors. Agonist activation of the receptor enhances its association with spinophilin that antagonizes arrestin functions both by diminishing receptor phosphorylation by G-protein-coupled receptor kinase 2 (GRK2) and by competing for receptor interactions with arrestin. Since spinophilin is highly enriched in dendritic spines, we investigated whether α_2 -adrenoceptor-induced antinociception as well as sex-related differences are modified in spinophilin knockout mice. We evaluated α_2 -adrenoceptor antinociception in a heat-evoked tail flick test in spinophilin wild type ($Sp^{+/+}$) and knockout ($Sp^{-/-}$) mice. Baseline tail flick latencies (TFLs) did not change between any groups. Interestingly, the α_2 -adrenoceptor agonist, clonidine, increased TFL in male and diestrous (low estrogen) $Sp^{-/-}$ as well as $Sp^{+/+}$ mice; in fact, this increase in TFL was significantly higher in $Sp^{-/-}$ male and diestrous groups than in their $Sp^{+/+}$ counterparts. This unexpected finding is consistent with enhanced α_2 -adrenoceptor-mediated sedation observed previously in $Sp^{-/-}$ mice, presumably due to accelerated endocytosis of desensitized receptors and recycling of refreshed receptors when arrestin is not competed for by spinophilin in $Sp^{-/-}$ mice. Despite modulation of α_2 -adrenoceptor effects in $Sp^{-/-}$ mice, sex-related differences were retained; thus, clonidine was ineffective in proestrous females (highest estrogen levels), in both $Sp^{-/-}$ and $Sp^{+/+}$ mice, reaffirming that estrogen suppresses α_2 -adrenoceptor-evoked antinociception. These findings show that elimination of spinophilin enhances α_2 -adrenoceptor-evoked antinociception in estrogen-deprived physiological settings, suggesting a role for spinophilin to suppress these effects, and yet this enhanced response cannot overcome the absence of antinociception with elevated estrogen levels.

Keywords

spinophilin; α_2 -adrenoceptors; sex differences; clonidine; pain; analgesia

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Sex-related differences have been reported in the perception and modulation of pain, such that women are noted to exhibit enhanced sensitivity to experimental pain and to experience a greater prevalence of pain disorders [5,15]. In laboratory models, more robust effects of G-protein coupled receptors (GPCRs) such as μ opioid receptor (MOR), opioid receptor like-1 (ORL1), and α_2 -adrenoceptors, in suppressing pain perception occur in males when compared to females as shown by us [10,16,26,27,36] and others [9,19,22,24,25,29,31,34]. Similar observations have also been made in humans with opiates [14,17]. Importantly, estrogen may itself suppress the anti-nociceptive effects and thus account for this observed absence of GPCR-induced antinociception in females [10,27,36]. Spinophilin (also known as neurabin II), is an adaptor protein present in synapses and highly enriched in dendritic spines [1]. Spinophilin moves to the cell surface in the presence of α_2 -adrenoceptor agonists [6] and associates with the receptor in an agonist-enhanced fashion, preferring the non-phosphorylated receptor for this interaction [30]. Because spinophilin interaction is with the α_2 -adrenoceptor-G-protein $\beta\gamma$ complex [38], spinophilin binding to the receptor blocks its interaction with G-protein-coupled receptor kinase 2 (GRK2) and thus results in diminished agonist-evoked receptor phosphorylation, association with arrestin, and arrestin-dependent functions, including agonist-evoked receptor internalization [38].

Interestingly, estrogen has been shown to enhance spinophilin immunoreactivity in hippocampus [7,21], suggesting the possibility that estrogen-induced increase in spinophilin may affect GPCR signaling and thus contribute to observed sex-related differences in the modulation of pain. Hence we hypothesized that estrogen-dependent, sex-related differences in α_2 -adrenoceptor-induced antinociception would be diminished in spinophilin knockout mice. To test this hypothesis, we investigated whether spinophilin affected α_2 -adrenoceptor-induced antinociception as well as sex-related differences utilizing spinophilin wild type and knockout mice.

Spinophilin wild type ($Sp^{+/+}$) and knock out ($Sp^{-/-}$) mice [13] were obtained from Dr. Paul Greengard (The Rockefeller University, New York, USA) via Dr Qin Wang, who bred these mice at the University of Alabama before housing at the Meharry Medical College. Both facilities are certified by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Heterozygotes were crossed to derive $Sp^{-/-}$ genotype. At Meharry, the mice are under a 12-hour light/dark cycle (lights on: 7am; lights off: 7pm). For these studies, food and water were available *ad libitum*. The experimental protocols for assessment of pain perception were approved by the Institutional Animal Care and Use Committee of Meharry Medical College and abided by the established guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals.

Estrous cycle stages were determined by the standard vaginal smear test. Nociceptive testing in female mice was conducted at pro- and diestrous stages after having established two regular ovarian cycles. Circulating estrogen levels peak during the proestrous phase and plummet during the diestrous phase of the estrous cycle.

Clonidine (0.3 mg/kg) [24], an α_2 -adrenoceptor agonist, and yohimbine (2 mg/kg; 5 min before clonidine) [12], a selective α_2 -adrenoceptor antagonist were injected subcutaneously (s.c.) in separate groups (n=3–5/group). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in sterile saline.

Thermal nociceptive thresholds were determined by the tail flick test [11] using warm water tail immersion method. After 30 min. acclimatization to the testing room, animals were gently wrapped in a soft cloth with the tail exposed and distal 2/3rd length of the tail was lowered in the bath. Latency to the first quick tail flick was manually recorded to the accuracy of 0.01 sec with a stop watch. The temperature of water was kept constant at 49°C using an immersion

circulator (Fisher Isotemp 2150, Fisher Scientific, PA) which yielded a baseline tail flick latency (TFL) between 3 and 5 sec. Three baseline readings were taken prior to s.c. drug injections at 10 min intervals and the testing continued for up to 60 min post injection. A cut off latency of 15 sec. was set to prevent the possibility of any heat-induced tissue damage. Animals were handled similarly but without tail immersion a day before tail flick test to reduce stress and to achieve stable TFL up to 2 hr (data not shown). Data were analyzed using SPSS (SPSS Inc., Chicago, IL). All measures were submitted to ANOVA, corrected for repeated measures, with appropriate between- (group, drug) and within- (time course) group factors, and dependent variable (TFL). A post-hoc (Bonferroni) test was performed for intergroup comparisons only where ANOVA yielded a significant main effect. Area under the curve (AUC) was calculated by trapezoid method using Prism (Graphpad Software, Inc., San Diego, CA) for time course plots to obtain a single measure of the overall response. A p -value of <0.05 was considered significant. Data were plotted as Mean $\pm$ SEM.

To determine whether baseline nociceptive thresholds are unaffected by mouse gender or by in vivo expression of spinophilin, we compared baseline TFLs in all experimental groups in both $Sp^{+/+}$ and $Sp^{-/-}$ genotypes which included male, proestrous (relatively high estrogen) and diestrous (relatively low ambient estrogen) females. As seen in Fig. 1, baseline TFLs to immersion in 49°C water were comparable between all groups [ANOVA conducted on baselines alone yielded no significant main effect of group ($F_{(2,76)}=1.83$; $p=0.18$)].

To affirm whether there are estrogen-dependent, sex-related differences in the α_2 -adrenoceptor mediated antinociception in the mouse, by analogy with previous studies in rat models [26, 27,36], we tested the effects of clonidine on thermal nociception in wild type $Sp^{+/+}$ male, diestrous (low estrogen) and proestrous (high estrogen) females. Clonidine (0.3 mg/kg, s.c.), significantly increased TFLs in the male and diestrous $Sp^{+/+}$ groups [Fig. 1A, C; ANOVA yielded significant main effects of group ($F_{(6,18)}=21.39$; $p<0.001$), time ($F_{(9,162)}=9.90$; $p<0.001$) and an interaction between the two ($F_{(54,162)}=2.37$; $p<0.001$)]. Post hoc comparisons indicated that clonidine-induced increase in TFLs was seen immediately following injection in males and 10 min after the injection in diestrous females, and lasted for 30 min (all $p<0.05$ compared to baselines). In contrast, no significant effect of clonidine was observed in proestrous $Sp^{+/+}$ females; thus, post-drug TFLs remained at baseline (pre-drug) levels (Fig. 1C) in females with maximal levels of physiological estrogen, as is characteristic of the proestrous phase of the estrous cycle. These findings, together, reveal the dramatic estrogen-dependent, gender-specific modulation of clonidine-induced antinociceptive actions in this mouse model.

We then evaluated whether elimination of spinophilin expression affects antinociceptive effects of clonidine using spinophilin knockout ($Sp^{-/-}$) mice. As seen in Fig 1B and D, clonidine was effective in increasing TFLs in $Sp^{-/-}$ male and diestrous groups [ANOVA yielded significant main effects of group ($F_{(6,20)}=60.14$; $p<0.001$), time ($F_{(9,180)}=46.74$; $p<0.001$) with an interaction between the two groups ($F_{(54,180)}=13.04$; $p<0.001$)]. In both groups, clonidine-induced increase in TFLs was seen immediately after the injection and lasted for 30 min in males and for 40 min in diestrous females (post hoc $p<0.05$ compared to baselines). In contrast, like for findings in $Sp^{+/+}$ proestrous females, clonidine failed to affect TFLs in $Sp^{-/-}$ proestrous females (Fig. 1D), i.e. in settings where ambient estrogen levels are at their highest in vivo in females.

Interestingly, the magnitude of clonidine-induced increase in TFLs was significantly more pronounced in $Sp^{-/-}$ mice than in $Sp^{+/+}$ male and diestrous female mice [ANOVA conducted on all groups ($Sp^{+/+}$ and $Sp^{-/-}$) yielded significant main effects of group ($F_{(13,38)}=39.37$; $p<0.001$), time ($F_{(9,342)}=45.52$; $p<0.001$) and an interaction between the two ($F_{(117,342)}=6.93$; $p<0.001$)]. Thus, the $Sp^{-/-}$ male group had significantly higher TFLs at time 0 and 20 min post-

injection, whereas $Sp^{-/-}$ diestrous group had significantly higher TFLs at time 0–30 min post-injection as compared to their respective $Sp^{+/+}$ counterparts (post hoc $p < 0.05$). These data also revealed that the effect of clonidine was observed earlier in $Sp^{-/-}$ diestrous group compared to its $Sp^{+/+}$ counterpart (time 0 vs 10 min post injection). Area-under-the-curve analysis was conducted on these time course data to obtain a single, readily comparable measure of the overall response, and as seen in Fig 2, it further affirmed the greater effects of clonidine in the $Sp^{-/-}$ mice than in wild type ($Sp^{+/+}$) mice ($F_{(3,15)} = 20.67$; $p < 0.001$; post hoc $p < 0.05$). These data demonstrate that clonidine antinociception per se is enhanced in $Sp^{-/-}$ mice, and yet sex-related differences in clonidine-induced antinociception are preserved, i.e. the enhanced effects in the diestrous and male mice in $Sp^{-/-}$ mice are not paralleled by any enhanced effect in proestrous mice.

To confirm that the effects of clonidine were due to activation of α_2 -adrenoceptors, as anticipated based on earlier findings in $Sp^{-/-}$ mice [20], we pretreated $Sp^{+/+}$ and $Sp^{-/-}$ male and diestrous animals with yohimbine, an α_2 -adrenoceptor antagonist (2 mg/kg; s.c.; 5 min prior to clonidine). As seen in Fig 1A ($Sp^{+/+}$) and B, D ($Sp^{-/-}$), yohimbine completely blocked the antinociceptive effect of clonidine in the male and diestrous groups, restoring the TFLs to baseline levels. Yohimbine alone did not result in any significant change in TFL from baseline in $Sp^{+/+}$ males (Fig 1A). These data are consistent with the interpretation that activation of the α_2 -adrenoceptor is involved in the antinociceptive effects of clonidine evaluated in these studies.

We demonstrate for the first time that α_2 -adrenoceptor-induced spinal antinociception is enhanced in male and diestrous but not in proestrous $Sp^{-/-}$ mice. A similar observation, although in male $Sp^{-/-}$ mice only and on a supraspinally mediated hot plate test rather than a spinally mediated tail flick test employed in the present study, has been recently reported [8] and is consistent with our findings in male $Sp^{-/-}$ mice. Previous findings would suggest that arrestin interaction with the receptor is simultaneously enhanced when the endogenous competitor for arrestin, spinophilin, is not expressed [38]. This enhanced antinociceptive effect in $Sp^{-/-}$ mice mirrors the enhanced sedative response to a structural analog of clonidine, UK 14,304, [38], and a reciprocal suppression of sedation in arrestin knock out ($arr3^{-/-}$) mice. One of the possible mechanisms underlying enhanced clonidine antinociception in the present study may be an accelerated endocytosis and recycling/reactivation of α_2 -adrenoceptors in $Sp^{-/-}$ mice. Although a direct evidence is lacking, we have previously provided evidence for a faster resensitization of epinephrine-mediated ERK42/44 signaling which was reversible by experimental blockade of receptor endocytosis in mouse embryonic fibroblasts (MEFs) from $Sp^{-/-}$ mice compared to that from $Sp^{+/+}$ [38]. In addition, several other possible mechanisms may also contribute to the observed enhancement in clonidine antinociception. Recently discovered arrestin functions and arrestin-mediated signaling pathways including GPCR confirmation-specific arrestin function [33], scaffolding of phosphatidylinositol 4-Phosphate 5-Kinase Ia [28] and ERK [23] may also be revealed in the absence of spinophilin which in turn may affect α_2 -adrenoceptor function. However, dissecting among these multiple mechanisms is beyond the scope of the present investigation and will require a series of future experiments. Furthermore, since the expression of spinophilin has been demonstrated to be enriched in the dendritic spines of neurons [9,21,35], these data imply that, at the cellular level, α_2 -adrenoceptor-induced antinociception is likely due to receptor-mediated signaling in the soma and dendritic spines of neurons in the pain pathway, rather than via autoreceptors at the terminals of these neurons. Deletion of spinophilin has also been shown to reduce AMPA and NMDA receptor currents in dissociated striatal and hippocampal neurons respectively [13]. Although similar changes in nociceptive neurons may affect basal nociception in $Sp^{-/-}$ mice, comparison of baseline TFL across all experimental groups ($Sp^{+/+}$ and $Sp^{-/-}$) did not yield a significant difference in the present investigation.

In contrast to the dramatic enhancement of the antinociceptive response to the α_2 -adrenoceptor agonist, clonidine, in $Sp^{-/-}$ mice, the estrogen-related differences in the α_2 -adrenoceptor mediated antinociception are not detectably modified. High levels of estrogens impair α_2 -adrenoceptor-induced antinociception when present endogenously (at proestrous stage) as well as when exogenously administered into ovariectomized rat females [26,27,36]. The molecular bases for these effects of estrogen have not been unequivocally established. However, estrogen receptors ($ER\alpha$ and $ER\beta$) are present in the dorsal horn of the spinal cord [2,3,32,37], and thus estrogen may affect α_2 -adrenoceptor-induced antinociception indirectly by altering the expression of α_2 -adrenoceptors [18], hindering α_2 -adrenoceptor – G protein (Gi/Go) coupling [4], or altering expression of other proteins involved in receptor trafficking or signaling, ultimately contributing to the ability of physiologically relevant changes in estrogen levels during the estrous cycle alter sensitivity to α_2 -adrenoceptor-mediated suppression of pain perception. If estrogen treatment enhances spinophilin expression in the cell types that participate in thermal pain perception in a manner analogous to reported estrogen-dependent increases in spinophilin expression and dendritic spines in the mouse hippocampus [21] and monkey prefrontal cortex and hippocampus [9,35], this might provide one molecular explanation for the ability of estrogen to suppress the anti-nociceptive effects of α_2 -adrenoceptors in the control setting. However, the effects of estrogen cannot solely be mediated by spinophilin, since sex-related differences are preserved in the $Sp^{-/-}$ mice.

While a complete understanding of mechanisms that underlie estrogenic modulation of α_2 -adrenoceptor-induced antinociception clearly warrant further investigation, our results provide the first insight into a probable role of spinophilin in modulating the α_2 -adrenoceptor-induced antinociceptive mechanism.

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References

1. Allen PB, Ouimet CC, Greengard P. Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines. *Proc Natl Acad Sci U S A* 1997;94:9956–9961. [PubMed: 9275233]
2. Amandusson A, Hermanson O, Blomqvist A. Colocalization of oestrogen receptor immunoreactivity and preproenkephalin mRNA expression to neurons in the superficial laminae of the spinal and medullary dorsal horn of rats. *Eur J Neurosci* 1996;8:2440–2445. [PubMed: 8950107]
3. Amandusson A, Hallbeck M, Hallbeck AL, Hermanson O, Blomqvist A. Estrogen-induced alterations of spinal cord enkephalin gene expression. *Pain* 1999;83:243–248. [PubMed: 10534596]
4. Ansonoff MA, Etgen AM. Receptor phosphorylation mediates estradiol reduction of alpha2-adrenoceptor coupling to G protein in the hypothalamus of female rats. *Endocrine* 2001;14:165–174. [PubMed: 11394633]
5. Berkley KJ. Sex differences in pain. *Behav Brain Sci* 1997;20:371–380. [PubMed: 10097000]
6. Brady AE, Wang Q, Allen PB, Rizzo M, Greengard P, Limbird LE. Alpha 2-adrenergic agonist enrichment of spinophilin at the cell surface involves beta gamma subunits of Gi proteins and is preferentially induced by the alpha 2A-subtype. *Mol Pharmacol* 2005;67:1690–1696. [PubMed: 15705742]
7. Brake WG, Alves SE, Dunlop JC, et al. Novel target sites for estrogen action in the dorsal hippocampus: an examination of synaptic proteins. *Endocrinology* 2001;142:1284–1289. [PubMed: 11181546]
8. Charlton JJ, Allen PB, Psifogeorgou K, Chakravarty S, Gomes I, Neve RL, Devi LA, Greengard P, Nestler EJ, Zachariou V. Multiple actions of spinophilin regulate mu opioid receptor function. *Neuron* 2008;58:238–247. [PubMed: 18439408]

9. Choi JM, Romeo RD, Brake WG, Bethea CL, Rosenwaks Z, McEwen BS. Estradiol increases pre- and post-synaptic proteins in the CA1 region of the hippocampus in female rhesus macaques (*Macaca mulatta*). *Endocrinology* 2003;144:4734–4738. [PubMed: 12960039]
10. Claiborne J, Nag S, Mokha SS. Activation of opioid receptor like-1 receptor in the spinal cord produces sex-specific antinociception in the rat: estrogen attenuates antinociception in the female, whereas testosterone is required for the expression of antinociception in the male. *J Neurosci* 2006;26:13048–13053. [PubMed: 17167094]
11. D'Amour FE, Smith DL. A method for determining loss of pain sensation. *J Pharmacol Exp Ther* 1941;72:74–79.
12. Dogrul A, Uzbay IT. Topical clonidine antinociception. *Pain* 2004;111:385–391. [PubMed: 15363883]
13. Feng J, Yan Z, Ferreira A, et al. Spinophilin regulates the formation and function of dendritic spines. *Proc Natl Acad Sci U S A* 2000;97:9287–9292. [PubMed: 10922077]
14. Fillingim RB, Gear RW. Sex differences in opioid analgesia: clinical and experimental findings. *Eur J Pain* 2004;8:413–425. [PubMed: 15324773]
15. Fillingim RB, Maixner W. Gender differences in the response to noxious stimuli. *Pain Forum* 1995;4:209–221.
16. Flores CA, Wang XM, Zhang KM, Mokha SS. Orphanin FQ produces gender-specific modulation of trigeminal nociception: behavioral and electrophysiological observations. *Neuroscience* 2001;105:489–498. [PubMed: 11672614]
17. Gear RW, Miaskowski C, Gordon NC, Paul SM, Heller PH, Levine JD. Kappa-opioids produce significantly greater analgesia in women than in men. *Nat Med* 1996;2:1248–1250. [PubMed: 8898754]
18. Karkanas GB, Li CS, Etgen AM. Estradiol reduction of alpha 2-adrenoceptor binding in female rat cortex is correlated with decreases in alpha 2A/D-adrenoceptor messenger RNA. *Neuroscience* 1997;81:593–597. [PubMed: 9316013]
19. Kiefel JM, Bodnar RJ. Roles of gender and gonadectomy in pilocarpine and clonidine analgesia in rats. *Pharmacol Biochem Behav* 1992;41:153–158. [PubMed: 1539065]
20. Lakhani PP, MacMillan LB, Guo TZ, et al. Substitution of a mutant alpha2a-adrenergic receptor via “hit and run” gene targeting reveals the role of this subtype in sedative, analgesic, and anesthetic-sparing responses in vivo. *Proc Natl Acad Sci U S A* 1997;94:9950–9955. [PubMed: 9275232]
21. Li C, Brake WG, Romeo RD, et al. Estrogen alters hippocampal dendritic spine shape and enhances synaptic protein immunoreactivity and spatial memory in female mice. *Proc Natl Acad Sci U S A* 2004;101:2185–2190. [PubMed: 14766964]
22. Loyd DR, Morgan MM, Murphy AZ. Morphine preferentially activates the periaqueductal gray-rostral ventromedial medullary pathway in the male rat: a potential mechanism for sex differences in antinociception. *Neuroscience* 2007;147:456–468. [PubMed: 17540508]
23. Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL, Lefkowitz RJ. Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proc Natl Acad Sci U S A* 2001;98:2449–2454. [PubMed: 11226259]
24. Mitrovic I, Margeta-Mitrovic M, Bader S, Stoffel M, Jan LY, Basbaum AI. Contribution of GIRK2-mediated postsynaptic signaling to opiate and alpha 2-adrenergic analgesia and analgesic sex differences. *Proc Natl Acad Sci U S A* 2003;100:271–276. [PubMed: 12496346]
25. Mogil JS, Wilson SG, Chesler EJ, et al. The melanocortin-1 receptor gene mediates female-specific mechanisms of analgesia in mice and humans. *Proc Natl Acad Sci U S A* 2003;100:4867–4872. [PubMed: 12663858]
26. Nag S, Mokha SS. Estrogen attenuates antinociception produced by stimulation of Kolliker-Fuse nucleus in the rat. *Eur J Neurosci* 2004;20:3203–3207. [PubMed: 15579177]
27. Nag S, Mokha SS. Activation of alpha2-adrenoceptors in the trigeminal region produces sex-specific modulation of nociception in the rat. *Neuroscience* 2006;142:1255–1262. [PubMed: 16934408]
28. Nelson CD, Kovacs JJ, Nobles KN, Whalen EJ, Lefkowitz RJ. Beta-arrestin scaffolding of phosphatidylinositol 4-phosphate 5-kinase Ialpha promotes agonist-stimulated sequestration of the beta2-adrenergic receptor. *J Biol Chem* 2008;283:21093–21101. [PubMed: 18534983]

29. Peckham EM, Traynor JR. Comparison of the antinociceptive response to morphine and morphine-like compounds in male and female Sprague-Dawley rats. *J Pharmacol Exp Ther* 2006;316:1195–1201. [PubMed: 16291875]
30. Richman JG, Brady AE, Wang Q, Hensel JL, Colbran RJ, Limbird LE. Agonist-regulated Interaction between alpha2-adrenergic receptors and spinophilin. *J Biol Chem* 2001;276:15003–15008. [PubMed: 11154706]
31. Shane R, Bernal SY, Rozengurtel S, Bodnar RJ. Estrus phase differences in female rats in morphine antinociception elicited from the ventrolateral periaqueductal gray. *Int J Neurosci* 2007;117:811–822. [PubMed: 17454245]
32. Shughrue PJ, Lane MV, Merchenthaler I. Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. *J Comp Neurol* 1997;388:507–525. [PubMed: 9388012]
33. Shukla AK, Violin JD, Whalen EJ, Gesty-Palmer D, Shenoy SK, Lefkowitz RJ. Distinct conformational changes in beta-arrestin report biased agonism at seven-transmembrane receptors. *Proc Natl Acad Sci U S A* 2008;105:9988–9993. [PubMed: 18621717]
34. Stoffel EC, Ulibarri CM, Folk JE, Rice KC, Craft RM. Gonadal hormone modulation of mu, kappa, and delta opioid antinociception in male and female rats. *J Pain* 2005;6:261–274. [PubMed: 15820914]
35. Tang Y, Janssen WG, Hao J, et al. Estrogen replacement increases spinophilin-immunoreactive spine number in the prefrontal cortex of female rhesus monkeys. *Cereb Cortex* 2004;14:215–223. [PubMed: 14704219]
36. Thompson AD, Angelotti T, Nag S, Mokha SS. Sex-specific modulation of spinal nociception by alpha(2)-adrenoceptors: Differential regulation by estrogen and testosterone. *Neuroscience* 2008;153:1268–1277. [PubMed: 18434028]
37. Vanderhorst VG, Gustafsson JA, Ulfhake B. Estrogen receptor-alpha and -beta immunoreactive neurons in the brainstem and spinal cord of male and female mice: relationships to monoaminergic, cholinergic, and spinal projection systems. *J Comp Neurol* 2005;488:152–179. [PubMed: 15924341]
38. Wang Q, Zhao J, Brady AE, et al. Spinophilin blocks arrestin actions in vitro and in vivo at G protein-coupled receptors. *Science* 2004;304:1940–1944. [PubMed: 15218143]

Figure 1.

Antinociceptive effects of clonidine, an α_2 -adrenoceptor agonist, are enhanced in spinophilin knockout mice whereas sex-related differences are preserved. **A.** Subcutaneous clonidine treatment resulted in increased tail flick latencies in spinophilin wild type males as compared to vehicle controls. This effect of clonidine was completely antagonized by yohimbine, an α_2 -adrenoceptor antagonist, given s.c., 5 min prior to clonidine. Yohimbine alone did not change TFLs. **B.** Similar, antagonist reversible antinociceptive effect of clonidine was also observed in spinophilin knockout male mice albeit it was significantly enhanced as compared to that in wild type males (A). **C.** The antinociceptive effect of clonidine varied with different stages of estrous cycle in wild type females. While clonidine was able to increase TFLs in the diestrous group (with low levels of estrogen), it failed to do so in the proestrous group (with high levels of estrogen). **D.** Similarly, clonidine only increased TFLs in spinophilin knock out

diestrous females but not in proestrous females. Yohimbine again completely blocked this effect of clonidine. However, as in spinophilin knock out males, the magnitude of clonidine's effect was significantly higher in spinophilin knock out diestrous group as compared to that in their wild type counterparts (C). Baseline TFLs remained between 3–5 seconds in all groups with no significant differences. The clonidine-induced increase in TFLs was generally apparent just after s.c. injection and subsided 40–50 min post-injection.

Figure 2.

Area under the curve (AUC) analysis of the time course plots presented in Fig.1 further corroborated that the overall effect of clonidine, presented as AUC, was indeed significantly higher in spinophilin knock out male and diestrous groups as compared with their wild type counterparts. $*p < 0.05$